Fusarin C Biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*

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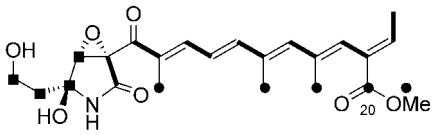
Fragments of polyketide synthase (PKS) genes were amplified from complementary DNA (cDNA) of the fusarin C producing filamentous fungi Fusarium moniliforme *and* Fusarium venenatum *by using degenerate oligonucleotides designed to select for fungal PKS* C*-methyltransferase (*CMeT*) domains. The PCR products, which were highly homologous to fragments of known fungal PKS CMeT domains, were used to probe cDNA and genomic DNA (gDNA) libraries of* F. moniliforme *and* F. venenatum. *A 4.0 kb cDNA clone from* F. venenatum *was isolated and used to prepare a hygromycin-resistance knockout cassette, which was used to produce a fusarin-deficient strain of* F. venenatum *(kb = 1000 bp). Similarly, a 26 kb genomic fragment, isolated on two overlapping clones from* F. moniliforme*, encoded a complete iterative Type I PKS fused to an unusual nonribosomal peptide synthase module. Once again, targeted gene disruption produced a fusarin-deficient strain, thereby proving that this synthase is responsible for the first steps of fusarin biosynthesis.*

Introduction

Polyketides are compounds that occur in a wide range of organisms including bacteria, fungi and plants.[1] These compounds often possess desirable pharmaceutical or agrochemical properties and are key to modern medical advances.[2] Much of the progress in understanding and exploiting the biosynthesis of polyketides has occurred through the study of bacterial polyketide synthases (PKSs).[3] In bacteria, three main types of PKS have been discovered. These are classified as Type I (multifunctional, often very large, multienzymes), Type II (complexes of monofunctional discrete enzymes) and Type III (simple monofunctional systems).[3] The bacterial Type I PKSs have been exploited for the directed synthesis of new compounds because the *modular* arrangement of their component enzyme domains makes their manipulation at a genetic level relatively simple. Each module contains all the active sites required for a particular round of chain extension.

In fungi, the PKSs discovered to date are Type I systems. However, they differ from the very large bacterial modular PKSs in that the fungal systems are *iterative*, that is, a single module acts repetitively. Other differences include the way in which chain branches are introduced. In bacteria, the Type I PKSs normally use branched extender units (for example, methylmalonate), whereas fungi introduce branching methyl groups by using a *C*-methyltransferase (*C*MeT) domain and methyl groups from *S*-adenosylmethionine (SAM). In the past decade many Type I modular PKSs from bacteria have been discovered, but reports of the cloning of fungal PKS genes remain rare.

We have focussed our efforts in this area on developing a series of oligonucleotide PCR primers that are selective for fungal PKS genes.[4] These can be used to rapidly obtain specific clones of fungal PKS genes. We have already demonstrated the utility of primers based on the β-ketoacyl synthase (KAS) domain of fungal PKSs.[4] However, we have also developed primers based on the unique fungal PKS *C*MeT domains and here we describe their use in cloning biosynthetic genes involved in the biosynthesis of fusarin C (**1**; Scheme 1).



Fusarin C **1**

— Intact acetate
■ acetate, via Krebs cycle
● S-adenosylmethionine

Scheme 1. *Structure of Fusarin C and biosynthetic origin of the carbon atoms.*[6]

Fusarins have been isolated from a number of fungal species including *Fusarium moniliforme* (= *F. verticillioides*, teleomorph *Gibberella fujikuroi* = *G. moniliformis*)[5] and *Fusarium venenatum* (= *F. graminearum*, teleomorph *Gibberella zeae*).[6] These compounds are potent mycotoxins. Their biosynthesis has been studied by using classical feeding experiments with ^{13}C-labelled acetates (Scheme 1).[7] These experiments showed that seven intact acetates make the polyunsaturated side chain and two of the pyrrolidin-2-one carbon atoms. The other four carbon atoms were also labelled by acetate, but label scram-

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bling and a low level of incorporation suggested that these carbon atoms were derived from a Krebs cycle intermediate, possibly aspartate, oxaloacetate or their derivatives. The four side-chain carbon atoms are derived from SAM, as is the methoxy group in the methyl ester.

