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Elucidation of the Biosynthetic Gene Cluster and the Post-PKS Modification Mechanism for Fostriecin in *Streptomyces pulveraceus*

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SUMMARY

Fostriecin is a unique phosphate monoester antibiotic that was isolated from *Streptomyces pulveraceus* as a protein phosphatase 2A (PP2A) and PP4A selective inhibitor. However, its biosynthetic mechanism remains to be elucidated. In this study, a 73 kb gene cluster encoding a six modular Type I polyketide synthases (PKS) and seven tailoring enzymes was identified by cosmid sequencing from the producer. The functions of two tailoring enzymes were characterized by gene disruption and an in vitro enzyme activity assay. Remarkably, the isolation of three malonylated fostriecin analogs from post-PKS gene knockout mutants indicated malonylated-polyketide formation could be a normal biosynthetic process in the formation of the unsaturated six-membered lactone in fostriecin. Based on this study, a comprehensive post-PKS modification mechanism for fostriecin biosynthesis was proposed.

INTRODUCTION

Fostriecin (CI-920, PD110,161), PD113,270, and PD113,271 were originally isolated from a fermentation culture of *Streptomyces pulveraceus* and are a class of phosphate ester natural products that are structurally related to cytostatin and phoslactomycin (Figure 1) (Amemiya et al., 1994; Hokanson and French, 1985; Hu et al., 2005; Kohama et al., 1993). Studies suggest that fostriecin is a phosphatase inhibitor with a 10^4-fold specificity for PP2A/PP4 over PP1, enzymes that play an important role in signal transduction, cell growth, and cell division (Lewy et al., 2002; Walsh et al., 1997). Therefore, fostriecin has attracted intense interest as a lead compound and was under evaluation as an antitumor drug in clinical trials. Although phase I clinical trials of fostriecin were halted due to its unpredictable chemical purity and storage instability, the comprehensive chemical synthesis of fostriecin and its analogs is under development to address these limitations and to further develop this specific antitumor agent (Lewy et al., 2002).

Polyketides represent a major class of antibiotics that are generated on multimodular enzymatic assembly lines. The modularity of the scaffold biosynthesis, together with the plurality of post-assembly modifications by tailoring enzymes, offers the prospect of creating various antibiotics with optimized activity, production, and pharmacokinetics. Phoslactomycins, antibiotics that are structurally related to fostriecin, were isolated from *Streptomyces sp.* HK803, and their biosynthesis mechanism was investigated (Palaniappan et al., 2003). However, the biosynthetic mechanism of fostriecin remains to be elucidated. The principle differences between phoslactomycin and fostriecin are the replacement of the C-8 methyl group by ethylamine, by methyl group at C-4 and the substitution of the terminal allelic alcohol by a cyclohexane ring in the phoslactomycin structure. Importantly, phoslactomycins are potent and highly selective inhibitors of PP2A (IC_{50} values ranging from 3.7–5.8 uM) compared to PP1 (IC_{50} > 1 mM). Recently, the PP2A inhibitory activity of phoslactomycin has been shown to inhibit tumor metastasis through the augmentation of natural killer cells. The unique and selective biological activity of this class of natural products has attracted considerable interest in recent years.

Although some analogs of fostriecin have been synthesized, providing useful structure-activity relationship data, the complexity of these molecules precludes the synthesis of many potentially desirable analogs. The details of the biosynthesis mechanism of fostriecin and phoslactomycin are being pursued to overcome the limitations of their chemical activity and to further develop this class of specific antitumor agents. The gene cluster for phoslactomycin has been cloned and characterized, but the hypothetical scheme for its complex biosynthesis events remains in need of further elucidation (Palaniappan et al., 2003). Here, we have cloned and characterized the fostriecin biosynthetic gene cluster and assigned putative biosynthetic roles for the encoded enzymes. Using comparative analysis of the gene cluster, in vitro enzyme activity assays and characterization of compounds produced by gene knockouts, we have proposed a plausible scheme for fostriecin biosynthesis. Our study, combined with the previous phoslactomycin reports, enables the rapid exploitation of these molecules and provides a framework for further studies of these complex pathways.

RESULTS

Isolation and Characterization of the Fostriecin Biosynthesis Gene Cluster from *Streptomyces pulveraceus*

There are numerous approaches for the identification and isolation of polyketide synthase (PKS) gene clusters from

