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Two Related Pyrrolidinedione Synthetase Loci in *Fusarium heterosporum* ATCC 74349 Produce Divergent Metabolites

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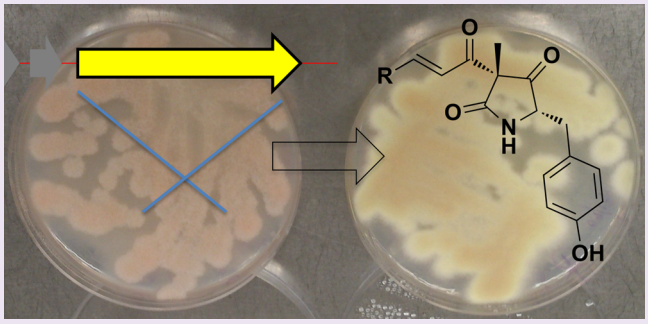
Supporting Information

ABSTRACT: Equisetin synthetase (EqiS), from the filamentous fungus *Fusarium heterosporum* ATCC 74349, was initially assigned on the basis of genetic knockout and expression analysis. Increasing inconsistencies in experimental results led us to question this assignment. Here, we sequenced the *F. heterosporum* genome, revealing two hybrid polyketide-peptide proteins that were candidates for the equisetin synthetase. The surrounding genes in both clusters had the needed auxiliary genes that might be responsible for producing equisetin. Genetic mutation, biochemical analysis, and recombinant expression in the fungus enabled us to show that the initially assigned EqiS does not produce equisetin but instead produces a related 2,4-pyrrolidinedione, fusaridione A, that was previously unknown. Fusaridione A is methylated in the 3-position of the pyrrolidinedione, which has not otherwise been found in natural products, leading to spontaneous reverse-Dieckmann reactions. A newly described gene cluster, *eqx*, is responsible for producing equisetin.

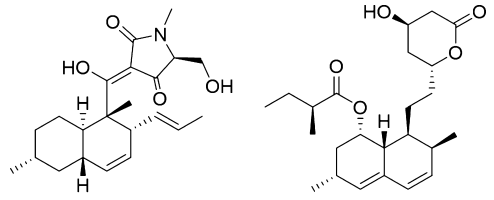


Filamentous fungi are prodigious producers of secondary metabolites. Within this diverse range of natural products, polyketides make up a large class, and their biosynthesis has been the subject of much study over the past decade.[1] A subgroup of polyketides is condensed with amino acids and cyclized to form 3-acyl pyrrolidinediones, also known as tetramic acids. These tetramic acids of natural origin are the product of a polyketide synthase (PKS) fused to a nonribosomal peptide synthetase (NRPS) module. In addition to tetramic acids, fungal PKS-NRPS proteins also lead to the synthesis of tetramic acid-like compounds, such as pyridinones and other derivatives that probably result from redox changes or pericyclic reactions.[2,3]

In such hybrid proteins, the PKS module belongs to the family of highly reducing PKSs (HR-PKS), which iteratively produce complex polyketides through mechanisms that are incompletely understood.[4,5] Typical HR-PKS domains are universally present in fungal hybrids, including ketosynthase (KS), acyltransferase (AT), ketoreductase (KR), dehydratase (DH), *C*-methyltransferase (MT), and acyl carrier protein (ACP). Together, these domains are capable of synthesizing polyketide-derived portions that are α-C-methylated and with carbons in varying reduction states, including ketone, hydroxyl, and olefin. In addition, an enoylreductase (ER) domain, which reduces double bonds, is often present either within the PKS or as an auxiliary protein.[5] In the case of lovastatin biosynthesis, the ER has been shown to collaborate with the core PKS in the production of a decalin ring rather than a linear polyketide.[6]

The NRPS module consists of an adenylation (A) domain that activates a specific amino acid, which is loaded onto the thiolation (T) domain. The covalently loaded amino acid is then condensed with the fully extended polyketide chain from the upstream PKS module by the condensation (C) domain. A Dieckmann cyclization domain (R*) catalyzes the final step that releases the intermediate to form a tetramate, such as pretenellin A, and prepseurotin A.[7−9]

Fusarium heterosporum ATCC 74349 produces a large amount of a tetramate, equisetin **1** (~2 g L^{-1} under some conditions).[10] Equisetin is similar to lovastatin **2** in its polyketide portion, but unlike lovastatin it appends an additional amino acid, serine (Figure 1). This pattern is very similar to products synthesized by fungal hybrid PKS-NRPS enzymes. Because of homology of equisetin to lovastatin, it is



equisetin **1** lovastatin **2**

Figure 1. Decalin-derived fungal polyketides. The decalin substructure is similar for equisetin **1** and lovastatin **2**. The major difference between the biosynthesis of equisetin and lovastatin would be the formation of the NRPS-derived tetramic acid ring of equisetin, which is not found in lovastatin.

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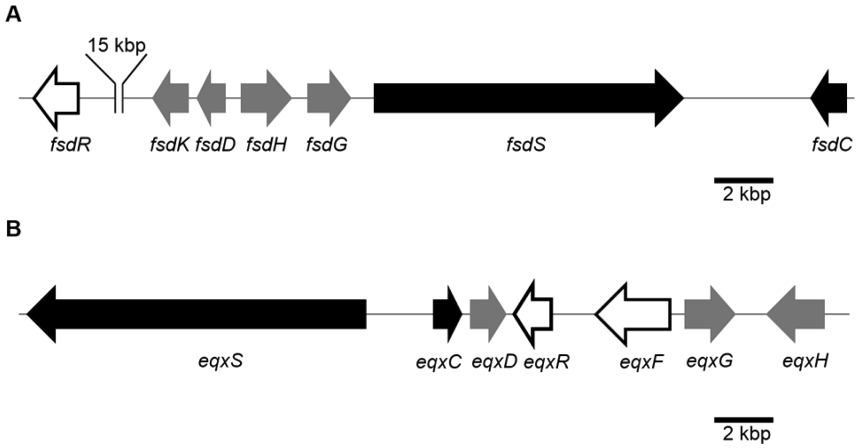