Results

Complementary DNA (cDNA) was prepared from *F. moniliforme* and *F. venenatum* mRNA. We prepared cDNA from mRNA obtained during the maximum rate of production of fusarins because most filamentous fungi possess multiple PKS genes (Figure 1). This strategy was designed to ensure the presence of fusarin biosynthetic mRNA while potentially reducing the number of possible competing sequences from non-fusarin biosynthetic genes. The cDNA from the two organisms was used as a template for PCR reactions with a pair of degenerate *C*MeT primers. These primers were designed to amplify fragments of fungal *C*MeT domains of approximately 320 bp (Figure 2). In both cases the PCR products were cloned and sequenced.

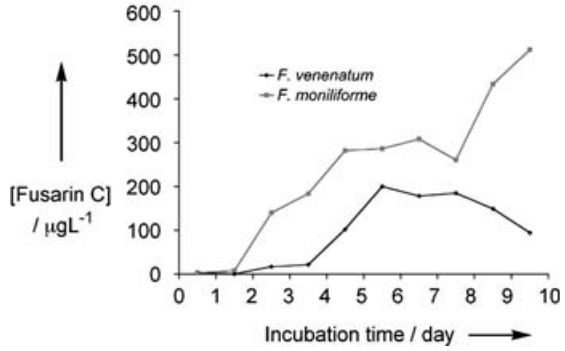


Figure 1. *Time-course of production of fusarin C by* F. moniliforme *and* F. venenatum. *Fusarin C production was monitored by LC-MS UV peak integration.*

Sequencing showed that some of the PCR products were closely related to fragments of other known PKS *C*MeT domains, such as those involved in lovastatin (**2**) biosynthesis in *Aspergillus terreus*[8] and those which we have previously cloned from a variety of fungi.[4] As expected, the products from the two organisms were also very similar to each other; they showed 59% nucleotide identity and 43% translated amino acid identity. These fragments were therefore deemed to be suitable probes for screening genomic DNA (gDNA) and cDNA libraries.

A cDNA λ library was constructed from *F. venenatum* mRNA isolated during fusarin production. This library was blotted onto Zeta-Probe membranes and the membranes were probed with the radiolabelled PCR products. Plaques which hybridised with the probes were purified and the DNA inserts were excised and sequenced. We thus isolated a 4.0 kb cDNA clone from *F. venenatum* which showed high homology to other highly reduced fungal PKSs, such as those responsible for lovastatin biosynthesis in *A. terreus* (kb = 1000 bp).[8]

The 4.0 kb *F. venenatum* PKS fragment, designated *venpks*1, was then used to probe both cDNA and gDNA libraries from *F. moniliforme*. A 2.5 kb sequence (*monpks*1) was obtained from the cDNA library and a 17 kb sequence (*monpks*2) was isolated from the gDNA library. These two sequences, while similar, did not encode the same PKS. Sequence comparison showed that the *monpks*2 genomic sequence was highly homologous to the *venpks*1 cDNA sequence, while the *monpks*1 cDNA clone was of lower homology.

In order to determine whether any of the three isolated PKS gene fragments was responsible for fusarin biosynthesis, we constructed knockout plasmids in which the hygromycin B resistance cassette, *hph* from the plasmid pAN7–1,[9] was flanked by or linked to fragments of each of the three PKS genes.

Transformation of *F. venenatum* with the *venpks*1–*hph* plasmid and selection on hygromycin B led to the isolation of eight hygromycin-resistant colonies. Each of these was grown