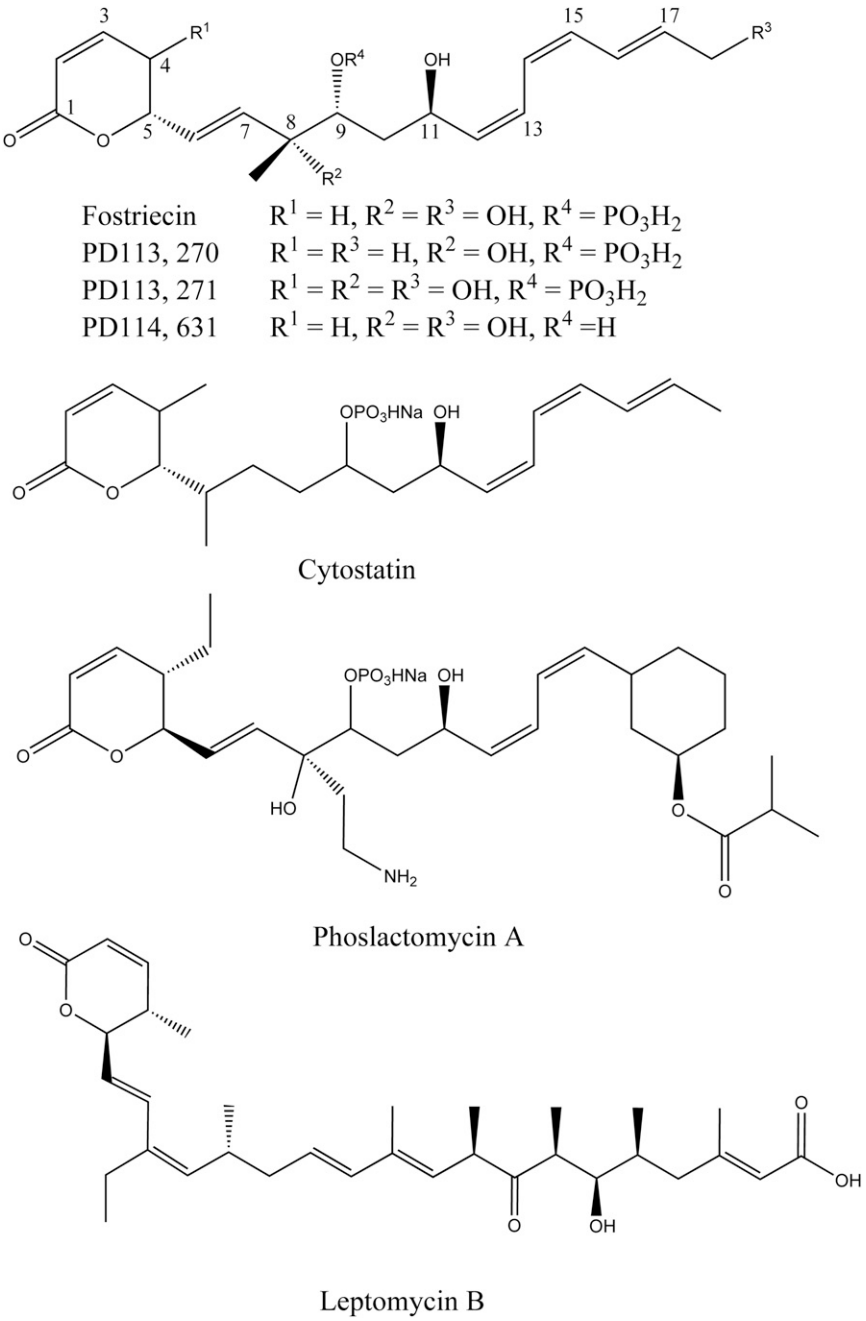

Figure 1. Chemical Structures of Fostriecin and Its Related Compounds
These structurally related compounds share the same feature, a six-membered lactone with a *cis*-$\Delta^{2,3}$-double bond.

various organisms. A simple method has been developed for the identification and isolation of modular (Type I) PKS genes from various organisms that involves sequencing the ends of cosmid inserts of a random genomic DNA library containing one or more modular PKS gene clusters. The PKS fragment identified from the sequencing effort can then be used as "a perfect probe" to isolate all of the modular PKS clusters in an organism (Hu et al., 2005; Santi et al., 2000). A modification of this method that allowed the rapid and facile cloning and identification of an interesting modular PKS gene cluster was developed in this study for the cloning of the entire fostriecin gene cluster.

A cosmid library from the *S. pulveraceus* ATCC 31906 genome was constructed on Supercos-1 (Stratagene, La Jolla, CA), and the end sequences from the inserts of ~1,000 cosmids were obtained and used for BLAST analysis. There were 28 cosmids containing Type I or Type II PKS sequences at either end or both ends. Restriction analysis of those sequences revealed that there were two PKS clusters. The cosmid Cfos-F2 from one PKS group that contained a unique P450 sequence at one end and a Type I PKS at the other end was selected to sequence first. The sequencing of Cfos-F2 revealed four complete PKS modules with one partial module, one P450 gene, and one homoserine kinase gene, which was consistent with the hypothetical biosynthesis of fostriecin. Three other overlapping cosmids, Cfos-F1, Cfos-F3, and Cfos-F4, which are in the same group as Cfos-F2, were individually and completely sequenced using a shotgun cloning strategy. A total of 73 kb of contiguous DNA was cloned and sequenced to provide the complete DNA sequence for the fostriecin gene cluster (Figure 2). The sequence was deposited in the GenBank database under the accession number HQ434551.

Organization of the Fostriecin Biosynthetic Gene Cluster

The DNA fragment that spans ~73 kb from four cosmids (Cfos-F1–F4) and contains all of the putative biosynthetic genes that are required for fostriecin biosynthesis is shown to contain 21 open reading frames (ORFs). The deduced gene organization within this region is shown in Figure 2. The presumed functions of the open reading frames for post-PKS in the fostriecin biosynthetic gene cluster is listed in Table 1.

The organization of the fostriecin modules encoding the multifunctional PKS is more complex than the known polyketides, such as tautomycetin, leptomycin, and erythromycin (Hu, et al., 2005; Li, et al., 2009), in which the PKS modules are tfcgenerally found squeezed into their genomes. The PKS modules in the fostriecin gene cluster are concentrated at two positions, with six additional ORFs that may encode post-PKS

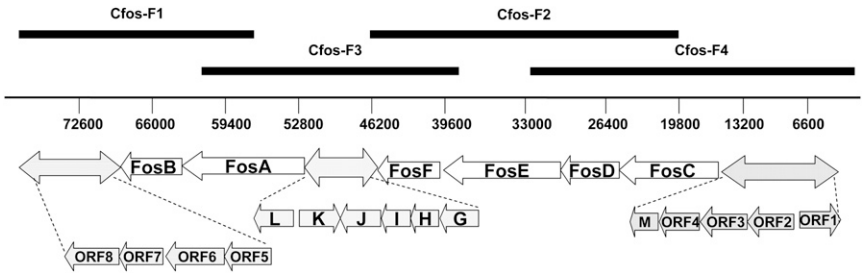

Figure 2. Distribution of Sequenced Cosmids and Organization of the Fostriecin Biosynthetic Gene Cluster

The sequenced 73 kb DNA region from *S. pulveraceus* ATCC 31906 as represented by the four overlapping cosmids Cfos-F1-F4. The PKS modules for the fostriecin locus at two positions, with six additional tailoring enzymes centralized in the PKS. The orientation of the arrows indicates the direction of transcription.

modification enzymes centralized in the PKS as in the phoslactomycin gene cluster.

Speculation as to the domain functions was based on the sequence homology to known PKS domains. As shown in Figure 3, a total of six large ORFs encode the core Fos-PKS, which contains a total of nine modules, including FosA, which has a loading module, and FosF, which has a thioesterase domain (TE). This organization is consistent with a role in catalyzing the last step of chain elongation and the subsequent release of the PKS chain. However, the formation of an unsaturated six-member lactone differs from many TE domains of Type I PKS that have been studied to date. Similar cases have only been demonstrated in phoslactomycin and leptomycin biosynthesis. The product of the last module, which contains a *cis*-$\Delta^{2,3}$-double bond, should consist of a keto reductase (KR) domain, followed by a dehydration (DH) domain. However, the DH domain is not found in the last module of fostriecin, phoslactomycin or leptomycin (Figure 3). Although Module 1 has a solo enoyl reductase (ER) domain, the conjugated triene property of fostriecin indicates that this ER is inactive. Otherwise, there should be a saturated bond between C16 and C17.