Figure 2. PKS-NRPS gene clusters found in the *F. heterosporum* genome. (A) The *fsd* cluster for fusaridione A (previously known as the *eqi* cluster). (B) The *eqx* cluster for equisetin biosynthesis. Genes including PKS domains are shown in black, including the PKS-NRPS genes *fsdS* and *eqxS* and the *trans*-ER-like genes *fsdC* and *eqxC*. Regulators *fsdR*, *eqxR*, and *eqxF* are shown in white. Other putative enzymes and transporters are shown in gray, including methyltransferases (*fsdD* and *eqxD*), transporters (*fsdG* and *eqxG*), oxidases (*fsdH* and *eqxH*), and a prenyltransferase (*fsdK*). (Note: overexpression of *fsdR* did not alter *fsd* expression, indicating that it may not be part of the pathway; it lies ~15 kbp from the other pathway genes.).

expected that, in addition to the core PKS-NRPS protein, an auxiliary ER would be required.[5,6] Equisetin is also amide N-methylated, which would most likely require an additional, dedicated MT. Using degenerate PCR and knockout mutation analysis in *F. heterosporum*, a previous study identified three reducing PKS genes, *deg1*, *deg2*, and *deg3*.[10] Through knockout mutagenesis, *deg2* was assigned as being responsible for equisetin production. This PKS-NRPS hybrid gene was thus renamed *eqiS*. The *deg2* cluster also contained genes potentially involved in equisetin biosynthesis, such as additional candidate ER and MT genes.

A key feature of equisetin biogenesis was that equisetin was produced at high levels (2 g L^{-1}) only on corn grit agar (CGA), but no detectable equisetin was produced on many other types of media.[10] In later studies, we sought to exploit this feature for heterologous expression of fungal genes. Several failed attempts led us to question whether the equisetin biosynthetic genes had been correctly identified. Here, we show through genomic, biochemical, genetic, and chemical studies that the previously characterized *eqiS* is not essential for equisetin production but instead produces a novel tyrosine-derived 2,4-pyrrolidinedione, which we named fusaridione A. A second PKS-NRPS gene found in the genome, *deg3*, instead produces equisetin. We renamed the *deg2* cluster *fsd* and the *deg3* cluster *eqx*.

RESULTS AND DISCUSSION

***fsd* Does Not Encode the Equisetin Synthetase.** The *deg2/eqiS/fsdS* knockout vector, TOPO-deg2KO, contained elements of the PKS *fsdS* flanking a phleomycin resistance marker. Homologous recombination in *F. heterosporum* produced a double crossover mutant, FusKO474, as verified by PCR. In addition, the previously described *eqiS/fsdS* hygroymcin resistance-based knockout, JWS19,[10] was resurrected from freezer stocks. In previous experiments, JWS19 was reported to produce no equisetin on CGA after 10 days, while wild-type *F. heterosporum* synthesized abundant equisetin.[10] However, here we found that both FusKO474 and JWS19 produced abundant equisetin equivalent to that produced by the wild-type strain on CGA after 21 days of growth (Supplementary Figure S1). Therefore, *fsdS* is not involved directly in equisetin biosynthesis.

Two PKS-NRPS Clusters Identified in the *F. heterosporum* Genome. To rapidly identify the correct equisetin pathway, the whole genome of *F. heterosporum* ATCC 74349 was sequenced by paired-end Illumina. Automated assembly availed a genome of 39 Mbp in length with a calculated GC content of 47.7% on 120 contigs (75% of the genome was on 17 contigs). These genome statistics are very similar to those for other *Fusarium* species that have been sequenced, such as *F. graminearum* (36 Mbp, 48.3% GC) and *F. verticillioides* (42 Mbp, 48.7% GC).[11] *De novo* annotation software, Maker2, was employed to predict a total of 12,448 proteins using the gene prediction model provided by *F. graminearum*.[12]

BLAST analysis of the whole genome (both assembled and raw reads) identified only three PKS genes with homology to the lovastatin nonaketide synthase, which were identical to the genes previously identified by PCR.[10] In addition, 9 further PKS genes were present in the genome but lacked any features consistent with equisetin production (Supplementary Figure S5). Of these three genes, *fsdS* and *eqxS* were PKS-NRPS genes, whereas *deg1* lacked an NRPS portion. *fsdS* and *eqxS* were very similar in domain order and content, although their protein sequence identity was only 47%. We employed the SMURF program to analyze fungal secondary metabolite genes clustered about these PKS-NRPS genes, revealing that both *fsd* and *eqx* clusters encoded at least one regulatory protein, a MT, a reductase that may function as an ER, and an exporter (Figure 2, Supplementary Tables S5 and S6).[13] Therefore, without functional analysis, these clusters were difficult to differentiate. Since *fsdS* was ruled out as the equisetin synthetase, and since abundant evidence implicates PKS-NRPS genes in tetramic acid synthesis, *eqxS* was a likely candidate.