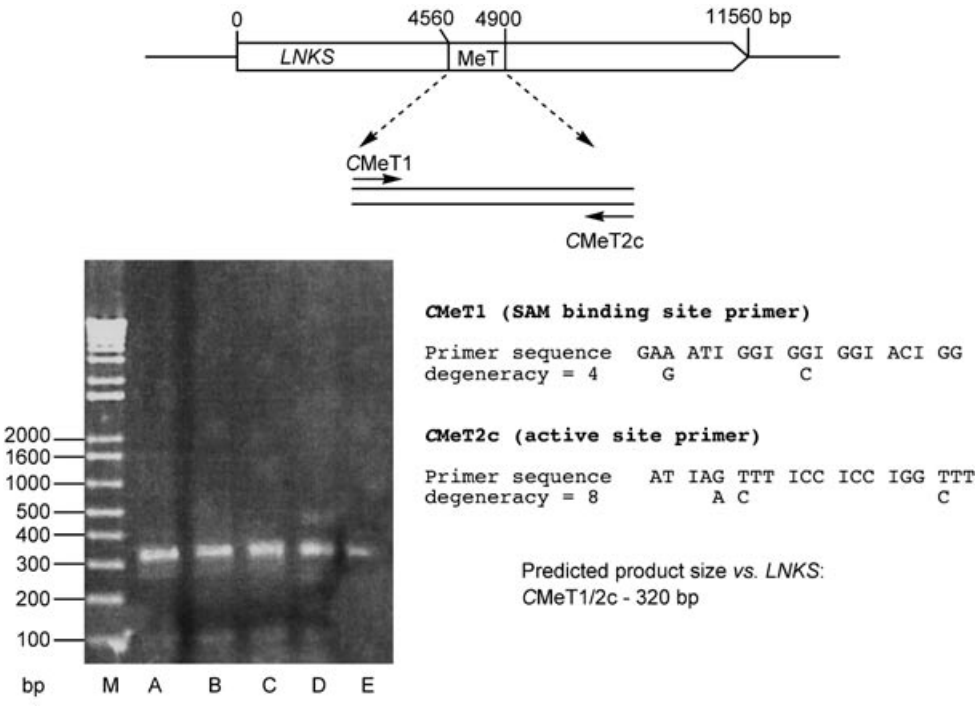


Figure 2. *PCR amplification of PKS CMeT domains from* F. venenatum *and* F. moniliforme. *Degenerate PCR primers, made with a combination of mixed bases and inosines (I) as shown, were based on conserved PKS CMeT domains. The cDNA templates were obtained during fusarin production. Lanes A–C:* F. venenatum *(at days 4, 5 and 6 of culture, respectively); lane D:* F. moniliforme *(at day 3); lane E: positive control (LNKS amplified from* A. terreus*).*

in production medium and then extracted and examined for fusarin production by LC-MS (Table 1). Of the eight strains tested, five showed no fusarin production, while the other three showed much reduced production (Table 1). In parallel, the total gDNA from each clone was fully digested with *Bgl*II, and Southern blotting showed that *hph* had inserted at the homologous position in all the clones (Figure 3). Four modes of *hph* insertion are possible when using circular plasmids with the selectable marker flanked by homologous sequences (Figure 3B): simple replacement (R) by double crossover, insertion at the 5′ end (I-5) by single crossover, insertion at the 3′ end (I-3) by single crossover, and insertion at both ends (I-B, not shown in Figure 3B). All four modes of insertional knockout were observed in the Southern blot experiment (Figure 3C). For example, the autoradiographs show that strains 6–8 now have gDNA that does not hybridise with the dPKS probe but which does hybridise with the HYG probe; this indicates replacement by double crossover (R). Strains 3–5 have undergone single crossover at the 3′ site (I-3), strain 2 has undergone

Table 1. *Analysis of Fusarin C production by wild-type (WT) and knockout clones of* F. venenatum. *Mutation type refers to the different modes of* hph *insertion shown in Figure 3. The HPLC detection limit is 3.0 $\mu g L^{-1}$. Clones 2 and 4 are not significantly above this. Clone 7 did show a weak HPLC peak corresponding to fusarin C but fusarin C was not detected by MS in this case.*

Clone	1	2	3	4	5	6	7	8	WT
fusarin C [$\mu g L^{-1}$]	<3	10.5	<3	4.8	<3	<3	34.8	<3	533
mutation type	I-B	I-5	I-3	I-3	I-3	R	R	R	–