The fostriecin gene cluster also contains genes that encode the set of tailoring enzymes FosM, FosH, FosJ, and FosK as outlined in Figure 2. FosM (named ORF4 in previous research) (Palaniappan et al., 2008), was deduced to be a homologous NAD-dependent epimerase/dehydratase family protein with up to 42% sequence similarity to the phoslactomycin gene *plmT2*, which facilitates the formation of *cis*-$\Delta^{2,3}$-double bonds even if the targeting compound M-PLMB could transfer double bonds to PLM B without the function of PlmT2. FosH, which is homologous to PlmT5 as a homoserine kinase family member, is likely responsible for phosphorylation of the hydroxyl group at the C-9 position. FosG, FosJ, and FosK share sequence similarities to cytochrome P450 hydroxylase, and may contribute to the oxidation of C-4, C-8, and C-18. FosK is homologous to the PlmS2 in phoslactomycin, which is involved in a cyclohexyl-modifying hydroxylation at the C-18 position. There is no hydroxylation at the C-8 in phoslactomycin biosynthesis. The fostriecin gene cluster also contains other ORFs that remain to be elucidated.

Gene Disruption of *fosJ* and Isolation of Intermediates

Analysis of the fostriecin biosynthetic gene cluster and the chemical structure of fostriecin indicated that there are three steps of hydroxylation (at C-4, C-8, and C-18) after the polyketide chain is released that likely involve the *fosK*, *fosJ*, and *fosG* genes, which encode proteins highly homologous to the cytochrome P450 monooxygenases. To better understand fostriecin biosynthesis and characterize the enzyme functions, the *fosJ* gene was disrupted to block one of the hydroxylations in the biosynthesis.

The loss of the *fosJ* gene was achieved through double crossover using REDIRECT technology (Gust et al., 2003). The target gene, which was identified on the Cfos-F3 cosmid, was replaced by the *aac(3)IV/oriT* (red) cassette using PCR targeting strategies, yielding a mutated cosmid Cfos-F3-J. The mutated cosmid was introduced into *Escherichia coli* ET12567/pUZ8002 by electroporation and was subsequently transferred into the target strain by conjugation (Kieser et al., 2000). The desired double crossover mutants, which were selected by their apramycin-resistant and kanamycin-sensitive phenotype, was isolated as STQ0701 (Δ*fosJ*) (Figure S1A available online) and confirmed by PCR analysis (Figure S1B).

Using the fermentation and isolation method, the Δ*fosJ* mutant strain was fermented and processed along with the wild-type strain as a positive control. Following wild-type fermentation, fostriecin is the predominant product, accompanied by the major analog PD113,271 and the minor analog PD113,270, as expected (Figure 4A). However, fostriecin, along with PD113,270 and PD113,271, were completely abolished in the Δ*fosJ* mutant. Instead, a series of analogues (**1**–**5**) were isolated from the Δ*fosJ* mutant (STQ0701) (Figure 4A). The chemical structures of compounds **1**–**5** were characterized by high-resolution mass spectrometry (HR-MS) and nuclear magnetic resonance (NMR) analysis (Figure 4B), with the exception that the ^{13}C NMR analysis of compound **5** was not completed due to its chemical instability and the difficulty in isolating it (shown in Tables S3–S6) (Boger et al., 1997; Stampwala et al., 1983). The structure details are described in Text S1.

As shown in Figure 4B, all of the fostriecin intermediates (**1**–**5**) that were isolated from STQ0701 lack the C-8 hydroxyl group, indicating that FosJ may be responsible for C-8 hydroxylation. Compounds **1**, **2**, **4**, and **5** also lack hydroxyl groups at the C-4 and C-18 positions. Compound **3** has a hydroxyl group at both C-4 and C-18, respectively, and also has a phosphoryl group at C-9. This result suggests that FosJ must be active early in the pathway with relaxed substrate specificity and can be bypassed by enzymes for downstream modification steps during fostriecin biosynthesis.

Complementation of the STQ0701 mutant with an expression construct coding the *fosJ* gene on a pSET152 vector under the *ermE** promoter through recombinant conjugation resulted in a complemented strain, STQ0702 (Δ*fosJ* + *fosJ*). The resulting strain has restored production of fostriecin, PD113,270, and PD113,271 (Figure 4A) as in the wild-type, but it still accumulated a small amount of compound **3**. Compound **3**, the

Table 1. Deduced Functions of Open Reading Frames for Post-PKS in the Fostriecin Biosynthetic Gene Cluster

Gene	Size[a]	Putative Function	Closest Homolog	Identity/Similarity
fosG	409	cytochrome P450 monooxygenase	*S. lavendulae* ORF4	47/68
fosH	316	homoserine kinase	*S. HK803* PlmT5	44/62
fosI	424	ABC transporter	*S. HK803* PlmS4	51/66
fosJ	420	cytochrome P450 monooxygenase	*S. HK803* PlmT4	55/72
fosK	398	cytochrome P450 monooxygenase	*S. HK803* PlmS2	59/72
fosL	290	putative hydrolase	*S. HK803* PlmS3	50/66
fosM	313	NAD-dependent epimerase/dehydratase family protein	*S. HK803* PlmT2	42/56
orf1	284	methyltransferase	*S. violaceusniger* Tu4113	71/80
orf2	165	MarR-family transcriptional regulator	*Catenulispora acidiphila* DSM 44928	63/83
orf 3	213	metallo-β-lactamase superfamily protein	*S. hygroscopicus* ATCC53653	50/63
orf 4	958	LuxR-family transcriptional regulator	*Saccharopolyspora erythraea* NRRL 2338	32/44
orf 5	429	GH3-superfamily	*Planctomyces limnophilus* DSM 3776	28/46
orf 6	722	3-hydroxy-3-methylglutaryl coenzyme A reductase	*Photorhabdus luminescens subsp.* plu4507	38/57
orf 7	258	thioesterase	*S. avermitilis* AveG	51/67
orf 8	633	heat shock protein	*S. coelicolor* HtpG	80/89

[a]Number of amino acids.

major intermediate in the *fosJ* mutant, was expected as the post-PKS process persisted successfully in the absence of FosJ. Fostriecin production in STQ0702 was confirmed by coinjection with purified authentic standards and by mass spectrometric analysis. The results of the complementation experiment indicate that the five intermediates that were isolated from the *fosJ* mutant, as in a different stage of biosynthesis, are not the result of a polar effect of *fosJ* mutation. The lack of the C-8 hydroxyl group may impact downstream enzymes to some degree.

Gene Disruption of *fosH* and Isolation of Intermediate

Homology analysis showed that *fosH* shares 43% sequence identity with *plmT5,* which was derived from the phoslactomycin biosynthetic gene cluster. Therefore, we proposed that the *fosH* gene, which is located downstream of the *fosG* gene in the gene cluster, may be in charge of the phosphorylation of C9-OH in fostriecin biosynthesis. To investigate this hypothesis, a *fosH* deletion mutant was constructed using the REDIRECT technology as above. The target gene, *fosH*, which was identified on the Cfos-F3 cosmid, was replaced by the *aac(3)IV/oriT* (red) cassette using PCR targeting strategies and yielded a mutated cosmid, Cfos-F3-H. The mutated cosmid was introduced into *E. coli* ET12567/pUZ8002 by electroporation and was subsequently transferred into a fostriecin producer by conjugation (Kieser et al., 2000). The desired double crossover mutants, selected by their apramycin-resistant and kanamycin-sensitive phenotype, were isolated as STQ0703 (Δ*fosH*) (Figure S1A) and confirmed by PCR analysis (Figure S1C).