Pyrophosphate Exchange Assay of FsdS and EqxS. We reexamined the amino acid substrate specificity of both the *fsdS* and *eqxS* proteins. A series of His-tagged, adenylation domain-containing constructs were synthesized to contain varying combinations of NRPS domains and expressed in *E. coli*. The most stable, folded constructs (*eqxS* A and ATR domains; *fsdS* ACP-CATR domains) were purified and used in the pyrophosphate exchange assay[14] using all 20 proteinogenic amino acids and *N*-methyl-L-serine. The major amino acid activated by *fsdS* was L-tyrosine, while that activated by *eqxS*

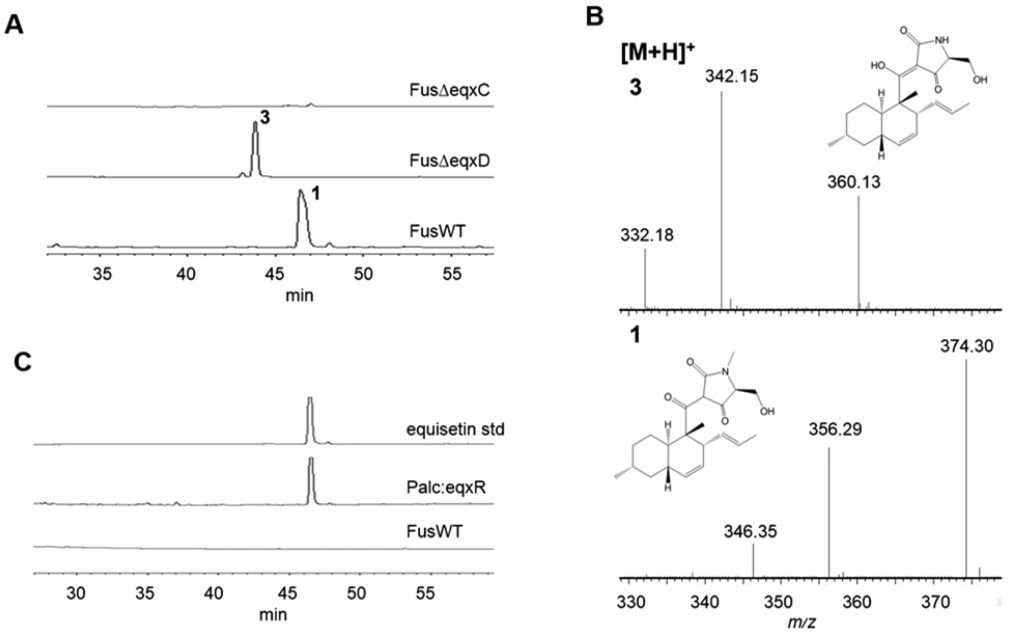

Figure 3. Knockouts and overexpression of *eqx* pathway genes. (A) Analytical HPLC of crude extracts of *eqxC* and *eqxD* knockouts compared to the wild-type strain (monitored at λ 293 nm). The *eqxD* knockout mutant does not produce equisetin **1** but instead produces an earlier eluting compound **3**. However, knocking out *eqxC* abolishes equisetin production, and no new metabolites are observed. (B) LC−MS shows that compounds **1** and **3** differ by 14 Da. (C) Analytical HPLC-DAD analysis of crude extracts of PDB cultures of *F. heterosporum* WT and the Palc:eqxR mutant. Overexpression of *eqxR* induces equisetin production (top trace shows equisetin standard).

was L-serine (Supplementary Figures S3A−D). This implicated *eqx* in equisetin synthesis, since equisetin contains Ser, while *fsdS* would likely produce a tyrosine-derived molecule.

Mutagenesis of *eqx* Cluster. Vectors were constructed to knock out *eqxS* and clustered genes N-MT *eqxD* and *trans*-ER *eqxC*. Initially, all vectors were constructed using native sequences flanking a hygromycin resistance cassette. These vectors were transformed into *F. heterosporum*, and transformants were screened for homologous recombination by PCR (Supplementary Figure S2). While multiple knockout mutants of *eqxC* and *eqxD* were obtained on the first attempt, concomitant knockout of *eqxS* was unsuccessful. Indeed, over 15 attempts were made to knockout *eqxS* using three different vectors, using both hygromycin and phleomycin as selection markers. By comparison, simultaneous attempts to introduce other genes were successful, usually in the first attempt, using the same materials. Speculatively, this may result from an untoward phenotype resulting from manipulation of *eqxS*.

In addition, predicted *eqx* regulators *eqxR* and *eqxF* were cloned into a phleomycin selection vector so that their expression could be controlled by the *Aspergillus nidulans alcA* inducible promoter and inserted into the genome by ectopic integration.[15−17]

***N*-Methyltransferase *eqxD* Plays a Role in Equisetin Biosynthesis.** *eqxD* knockout FusΔeqxD was grown on CGA for 21 days. Extraction with acetone followed by analytical HPLC showed that equisetin production was abolished. Instead, high-level production of an earlier eluting compound was observed (Figure 3A). By DAD-HPLC, the compound had the same chromophore as equisetin, and the LC−MS spectrum showed that the compound was 14 Da lighter than equisetin (Figure 3B). Purification on C_{18} column with a methanol/water gradient afforded pure compound **3** (6 mg; isolated yield 240 mg **3** per kg media). The molecular formula of **3** was determined by FT-ICR ESIMS to be $C_{21}H_{29}NO_4$ (m/z 360.21698 $[M + H]^+$, Δ 0.14 ppm). 1D proton NMR supported the absence of the *N*-methyl singlet at δ 3.07 observed for equisetin.[18] The 1D and 2D NMR spectra were essentially identical with those for the previously reported metabolite trichosetin, a tetramate produced by *Trichoderma harzianum* in dual culture with *Catharanthus roseus*.[19] For example, the ^{13}C spectrum of **3** was essentially identical to that shown in the previous manuscript. Moreover, all of the analyzed 2D data were consistent with the proposed structure for trichosetin.[19] Trichosetin is synonymous with *N*-desmethylequisetin. The chemical specificity of this result provided positive evidence associating the *eqx* cluster with equisetin production.