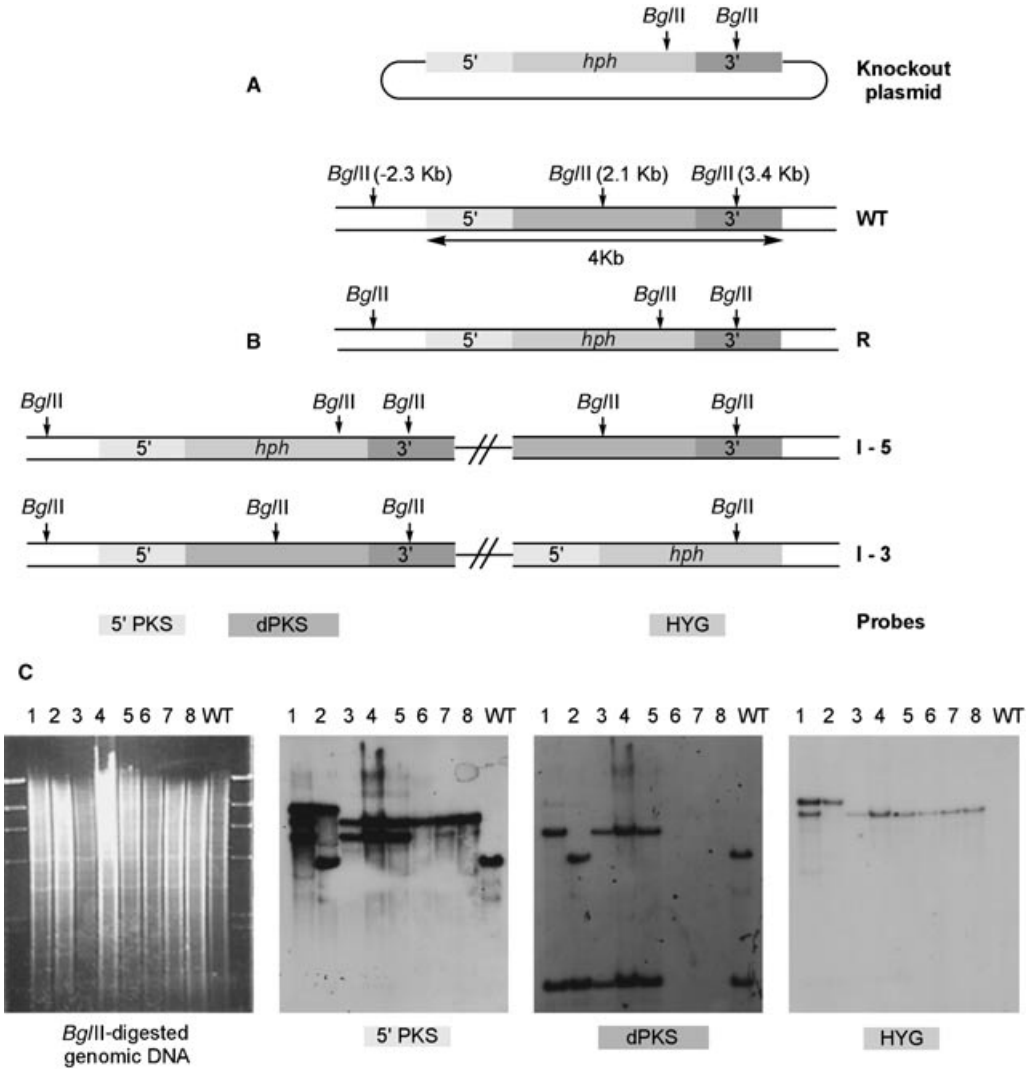


Figure 3. *Fusarin C gene knockout in* F. venenatum. *A) The* venpks*1*–hph *knockout plasmid constructed by flanking the hygromycin-resistance cassette (*hph*) with approximately 1 kb 5′ and 3′ PKS fragments from the* F. venenatum *cDNA-derived* venpks*1. B) Four modes of insertional inactivation result from homologous recombination of* venpks*1*–hph *into the genome of wild-type (WT)* F. venenatum*: replacement by double crossover (R); single crossover at the 5′ fragment (I-5); single crossover at the 3′ fragment (I-3); and dual single crossovers in both the 5′ and 3′ fragments (I-B, not shown). C) Southern blot analysis of hygromycin-resistant transformants. Lanes 1–8: gDNA digested with* Bgl*II; flanking lanes contain size markers (λ DNA digested with* Hind*III: (from top) 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 kb). Panels from left: ethidium bromide stained gel; autoradiographs of membrane probed with 5′ PKS fragment, central PKS fragment and* hph. *The membrane was stripped between hybridisations.*

crossover at the 5′ site (I-5), and strain 1 has undergone dual crossover (I-B). As expected, the wild-type (WT) strain gave gDNA that did not hybridise to the HYG probe but gave two *Bgl*II fragments that hybridised to the dPKS probe and one *Bgl*II fragment that hybridised to the 5′ PKS probe.

Transformation of *F. moniliforme* with the knockout plasmid (*monpks*2–*hph*) constructed from the *F. moniliforme* gDNA library fragment similarly resulted in the isolation of strains that were incapable of fusarin production. On the other hand, *monpks*1–*hph* disruption of the gene corresponding to the *F. moniliforme* cDNA clone did not lead to a reduction in fusarin production. In both cases homologous recombination was confirmed by Southern blot analysis of *Hin*dIII-digested genomic DNA (not shown).

In order to obtain further sequence data, PCR was used to amplify an 800 bp fragment from the 3′ end of *monpks*2, and this fragment was then labelled and used to probe the *F. moniliforme* gDNA library. A new clone was isolated which overlapped and extended the 3′ end of the original cloned DNA, to give a total of 26 kb of continuous sequence (Figure 4).