The Δ*fosH* mutant (STQ0703) was fermented and processed along with the wild-type strain as previously described. The production of fostriecin, along with PD113,270 and PD113,271, were also completely abolished in the Δ*fosH* mutant. However, an analogue (**6**), found in significant amounts, was isolated from the Δ*fosH* mutant. When the fermentation period was extended, a minor amount of compound (**7**) began to accumulate (Figure 4A). The chemical structures of compounds **6** and **7** were characterized by HR-MS and NMR analysis as shown in Figure 4B. Moreover, a 2D-NMR analysis of compound **6** was measured to establish its absolute configuration completely despite its chemical instability (Tables S3–S6). The structure details are described in Text S1.

The Δ*fosH* mutant predominantly produces compound **6,** suggesting that the *fosH* gene plays an essential role in phosphorylation in fostriecin biosynthesis and that the occurrence of C-9 phosphorylation has an obvious impact on the downstream tailoring enzymes. However, *fosH* lies downstream of *fosG* and upstream of *fosJ* in the gene cluster. The *fosK* gene lies in the opposite transcriptional direction near fosJ, as shown in Figure 2. Considering the existence of the C-8 hydroxyl group in compound **6**, FosH inactivation should not have a polar effect on its downstream enzymes.

Characterization of the Homoserine Kinase FosH In Vitro by Heterologous Expression in *E. coli*

To further investigate the function of FosH in the biosynthesis pathway, an enzyme activity assay was performed in vitro. A FosH heterologous expression system was constructed on pET28b and introduced into *E. coli* BL21 to produce the target protein. The expression construct was confirmed by DNA sequencing and the target protein (labeled as EH1) was purified as described in the Experimental Procedures and analyzed by SDS-PAGE analysis (Sambrook et al., 1989) (Figures S2 and S3).

As shown in Figure 5, compounds **1** and PD114,631 (Hokanson and French, 1985) could be converted to compounds **2** and fostriecin, respectively, by EH1 in vitro. This result supports the hypothesis that FosH is responsible for the phosphorylation of fostriecin and that its function can be processed whether hydroxylation at C-8 and C-18 occurred or not. Meanwhile, compound **1** can also be phosphorylated by EH1 to yield compound **2** up to 95% of the time under adequate reaction conditions. Combined with the isolation of compound **5** in Δ*fosJ* mutants, FosH could convert PD114,631, compound **1**

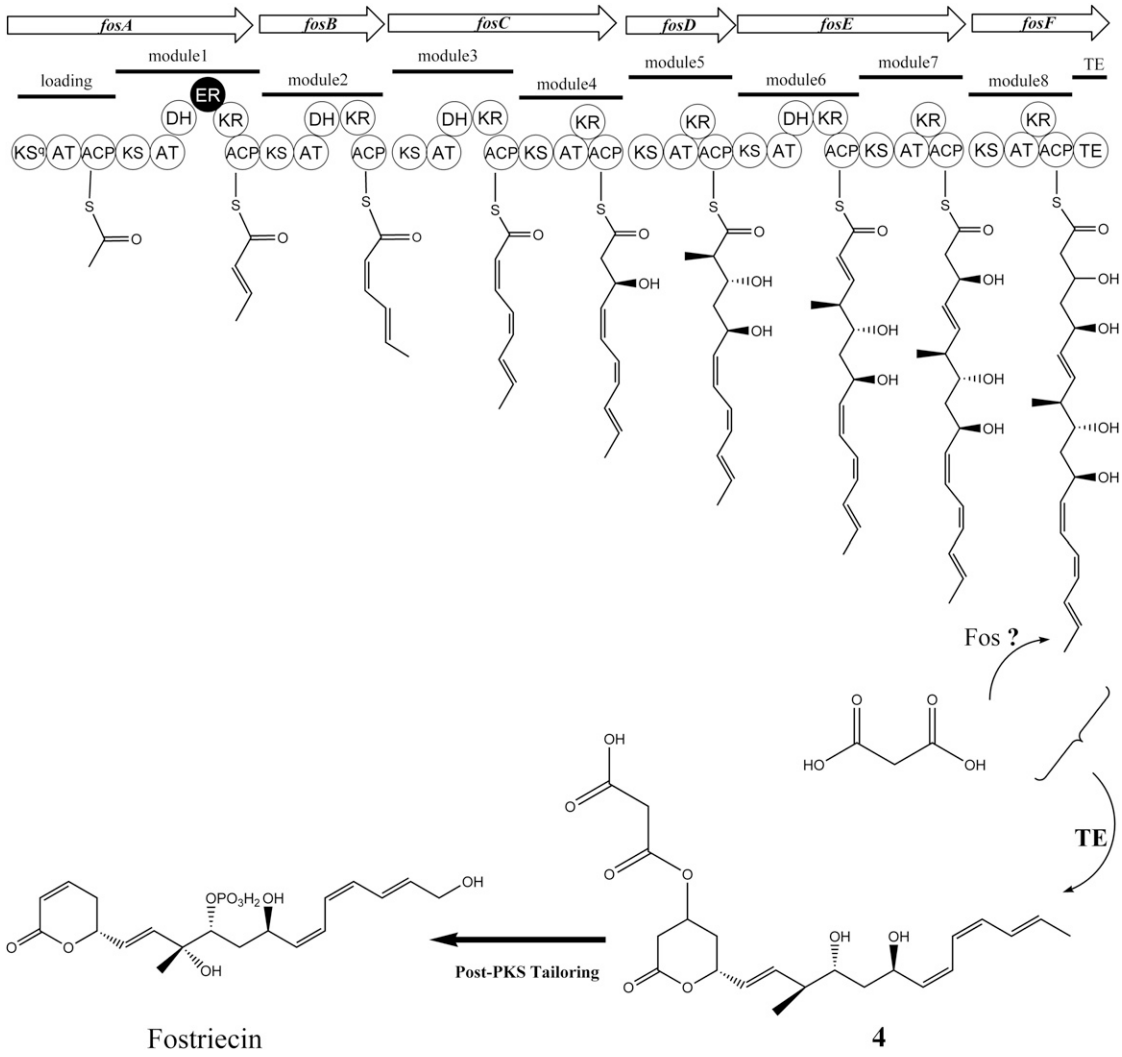

Figure 3. Proposed Fostriecin Polyketide Domain Organization and Biosynthetic Pathway in *Streptomyces pulveraceus*

Fostriecin polyketide biosynthesis uses a conventional PKS biosynthesis rule except that the ER domain in Module 1 is inactive. ACP, acyl carrier protein; AT, acyl transferase; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; KS, ketosynthase; TE, thioesterase.

and compound **4** into fostriecin, compound **2** and compound **5** in vitro or in vivo, indicating that FosH has a relaxed substrate specificity.