Equisetin Biosynthesis Requires *trans* ER (*eqxC*) Activity. The *eqxC* knockout, FusΔeqxC, was grown on CGA for 21 days. Extraction followed by analytical HPLC showed that equisetin was not produced, while the wild-type strain produced abundant equisetin (Figure 3A). Since a *trans*-ER is an integral part of fungal HR-PKS proteins,[5] this result strongly implicated the *eqx* PKS in equisetin synthesis.

Altered Regulation of *eqx* Cluster Allows High-Level Production of Equisetin in Broth Culture. Other than CGA culture for equisetin production employed by our lab, one other defined solid-state medium has been reported to induce *F. heterosporum* to biosynthesize equisetin.[20] Our attempts to induce the wild-type strain to produce equisetin in liquid culture by altering both carbon and nitrogen sources were unsuccessful (unpublished data). To achieve controllable expression in liquid culture, we cloned the two *eqx* putative regulators downstream of the inducible *A. nidulans alcA* promoter to make plasmids alcAeqxF and alcAeqxR. Independent transformation of these plasmids into FusWT with subsequent selection on phleomycin led to isolation of transformants Palc:eqxR and Palc:eqxF, which were verified by PCR to carry the expression cassette. Spores were prepared

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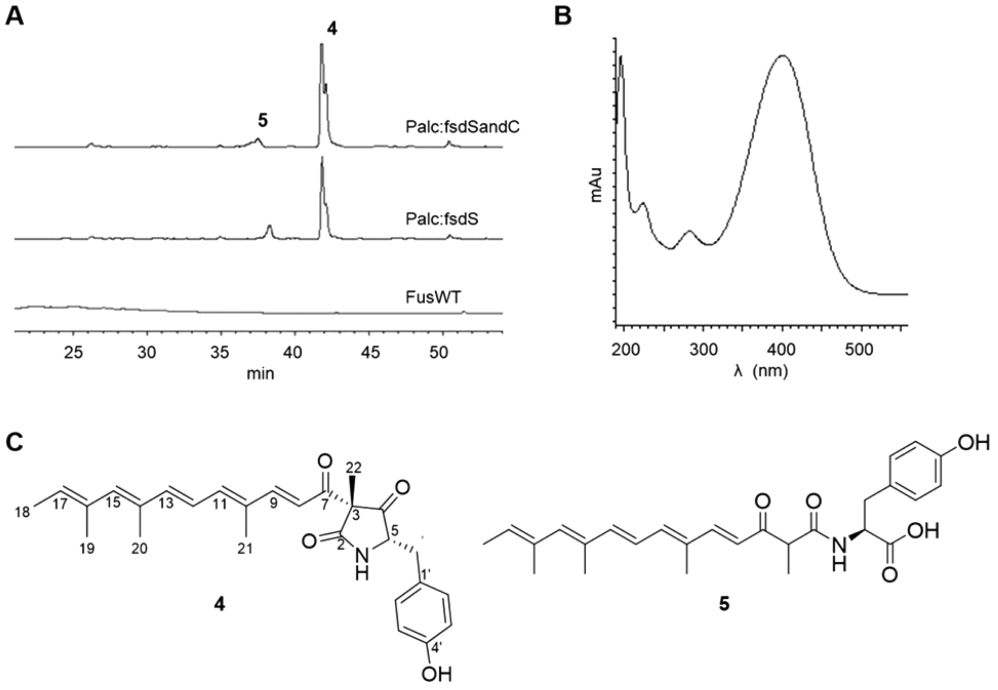

Figure 4. Overexpression of *fsd* pathway genes. (A) Analytical RP HPLC of crude mycelial extracts of 7 day PDB cultures of *F. heterosporum* transformed with *fsdS* and *fsdC* under control of *A. nidulans alcA* promoter (monitored at λ 380 nm). Two major new metabolites (**4** and **5**) were produced by the Palc:fsdS mutant. Introduction of the *fsdC* gene into Palc:fsdS to make the mutant Palc:fsdSandC did not lead to other products. (B) UV spectrum of **4** measured by DAD showing maxima at λ 402 nm, λ 280 nm, and λ 224 nm. (C) Structures of **4** and **5** were determined by 1D and 2D NMR. Metabolite **4** is a novel pyrrolidinedione, and **5** is its ring-opened form.

from each of the mutants and cultured in PDB for 18 h at 30 °C followed by cyclopentanone induction and further incubation for 4 days. DAD-HPLC analysis of the crude extracts from mutant cultures compared to the wild-type strain showed that Palc:eqxR synthesized equisetin at 207 mg L^{-1} under these conditions. By contrast, neither Palc:eqxF nor FusWT produced detectable equisetin in liquid broth (Figure 3C). Therefore, *eqxR* is a positive regulator of the *eqx* cluster. Since regulatory elements are often clustered with fungal biosynthetic pathways, this provided strong positive evidence for the involvement of *eqx* in equisetin production.

***fsd* Pathway Produces a Novel Tyrosine-Derived 2,4-Pyrrolidinedione.** We could not observe any evidence for production of putative *fsd* pathway products in a wide variety of media. In these experiments, we compared the metabolite expression patterns in the *fsdS* knockout (FusKO474) with those of the wild-type strain. This led us to explore another strategy to determine the secondary metabolite associated with potential *fsdS* activity. The promoter of *fsdS* was swapped for the *A. nidulans alcA* promoter to make the fungal expression plasmid alcAfsdS. Transformation of *F. heterosporum* with alcAfsdS led to isolation of a hygromycin-resistant mutant, Palc:fsdS. A possible *trans*-ER, *fsdC*, was also cloned into an *alcA*-controlled expression plasmid to generate alcAfsdC, which was then transformed into Palc:fsdS with phleomycin selection to generate the cotransformed mutant Palc:fsdSandC. Transformation resulted in colonies that were bright yellow on PDA, while wild-type *F. heterosporum* is pink (Supplementary Figure S4). Both mutants were cultivated in PDB with and without cyclopentanone induction. The filtered broth was analyzed by DAD-HPLC. Two new metabolites were produced by both the Palc:fsdS and Palc:fsdSandC mutants, but the metabolites were not detected in the FusWT extract (Figure 4A).