DISCUSSION

Fostriecin Biosynthesis Gene Cluster from *Streptomyces pulveraceus*

Inspired by the unusual biological activity and specific chemical structure (the *cis*-$\Delta^{2,3}$-double bond, phosphate ester, and conjugated triene), we developed a cloning strategy to isolate the fostriecin gene cluster for elucidating its biosynthesis mechanism. As summarized in Table 1, the fos gene cluster was found to consist of 21 ORFs encoding six modular Type I polyketide synthases (FosA-F) and seven tailoring enzymes, including three cytochrome P450 (FosG, FosJ, and FosK), one homoserine kinase (FosH), one transport protein (FosI), one putative hydrolase (FosL), and one NAD-dependent epimerase/dehydratase family protein (FosM). The fos gene cluster boundaries were precisely determined based on sequencing of the entire fos gene cluster. Different ORFs with varying functions may represent a possible fostriecin biosynthesis mechanism that has not been previously reported. This work provides a molecular basis for understanding the complex fostriecin biosynthetic mechanism and for generating various fostriecin analogs via combinatorial biosynthesis.

Functional Analysis of the Tailoring Enzymes FosJ and FosH in the Biosynthesis of Fostriecin

Inactivation of one of the tailoring enzymes, FosJ, not only results in the abolishment of the biosynthesis of fostriecin along with its natural analogs PD113,721 and PD113,720 but also allows the accumulation of five intermediates in the Δ*fosJ* mutant. All five intermediates lack a C-8 hydroxyl group. When the Δ*fosJ* mutant

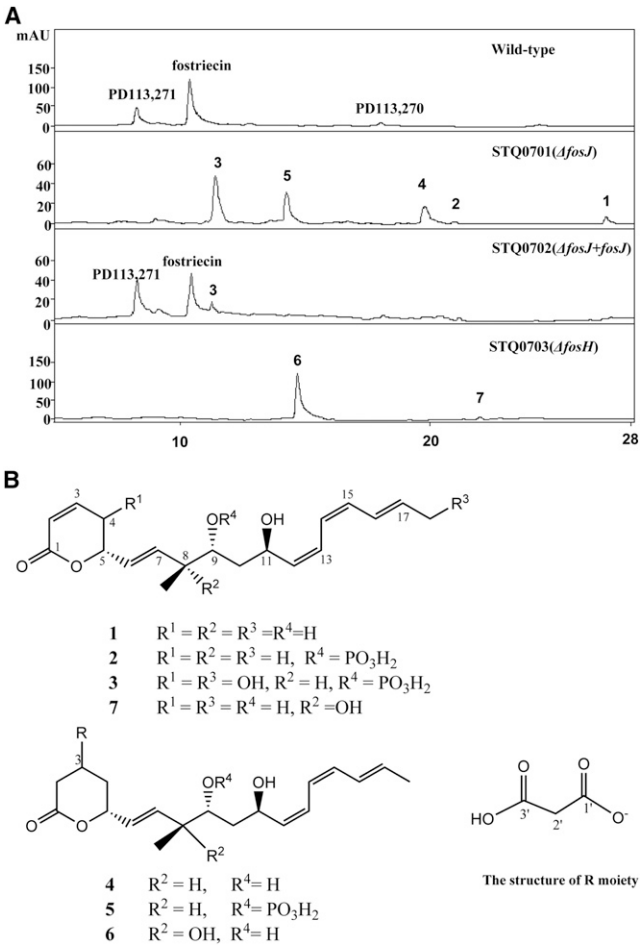

Figure 4. Disruption of the *fosJ and fosH* Genes and the Structure of Isolated Intermediates

(A) HPLC chromatograms of culture extract for Δ*fosJ* and Δ*fosH* gene disruption mutants, Δ*fosJ* complementation mutant, and wild-type strain. The mutant constructs are described in Figure S1 and Tables S1 and S2.

(B) The chemical structure of compounds **1**–**7** from Δ*fosJ* and Δ*fosH* mutant strains. NMR data are listed in Tables S3–S6, and the detailed structure elucidations are described in Text S1.

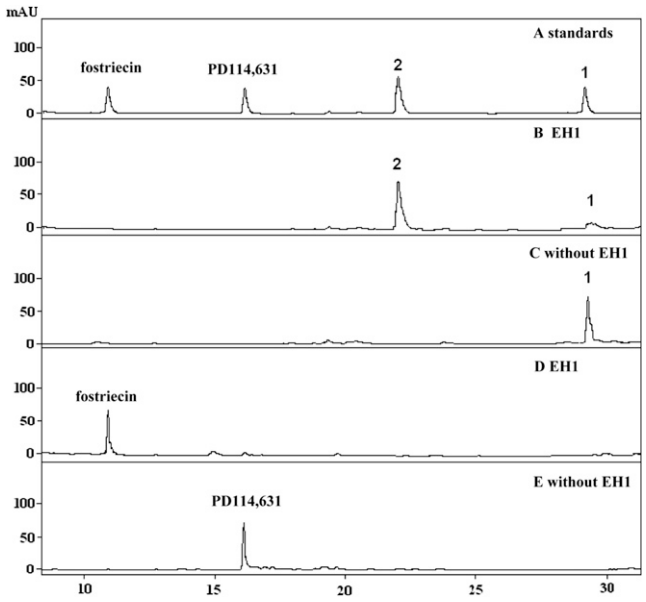

Figure 5. In Vitro Enzyme Activity Assay of Purified FosH Protein: EH1

(A) Four standard compounds in a coinjection.

(B) EH1 enzyme catalyzing reaction analysis of compound **1**.

(C) The control substrate reaction analysis of compound **1** without EH1 protein.

(D) EH1 enzyme catalyzing reaction analysis of PD114,631.

(E) The control substrate reaction analysis of PD114,631 without EH1 protein.

The construct and confirmation of FosH heterologous expression system are shown in Figures S2 and S3.

was reconstructed by introduction of the *fosJ* gene into the mutant strain (Δ*fosJ* + *fosJ*), the fostriecin production was restored. Together, these results confirmed that *fosJ* is responsible for C-8 oxidation in fostriecin biosynthesis. However, compound **3** still contains two hydroxyl groups at C-4 and C-18 and a phosphoryl group. This suggests that the loss of the C-8 hydroxyl group has little impact on the downstream post-PKS modification process.

FosH was demonstrated to be responsible for C-9 phosphorylation of fostriecin based on gene inactivation and an in vitro enzyme activity assay. The genetic loss of *fosH* in the wild-type producer completely blocked fostriecin production, but compound **6** accumulated as the major intermediate, indicating that FosJ is functions to perform hydroxylation at the C-8 in the Δ*fosH* mutant, suggesting that there is not a polar effect in the *fosH* disruption mutant. In other words, lack of phosphorylation at the C-9 could have a significant impact on the activities of downstream enzymes, but FosH-mediated phosphorylation in vitro may have relaxed specificity.

cis-$\Delta^{2,3}$-Double Bond Formation in Fostriecin Is from Malonylated Intermediates

The isolation and characterization of three malonylated compounds (**4**, **5**, and **6**) from Δ*fosJ* and Δ*fosH* mutants provides a further insight for *cis*-$\Delta^{2,3}$-double bond formation in fostriecin biosynthesis. General PKS chain elongation is terminated or cycled to form a lactone ring that is liberated by the TE enzyme and followed by a series of post-PKS modification steps in common polyketide biosynthesis. Because the predicted dehydratase (DH) domain in the last module is absent in fostriecin, phoslactomycin, and leptomycin, the polyketide chain of the six-membered lactone should contain a hydroxyl group at the C-3 site (Khosla et al., 1999). However, unexpectedly, the *cis*-$\Delta^{2,3}$-double bond in the unsaturated six-member lactone moiety is formed in fostriecin, phoslactomycin, and leptomycin biosynthesis, and several of the malonylated compounds are found in the fostriecin mutants (Δ*fosJ*, Δ*fosH*) and the phoslactomycin mutant (Δ*plmT2*), in which the C-3 hydroxyl group is esterified with malonic acid to create malonylated compounds (Palaniappan et al., 2008). The function of PlmT2 or FosM (named ORF4 in the phoslactomycin report) has been proposed following plasmid-based expression of *plmT2* or *fosM* that facilitates the eventual formation of the unsaturated lactone. Our data support the idea that similar dehydratase-independent pathways, which process the malonylation and

decarboxylative elimination, also function in fostriecin and possibly leptomycin formation, as well as in phoslactomycin biosynthesis, to generate the *cis*-$\Delta^{2,3}$-double bonds in the six-membered lactone moieties. To this point, isolation of malonylated compound **4** from the Δ*fosJ* mutant suggests that the malonylation process occurred at an early stage, most likely preceding the release of the full length polyketide intermediate from Fos-PKS (Figure 3).

Post-PKS Modification Pathway in Fostriecin Biosynthesis

Structurally, the natural fostriecin has four varying functionalities that are absent from the molecule generated from PKS, including two hydroxyl groups at the C-8 and C-18 positions, a phosphoryl group at the C-9 position and an unsaturated double bond at the C2-C3 site. After PKS elongation and liberation from PKS by the TE enzyme, the nascent PKS chain undergoes some or all of the normal postmodification steps that are typically associated with fostriecin natural product biosynthesis, such as hydroxylation, phosphorylation, and others.

A biosynthesis pathway featuring specific chemistry for unsaturated six-membered lactone has been proposed based on our findings in this study from the sequencing of the *fos* gene cluster, the deduced function of the ORFs within the *fos* gene cluster and the in vivo/in vitro analysis of selected genes by targeted gene inactivation, mutant complementation, and enzyme activity assays. This proposal is also based on the structural characterization of the isolated intermediates.

The isolation of compound **4** from the *fosJ* deletion mutant indicates that malonic acid is linked to the PKS chain at module eight during PKS elongation; most likely, it precedes the release of the full length polyketide intermediate from Fos-PKS. A series of malonylated compounds from the Δ*fosJ* and Δ*fosH* mutants also suggest that malonylation is an efficient process and occurs in the early stage, following the post-PKS modification steps in fostriecin biosynthesis and providing the primary basis for following the post-PKS modification pathway in fostriecin biosynthesis.

A rational post-PKS processing pathway is proposed. The malonylated compound **4**, liberated from PKS elongation by TE, was catalyzed by FosJ to form compound **6**. Then, fosH could catalyze the phosphorylation of compound **6** to create the potentially malonylated compound **8**. Compounds **8** and **9** were isolated from FosK and FosM mutants to support this proposal (unpublished data). The oxidation of C-18 by FosK may proceed before C-4 oxidation by FosG. We believe that C-4 oxidation must follow the unsaturated 6-membered lactone ring formation. Therefore, *cis*-$\Delta^{2,3}$-double bond formation should be the last step in post-PKS tailoring of fostriecin biosynthesis to yield fostriecin (Figure 6). Finally, FosG modification occurs on fostriecin to produce PD 113,271.

It is worth mentioning that FosJ could be bypassed in the Δ*fosJ* mutants, leading to the accumulation of compound **3**. The small amount of nonmalonylated compounds **1** and **2** that were isolated from the Δ*fosJ* mutant, and compound **7** from the Δ*fosH* mutant and PD113270 from the wild-type could be derived from the unstable decomposition of compounds **4**, **5**, **6**, and **8,** respectively.

SIGNIFICANCE

Fostriecin is a natural phosphate ester product with potent antitumor activity. The fostriecin biosynthetic gene cluster described here provides the molecular basis for understanding the complex fostriecin biosynthetic mechanism. Gene disruption of *fosJ* or *fosH* and an in vitro enzyme activity assay of heterologously expressed FosH establish that FosJ mediates oxidation at the C-8 position, whereas FosH drives phosphorylation at the C-9 position. The intermediates isolated from the mutants indicate that malonylated polyketide formation could be a normal biosynthetic process in the eventual formation of unsaturated six-membered lactones. The proposed pathway for fostriecin post-PKS modification sets the stage for understanding the mechanisms that build these structurally unique and pharmaceutically important agents and contributes to the general field of combinatorial biosynthesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Reagents

E. coli DH5α was used as the host for standard cloning experiments. *E. coli* ET12567/pUZ8002 was used as cosmid donor host for *E. coli-Streptomyces* conjugation. *E. coli* BW25113/pIJ773 was provided by John Innes Center (Norwich, UK) as part of the REDIRECT Technology kit. SuperCos1 (Stratagene) and *E. coli* DH5α were used to construct the *S. pulveraceus* genomic library. The *Streptomyces pulveraceus* ATCC 31906 was purchased from ATCC. *E. coli* BL21(DE3) and pET28b(+) (Novagen) was used for heterologous expression of *fosH* gene.