The major new metabolite was purified by C_{18} chromatography to yield a bright yellow compound, fusaridione A (**4**, 8 mg). The molecular formula was determined by FT-ICR ESIMS to be $C_{27}H_{31}O_4N$ (m/z 434.23267 $[M + H]^+$, Δ 0.21 ppm). All proton signals except for two exchangeable protons could be detected in the 1D ^1H NMR spectrum. From the HMQC, HSQC, and HMBC spectra, all 27 carbons were accounted for: 9 quaternary, 5 methyl, 1 methylene, and 12 methine. Consistent with the pyrophosphate exchange assay data showing that FsdS activates tyrosine, typical NMR shifts for tyrosine were identified, including a *p*-substituted phenol (δ 7.06 (d) and δ 6.76 (d)) and a benzylic methylene group (δ 3.06(d) and δ 2.84(d)). The α-proton (H-5) of tyrosine was observed at δ 4.29 and correlated to the phenol ring by TOCSY via W-coupling. UV maxima were observed at λ 280 nm (phenol) and at λ 402 nm for an additional conjugated moiety that was determined to be a pentaene by NMR analysis (Figure 4B). Analysis of the NMR data revealed that the ring is not a tetramic acid, but instead a pyrrolidine-3-methyl-2,4-dione (Figure 4C). The structure was further confirmed by observing ^3J HMBC correlations from the C-22 methyl protons (H-22, δ 1.46) to the carbonyl carbons, the amide carbonyl (C-2, δ 172.0), and C-4 (δ 207.1) (Figure 5). H-5 and H-6 also showed HMBC correlations with ring carbonyls.

The pentaene side chain structure was determined by examination of COSY, TOCSY, and HMBC data, which unambiguously revealed the positions of vinylic methyl groups. The attachment of the polyene side chain to the 3-position of the ring was revealed through an additional ^3J HMBC signal from the C-22 methyl group to the C-7 carbonyl (δ 189.8). The connectivity shown for the polyene chain was determined from the HMBC, COSY and TOCSY spectra. From the NOESY spectra, the *trans* nature of the double bonds was confirmed.

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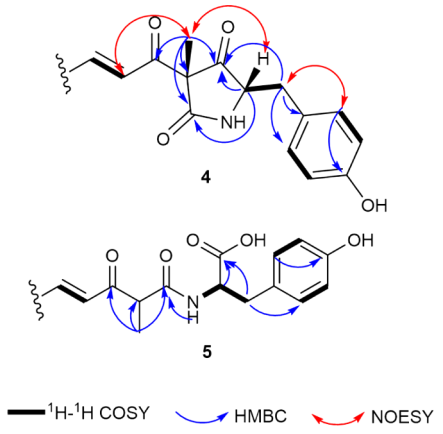

Figure 5. Key NMR data for **4** and **5**.

The methyl group at the 3-position of the pyrrolidinedione ring had NOESY correlations with H-5, fixing it *anti* to C-β of the tyrosine (Figure 5). The tyrosine *S*-configuration was determined by a variation of Marfey's method.[21]

A second major compound present in the *fsdS* expressions was purified as a bright yellow compound, **5** (3 mg), which had a molecular formula of $C_{27}H_{33}NO_5$ (*m*/*z* 452.24335 $[M + H]^+$, Δ 0.44 ppm), differing from **4** by the addition of H_2O. By DAD-HPLC, the compound had UV maxima at λ 280 nm and λ 386 nm, differing from **4** by a blue shift for the conjugated moiety that likely arises from cross-conjugation in comparison to **4**. Indeed, 1D and 2D NMR spectroscopy revealed that **5** was a ring-opened variant of **4** (Figure 4C). For example, chemical shift data of the tyrosine α-carbon (δ 51.6) was consistent with a linear, and not cyclic, molecule. A complicating factor was that the pentaene moiety was present as a mixture of two tautomers, which could not be resolved by changing the solvent or changing the temperature from −20 to +30 °C. HMBC data from both tautomers confirmed the side-chain structure of the molecule. Purification and concentration of **4** using TFA in the chromatography solvents afforded pure **5**, indicating that **5** is a degradation product of **4** that likely results from a reverse-Dieckmann reaction. Upon casual observation, the tautomer problem makes some spectra appear to contain mixtures, when in fact both **4** and **5** were obtained and analyzed as pure materials.

Conclusion. Filamentous fungal genomes contain numerous secondary metabolite gene clusters.[1,22] In recent years, it has become clear that most of these clusters are silent.[1,2,23,24] To fully exploit fungal pathways, currently the best strategy employs genome sequencing, followed by any one of several known methods of eliciting otherwise cryptic pathways.[16,17,25] Here, we sequenced the genome of *F. heterosporum* ATCC 74349 and then used a variety of functional approaches to elucidate the function of two hybrid PKS-NRPS gene clusters. We uncovered a novel PKS-NRPS cluster responsible for equisetin production and reassigned the previously ascribed equisetin synthetase gene to a novel pyrrolidinedione with unusual methylation.

Previously, the equisetin synthetase was assigned by knocking out *fsdS*, which is synonymous with *deg2* and *eqiS*, and finding that equisetin production was abolished in 10-day fermentations.[10] However, the data presented here definitively show that this was a misassignment and that the *eqx* gene cluster is responsible for equisetin biosynthesis. This previous study reinforces the well-known danger of relying on primarily negative data, such as knockout mutagenesis, for functional assignment of genes. It is remarkable that the *fsd* and *eqx* gene clusters are similar in terms of their biosynthetic content. This finding also explains some discrepancies in the literature. Analysis of fungal PKS-NRPS C domain phylogenies showed that the previously identified EqiS fell into a clade that uses aromatic amino acids, whereas EqiS was reported to use serine.[26] This information was interpreted to indicate polyphyly in the NRPS modules of fungal PKS-NRPS proteins. Based upon the evidence here, it is clear that C domain phylogeny is predictive of amino acid specificity.

The following lines of evidence support *eqx* as the equisetin biosynthetic gene cluster. (1) Only two PKS-NRPS genes (*fsdS* and *eqxS*) were present in the genome, and other biosynthetic genes did not have the correct functions for tetramate biosynthesis. (2) Two different *fsdS* knockouts did not inhibit equisetin biosynthesis. (3) Knockout of the PKS component ER *eqxC* abolished equisetin production. (4) Knockout of the MT *eqxD* prevented methylation and led to accumulation of desmethylequisetin. (5) Overexpression of regulator *eqxR* led to efficient synthesis of equisetin in 5 days in liquid culture, whereas otherwise 3 weeks on solid media is required. While activation of a regulator can sometimes activate a remote biosynthetic gene cluster as well as the adjacent cluster,[27] in this case strong induction led to a single product that was attributed to the adjacent cluster. Taken together, this provides both gain- and loss-of-function experiments demonstrating that *eqx* is crucial for equisetin production. Because *eqx* contains broadly similar genes to those found in *fsd*, the biosynthetic scheme is likely identical to that previously proposed (Figure 6).[10]

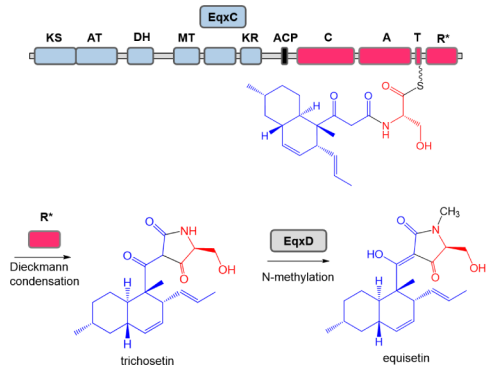

Figure 6. Proposed biogenesis of equisetin. The PKS module of EqxS together with the enoylreductase (EqxC) catalyze the formation of the polyketide unit (blue) which is then conjugated to L-serine (red) by the condensation domain of the NRPS module. Activity of the Dieckmann cyclase domain (R*) results in release of the intermediate as the tetramate, trichosetin. Subsequent N-methylation is carried out by EqxD to give equisetin.

fsdS was apparently silent since no metabolites could be attributed to this pathway, despite controlled experiments in which wild-type was compared to *fsdS* knockouts in multiple conditions. Alternatively, it is possible that the final products of the pathway are unstable or otherwise difficult to isolate. Upon induction under artificial conditions, *fsdS* led to production of the highly unstable product fusaridione A **4**. This product is likely not the natural product, as the other genes in the cluster were not coexpressed, except for the putative ER-like gene, *fsdC*. Other genes located in chromosomal proximity to *fsdS* encode proteins such as a potential prenyltransferase (FsdK)

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and a cytochrome p450 oxidase (FsdH). It is possible that the putative ER does not function as an enzyme in the *fsd* pathway. Alternatively, the products of some of the other proteins (such as FsdK or FsdH) may be the substrates of FsdC. It should be emphasized that FsdC is likely not an ER, as its sequence is only distantly related to other characterized *trans*-ER sequences.

Compound **4** bears structural similarity to products of other fungal PKS-NRPS proteins, such as the proposed prepseurotin A[9] and the known pretenellin A[28] metabolites. In addition, it is similar to the known fungal products, militarinones, for which the biosynthetic genes have not been characterized.[29] Therefore, the proposed biosynthesis of **4** is consistent with what is known about fungal PKS-NRPS metabolism (Figure 7). The

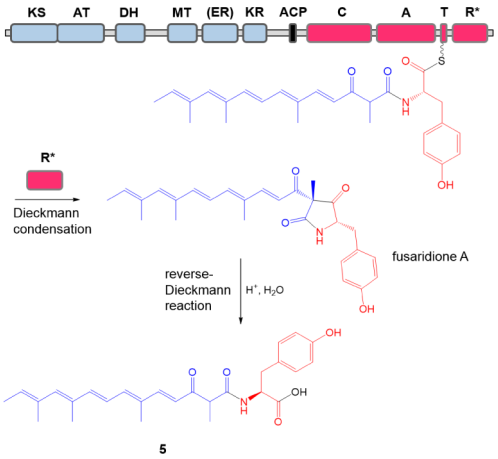

Figure 7. Proposed biogenesis of fusaridione A. The PKS module of FsdS catalyzes the formation of the polyketide unit (blue) which is then conjugated to L-tyrosine (red) by the condensation domain of the NRPS module. Activity of the Dieckmann cyclase domain (R*) results in release of the intermediate as fusaridione A. The unstable pyrrolidinedione ring of fusaridione A is opened through a reverse-Dieckmann reaction to afford **5**.

polyketide is synthesized by incorporation of the equivalent of seven acetate units. Every extension requires participation of the KS, AT, and ACP domains. KR and DH domains produce double bonds at every possible position except for C-7, which remains in the ketone oxidation state. As with other fungal polyketides, methyl groups would be introduced by a dedicated C-methyltransferase domain within the PKS, which acts at C-3, -10, -14, and -16. Subsequently, through the action of C, A, and T domains, tyrosine is activated and appended to the polyketide chain.