All reagents including biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were acquired from standard commercial sources. All growth media for *Streptomyces* and *E. coli* were prepared according to standard protocol.

DNA Isolation and Manipulation

DNA isolation and manipulation in *E. coli* and *Streptomyces* were carried out according to standard methods (Kieser, et al., 2000). PCR amplifications were carried out on a PCR machine (Bio-Rad) using Taq DNA polymerase. Primer synthesis and DNA sequencing were performed at TaKaRa (Dalian).

Construction of Genomic Library and Screening

Streptomyces sp. ATCC 31906 genomic DNA was partially digested with *Sau*3AI to yield a smear of ~30–40 kb, dephosphorylated with shrimp alkaline phosphatase, and ligated into SuperCos1, where it was pretreated with *Xba*I, dephosphorylated, and then digested with *BamH*I. The resulting ligation mixture was packaged using the Gigapack XIII (Stratagene) and transduced into *E. coli* DH5α to generate the genomic library. The transduced cells were spread onto LB plates containing ampicillin, and the plates were incubated at 37°C overnight.

Approximately 1,000 cosmids from the *E. coli* transductants were harvested from the above ligation mixture and were sequenced with two reactions using convergent primers T7cos (5′-CATAATACGACTCACTATAGGG) and T3cos-1 (5′-TTCCCCGAAAAGTGCCAC). After BLAST analysis, the sequences revealed 28 cosmids carrying DNA fragments that encoded Type I or Type II PKS genes at either end or both ends. Based on the relationships among these cosmids, four cosmids (Cfos-F1, Cfos-F2, Cfos-F3, and Cfos-F4), were selected to be investigated further for the fostriecin gene cluster.

DNA Sequencing and Analysis

The 73 kb regions from the four cosmids were sequenced using Big Dye Terminator chemistry (Applied Biosystems). The DNA was assembled with Sequencher 4.1 (Gene Codes), and the corresponding deduced protein sequences were analyzed with Mac Vector 6.5.3 (Accelrys) software and

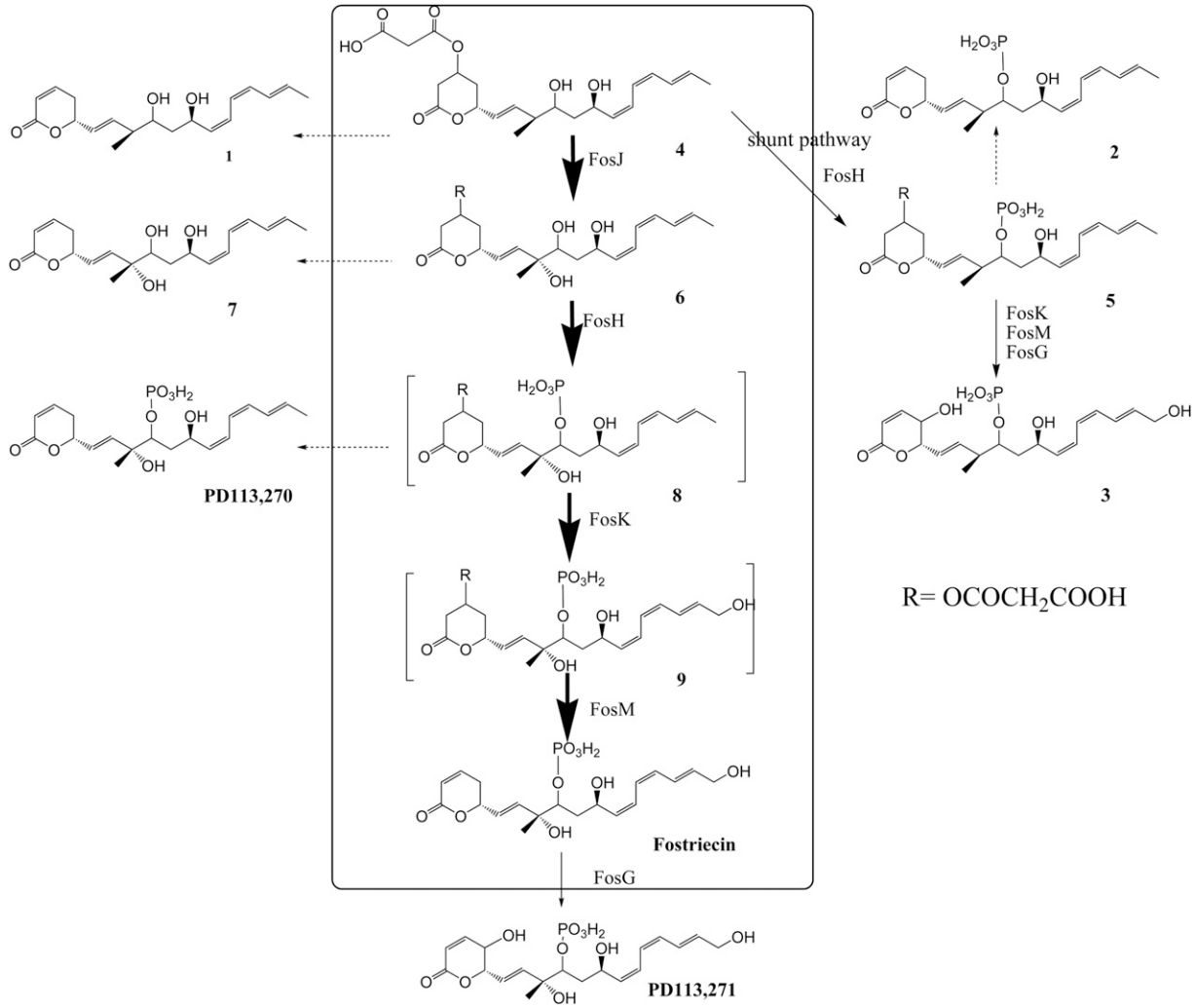

Figure 6. A Proposed Post-PKS Modification Pathway in the Biosynthesis of Fostriecin

The main post-PKS biosynthesis pathway is depicted in the box indicating by a solid arrow, then a shunt pathway occurs in the *fosJ* deletion mutant to produce compound **3,** as indicated with a simple arrow. Compounds **1**, **2**, and **7** and PD113,270 are derived from the decomposition of compounds **4**, **5**, **6**, and potentially compound **8**, respectively, as depicted with a dashed arrow. The isolation of compounds **8** and **9** was completed in the Δ*fosK* and Δ*fosM* mutants, respectively.

compared with sequences in the public databases using CLUSTAL W and BLAST programs available through the National Center for Biotechnology Information.

Gene Disruption of *fosJ* or *fosH* and *fosJ* Gene Complementation

The *fosJ and fosH* genes were disrupted using REDIRECT Technology according to the literature protocol (Gust et al., 2003). All primers for genetic manipulation were listed in Tables S1 and S2. Briefly, the apramycin gene *aac(3)IV/oriT* (red) cassette was used to replace an internal region of the target gene *fosJ* and *fosH*. The mutant cosmids Cfos-F3-J (Δ*fosJ*) and Cfos-F3-H (Δ*fosH*) were constructed and then introduced into *Streptomyces pulveraceus* by conjugation from *E. coli* ET12567/pUZ8002 according to the literature procedure. *Streptomyces* spores were suspended in 2× YT medium and heat shocked for 10 min at 50°C. Germinated spores were mixed with *E. coli* ET12567/pUZ8002 harboring pSET152 with different donor/recipient ratios from 1:1 to 10:1 and spread onto freshly modified MS plates. The plates were incubated at 30°C for 16–18 hr and then overlaid with 1 ml of water containing apramycin and nalidixic acid at respective final concentrations of 25 μg/ml. The plates were incubated further at 30°C for 72 hr, and the exconjugants appeared. The desired double crossover mutants, selected by their apramycin-resistant and kanamycin-sensitive phenotype, were isolated as STQ0701 (Δ*fosJ*) and STQ0703 (Δ*fosH*). The genotypes were confirmed by PCR analysis (Figure S1).

To construct expression plasmids for the genetic complementation experiment, the *fosJ* gene was amplified with the forward primer: 5′-GGACTAGTATGACCTCGACCGACGCCGT-3′, and the reverse primer: 5′-CGGAATTCTCACCAGGTGACGGGCAG-3′. The products were digested by *Spe*I and *EcoR*I and cloned into pSET152 to generate pSET152-J. It was introduced into the *fosJ* mutant strain STQ0701 by conjugation to yield the complemented strain STQ0702 in which the Δ*fosJ* mutation was complemented by the constitutive expression of functional copies of *fosJ* under the control of the *ermE** promoter.

Heterologous Expression and FosH In Vitro Activity Assay

A 1,084 bp DNA fragment was amplified from Cfos-F3 with 5′-AACATATGCCGGAATCGACCGACG-3′ and 5′-CGGAATTCTCAGAAAACCTGGAAACGGG-3′

primers. It was then ligated into the pET28b (+) vector and transformed into DH5α to generate pET28b-EH1 vectors. The resulting plasmid was confirmed by electrophoresis analysis as reported in Figure S3. It was then transformed into *E. coli* BL21 (DE3), yielding the BL21-EH1 strain. The overproduction and purification of EH1 were performed using the following protocol. The *E. coli* strain BL21-EH1 was cultivated with shaking at 28°C in LB medium containing 50 mg/ml kanamycin and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when the culture reached an OD600 of 0.5. After induction, incubation was continued for 5 hr. The cells were then harvested by centrifugation and suspended in 0.1 M PBS buffer. After suspension, the cells were lysed by lysozyme with a terminal concentration of 1 mg/ml and ultrasonic disruption. Cellular debris was removed from the lysate by centrifugation, and the protein was obtained. The EH1-His-tag protein was purified by affinity chromatography using Ni-NTA Superflow resin (GE). Fractions containing the desired protein were pooled and dialyzed using a Centrifugal Filter Device. All of the protein purification procedures were carried out at 4°C. The final cell pellet and the soluble and insoluble fractions were analyzed via SDS-PAGE to confirm the presence of the proteins as reported in Figure S4. The activity of EH1 was assayed in a mixture containing 0.5 mM dephosphated fostriecin analogs, 1 mM ATP, 5 mM $MgCl_2$, and FosH protein (EH1) in 75 mM Tris-HCl, pH 8.3. The catalysis process was sustained for 24 hr and monitored at different time points by high-performance liquid chromatography (HPLC).

In this study, compound **1** and dephosphoryl-fostriecin (PD114, 631) were used to determine FosH function. PD114,631 is prepared by the chemical method of dephosphorylating fostriecin according to a previously reported method (Hokanson and French, 1985).

Production, Purification, and Analysis of Fostriecin and Its Analogues from Wild-type and Mutant Strains

The *S. pulveraceus of* wild-type strain, Δ*fosJ* mutant strain (STQ0701), Δ*fosJ* mutant complementation strain (STQ0702, Δ*fosJ* + *fosJ*), and Δ*fosH* mutant strain (STQ0703) were fermented under the following conditions. The spore suspension was first inoculated into 30 ml of YPD medium in a 250 ml flask and incubated at 28°C and 220 rpm for 24 hr. The resulting seed culture was inoculated into fermentation medium (consists of 5% glycerol, 0.4% baker's yeast, 0.5% meat extract, 0.1% NaCl, 0.25% $CaCO_3$, 0.25% K_2HPO_4, pH 7.0) incubated at 28°C and 220 rpm for another 4 days for production. The fermentation broth was then centrifuged to remove the mycelia and the supernatant was loaded on the HP20SS equilibrated with 0.05 M phosphate buffer (pH 6.8), then the column was eluted with methanol, the fractions containing fostriecin or its analogs were collected, and evaporated to generate an oily residue. The oily residue was dissolved in CH_3OH and diluted to 10% methanol concentration with 0.1 M phosphate buffer (pH 6.8), then loaded on C18 chromatography with the methanol-buffer as mobile phase. A stepped gradient elution of methanol-buffer (10%, 20%, 30%, 40%, 50%, 60% methanol in buffer) was used to elute the column. All fractions containing fostriecin or its analogs were collected and concentrated in vacuum to produce an oily residue individually.

For compound purification, the samples were subjected to final purification by semipreparative chromatography (ZORBAX SB-C1 8,250 mm × 9.4 mm, 5 μm) to give pure objective compounds as follows: fostriecin, PD113,271, and PD 113,270 were from wild-type fermentation; compounds **1–5** from STQ0701, and compounds **6** and **7** from STQ0703.

All samples were subjected to HPLC analysis with a DAD detector (Agilent 1200). A gradient elution from 10% to 60% methanol over 25 min, followed a gradient from 60% to 90% methanol in 5 min was developed with 1 ml/min elution speed at 267 nm wavelength.

Structure Determination

The UV spectrums of the purified analogs were determined in an acetonitrile solution with DAD detector by Agilent 1200 HPLC. For NMR analysis, compounds were dried and dissolved in CD_3OD. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II400 spectrometer operating at 400 MHz and 100.61 MHz, respectively. The molecular mass was determined with Q-TOF-HRMS or API-ES-MS spectrometer (HP1100MSD).

The ^1H and ^{13}C NMR assignments of **1–7** were listed in Tables S3–S6, the detailed structure elucidation is provided in Text S1.

ACCESSION NUMBERS

The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number HQ434551.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, six tables, and text and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.10.018.

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