The major substantive difference between **4** and previously known compounds is the methyl group at C-3. Because of this methyl group, the product of the Dieckmann condensation **4** is readily degraded via the reverse-Dieckmann reaction in the course of fermentation or when treated with acid. Previously, it was shown that the R* domain in tetramic acid synthetases releases the Dieckmann product.[7,8] In the synthetic literature, it is reported that α-substituted, β-diketones readily undergo the reverse-Dieckmann reaction due to the lack of possible resonance stabilization.[30] Therefore, these results, in tandem with the known activity of R* domains, are consistent with an enzymatic release of **4**, followed by a nonenzymatic hydrolysis to yield the linear product **5**.

In conclusion, we have identified and assigned equisetin production by *F. heterosporum* to a new PKS-NRPS gene cluster, *eqx*. In addition, the product of the PKS-NRPS (FsdS) of the previously reported cluster has been characterized to be a novel compound, (3*R*,5*S*)-5-(4-hydroxybenzyl)-3-methyl-3-((2*E*,4*E*,6*E*,8*E*,10*E*)-4,8,10-trimethyldodeca-2,4,6,8,10-pentaenoyl)pyrrolidine-2,4-dione.

METHODS

Gene Cloning and Analysis Methods. DNA was obtained from *Escherichia coli* and yeast using the QIAprep Spin Miniprep Kit (Qiagen) and from *F. heterosporum* using the DNeasy Plant Mini Kit (Qiagen). DNA was amplified by PCR using high-fidelity enzymes Platinum HiFi Taq (Invitrogen) or Phusion Hot Start II HiFi (Finnzymes). Clones were analyzed using Platinum Taq (Invitrogen). Amplified fragments were cloned into destination vectors using yeast recombination in *Saccharomyces cerevisiae* BY4741, using a previously described lithium acetate method,[31] or by TOPO TA cloning (Invitrogen). The yeast-derived plasmids universally contained an *E. coli* replicon and resistance marker as well as the described[32] yeast plasmid elements. Plasmid rescue from yeast was performed using *E. coli* TOP10 (Invitrogen). Vectors used in this study, their construction methods, and primer sequences are provided in Supporting Information.

Fungal Mutagenesis. Vectors were cloned using hygromycin[33] or phleomycin[34] resistance markers. Gene knockout vectors were obtained by flanking resistance markers with ~2 kbp of *F. heterosporum* target DNA on each side. Overexpression vectors were obtained by fusing genes in frame with the cyclopentanone-responsive promoter *alcA*.[15,17] Linearized plasmids (10 μg) were transformed into *F. heterosporum* following a previously reported method,[10] except that the protoplasting buffer was modified to contain the following (in 20 mL): *Trichoderma* lysing enzyme (1 g), yatalase (100 mg), hemicellulase (30 mg), and β-glucuronidase (5 mg). Transformants were regenerated for 17 h at 30 °C on regeneration broth (1 M sucrose, 0.02% Difco yeast extract) and then plated on regeneration broth/1% agar with the appropriate selection agent (hygromycin 150 μg mL^{-1} or phleomycin 150 μg mL^{-1}). Colonies were screened by PCR to determine whether DNA was inserted via homologous recombination or ectopically. All screens for homologous recombination employed primer sets where the PCR product is ~8-fold larger if homologous recombination has occurred. Screens were employed both on colonies and on mature mycelia after weeks of growth to ensure integrity and purity of recombinant clones.

To knockout *fsdS*, phleomycin-resistance knockout vector TOPO-deg2KO was transformed into *F. heterosporum* by the protoplast method to yield FusKO474. *eqxC* and *eqxD* knockout vectors TOPO-OxoKO and TOPO-MTKO were synthesized similarly, except using the hygromycin resistance marker. Several *eqxS* knockout vectors were synthesized using both the hygromycin and phleomycin markers.

To overexpress proteins in *F. heterosporum* using *alcA*, transcription factors *eqxF* (alcAeqxF) and *eqxR* (alcAeqxR) were transformed into *F. heterosporum* using phleomycin selection, while *fsdS* (alcAfsdS) and *fsdC* (alcAfsdC) were transformed under hygromycin and phleomycin selection, respectively. A double mutant was constructed containing both alcAfsdS and alcAfsdC.

Genome Sequencing and Analysis. *F. heterosporum* DNA was sequenced at the University of Utah Huntsman Cancer Institute sequencing facility on an Illumina HiSeq 2000. A single lane was sequenced in a 100 bp paired-end sequencing run. The resulting raw reads were trimmed to provide reads >40 bp in length and with a PHRED quality score >30. The highest quality assembly was obtained using VELVET with the k-mer value of 61 and 235 x coverage.[35] The sequence was autoannotated with Maker2 using Augustus *de novo* gene prediction parameters, with *Fusarium graminearum* as the model organism.[12,36] BLASTx analysis was performed using a variety of polyketide genes as queries; to detect the closest relatives to lovastatin, residues 9−445 of the lovastatin nonaketide synthase (AAD39830.1)

were used in the search. Supplementary Tables S3 and S4 provide genome statistics and e-values for BLAST results.

Pyrophosphate Exchange Assay. The *eqxS* A and ATR domains, as well as the *fsdS* ACP-CATR, were constructed as N-terminal His-tagged sequences, as described in Supporting Information. The *eqxS* constructs were expressed in *E. coli* Rosetta 2DE3 (Novagen). The cultures were grown in 2XYT broth supplemented with ampicillin and chloramphenicol at 225 rpm and 30 °C until an OD_{600} of 0.4 was attained. The temperature was then reduced to 18 °C, IPTG (100 μM) was added, and the cultures were incubated for 18 h. *fsdS* ACP-CATR was expressed in *E. coli* BL21(DE3), to which the chaperone plasmid pG-KJE8 (Takara Bio Inc.) had been added. Protein expression was performed as above, except that the medium was Luria–Bertani (LB) broth, and the inducers were L-arabinose (2 mg mL^{-1}) and tetracycline (2 ng mL^{-1}) in addition to IPTG (100 μM).

Cells were harvested, flash frozen, and stored at −80 °C. Thawed cell pellets were resuspended in lysis buffer (5 mL g^{-1} cell pellet; 50 mM Tris, 200 mM NaCl, 10 mM imidazole, 5% glycerol, pH 7.5) and stirred on ice for 1 h with lysozyme (600 μg mL^{-1}). Cells were sonicated at 30% amplitude for 3 min with 30 s on/off cycles on the VibraCell 750 instrument and then incubated on ice for 30 min with DNase (20 μg mL^{-1}) and 10 mM $MgCl_2$. Following centrifugation at 20,000*g* for 45 min, the supernatant was filtered and loaded onto Ni-NTA resin. The resin was washed twice (1 M NaCl, 30 mM imidazole, pH 8.0), and proteins were eluted (1 M NaCl, 200 mM imidazole, pH 8.0) and dialyzed (25 mM HEPES, 500 mM NaCl, pH 8.0). *fsdS* ACP-CATR was further purified by FPLC on an SD300 column attached to an AKTA purifier, using the dialysis buffer. Purified proteins were analyzed by SDS-PAGE and flash frozen at −80 °C for storage.

The enzymes were used in the pyrophosphate exchange assay following a previously published protocol.[14] Enzymes were used at 1 μM, and all 20 proteinogenic amino acids and *N*-methyl-L-serine were used at 2 mM. A control reaction was performed with no amino acid added. Experiments were carried out in duplicate, and background was subtracted using the no amino acid control.

Chemical Analysis of Fungal Transformants. HPLC was performed using a Hitachi LaChrom Elite System with diode array detection using the Agilent Eclipse XDB C_{18} column (4.6 mm × 150 mm, 5 μm) for analytical purposes and the Discovery HS C_{18} column (25 cm × 10 mm, 5 μm) for preparative purification. Analytical mass spectrometry was performed using the Agilent ZQ, while high-resolution FT-ICR experiments were performed at the University of Utah Mass Spectrometry Core Facility using the LTQ-FT (Thermo-Electron) instrument. NMR data was acquired on a Varian INOVA 500, except for an HMBC spectrum of **4** acquired on a Varian INOVA 600 equipped with a cryoprobe.

Verified transformants were grown on PDA (Difco) for 4 days to generate spores, which were harvested by suspending the spores in sterile water with an inoculating loop followed by filtration through a cotton plug.[10] Fresh spores were used to inoculate media for all fungal expression experiments. In all analytical experiments, wild-type strains were used as simultaneous controls.

Spores from knockout mutants were transferred to CGA[10] and incubated at RT for 21 days prior to harvesting. Cultures were harvested by extracting three times with acetone (200 mL per 100 g media). The acetone was dried by rotary evaporation, leaving an aqueous residual. For analytical HPLC and LC–MS, this residual was desalted over a plug of end-capped C_{18} resin and analyzed directly. To purify compound **3**, a 100 g CGA culture was used, and the residual water was acidified to pH 2.0 with H_2SO_4 (conc) and extracted four times with hexanes (100 mL).[37] The extract was dried by rotary evaporation, resuspended in boiling hexanes (100 mL), and filtered. The hexanes fraction was washed six times with ethanol (2 mL) and dried by rotary evaporation; 25% of the residue was further purified by flash chromatography on end-capped C_{18} using a methanol/H_2O gradient, with TLC analysis employing a ferric chloride stain. Fractions containing **3** (90% methanol elution) were dried by rotary evaporation to give known compound trichosetin **3** (6 mg):[19] ^1H and ^{13}C NMR, see Supplementary Table S7; HRMS, see Supplementary Figure S22.

Spores from overexpression mutants were transferred to PDB (Difco) and incubated for 18 h with shaking at 200 rpm at 30 °C. To induce *alcA*, cyclopentanone (30 mM final concentration) was added, and the cultures were incubated for 6 d. Cultures were filtered through MiraCloth and subsequently extracted. For *eqxF* and *eqxR* overexpression, the filtered broth (250 mL) was extracted with ethyl acetate (2 × 250 mL) containing 1% acetic acid. The solvent was removed by vacuum, and the dried residue was subjected to analytical HPLC. The amount of equisetin **1** in the crude extract of the Palc:eqxR culture was quantified by HPLC using a standard curve generated with pure equisetin. For *fsdS* and *fsdS-fsdC* overexpression experiments, mycelia were extracted with acetone (200 mL), and the dried residue after solvent removal was purified by flash chromatography on end-capped C_{18} using an acetonitrile/water gradient, with TLC analysis employing iodine on silica as a stain. One fraction containing **4** was dried by vacuum to give pure compound fusaridione A **4** (8 mg). The filtered broth (4 L) was extracted with ethyl acetate (2 × 2 L) acidified with 1% acetic acid. The separated organic layer was dried under vacuum, and a portion of the residue was subjected to analytical HPLC. Flash chromatography on end-capped C_{18} using a methanol/H_2O (0.1% TFA) gradient was employed to isolate **5**. Fractions containing **5** were pooled, and solvent was removed under vacuum. Final purification was done with several rounds of preparative HPLC to afford pure compound **5** (3 mg). For ^1H and ^{13}C NMR data of **4** and **5**, see Supplementary Table S8; for HRMS, see Supplementary Figures S23 and S24.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The *fsd* and *eqx* gene clusters were deposited in GenBank, accession numbers KC439347 and AY700570.

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Notes

The authors declare no competing financial interest.

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