Detailed sequence analysis based on homology to known fungal sequences, searches for conserved sequence motifs and the discovery of frame-shifts in potential open reading frames (ORFs) allowed us to identify five possible introns in the 26 kb gDNA sequence. This work was aided by comparison with introns discovered in the *F. moniliforme* trichothecene biosynthetic gene cluster.[10] In all five cases, the 5′ end of the intron contained the conserved splice sequence GTXXG, while the conserved sequence at the 3′ end was AG.

Comparison of the *F. venenatum* cDNA clone and the *F. moniliforme* gDNA clone also identified a highly unusual 546 bp intron in the *F. moniliforme* sequence. This observation was confirmed by amplifying the corresponding region from *F. moniliforme* cDNA and sequencing the PCR product. The 5′ splice site (GTGTA) of this intron does not match those of the other introns identified here. Intriguingly, *within* this long intron sequence lies one of the previously discovered introns which does show the conserved 5′ and 3′ splice sequences. Sequence analysis of the 546 bp intron showed it to be homologous to enoyl reductase genes (see below).

After removal of the introns from the gDNA sequence, we identified six putative ORFs (Figure 4). At the 5′ end there is a 1316 bp ORF (ORF1) corresponding with no known function. 1.7 kb downstream from this is a 1.26 kb ORF (ORF2) which shows highest homology to known hydrolases. Another 940 bp downstream lies an 11.9 kb ORF (ORF3), which we name *fus*A.

Sequence analysis of *fusA* showed the presence of β-ketoacylsynthase (KAS), acyl transferase (AT), dehydratase (DH) and *C*MeT domains. Downstream from the *C*MeT domain are two regions of sequence showing moderate homology to enoyl reductase (ER) domains. One of these regions corresponds to the 546 bp intron and is thus excised from the mature *fusA* mRNA. The second ER region shows low homology to known ER domains, and the observation that the conserved reduced nicotinamide adenine dinucleotide phosphate (NADPH) binding motif (LIHXXXGGXG) shows several significant mutations (that is, LAISGVNRSK) suggests that the ER is likely to be inactive. The next domain in *fusA* is a keto-reductase (KR) and this is followed by the acyl carrier protein (ACP).

Downstream of the PKS domains, and part of the same ORF, further modules are present which show high homology to catalytic domains from fungal and bacterial nonribosomal peptide synthases (NRPSs). Clear condensation (C), adenylation (A) and thiolation (peptidyl carrier protein, PCP) domains are present. The final domain corresponds to a thiolester reductase (R).

At the 3′ end of the cloned genomic segment, and 1.7 kb downstream of *fusA*, is a putative 848 bp ORF (ORF4) with no obvious function. Further beyond this lies a 1.4 kb ORF (ORF5) with high homology to known monocarboxylate transporter genes. The final ORF (ORF6) is a 1.8 kb 5′ fragment of a multidrug resistance gene, a so-called ABC transporter system.[11]

Discussion

Fusarin C (**1**) is one of a class of acyl-tetramic acids which are found in slime moulds, terrestrial fungi, marine fungi and marine sponges. The class includes compounds such as fuligorubin (**3**) from a slime mould,[12] zopfiellamide A (**4**) from a marine fungus,[13] and pramanicin (**5**) from a terrestrial

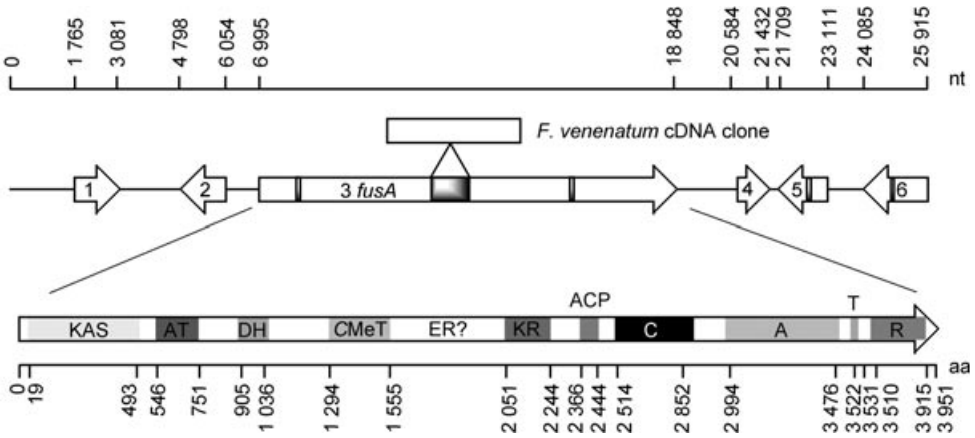
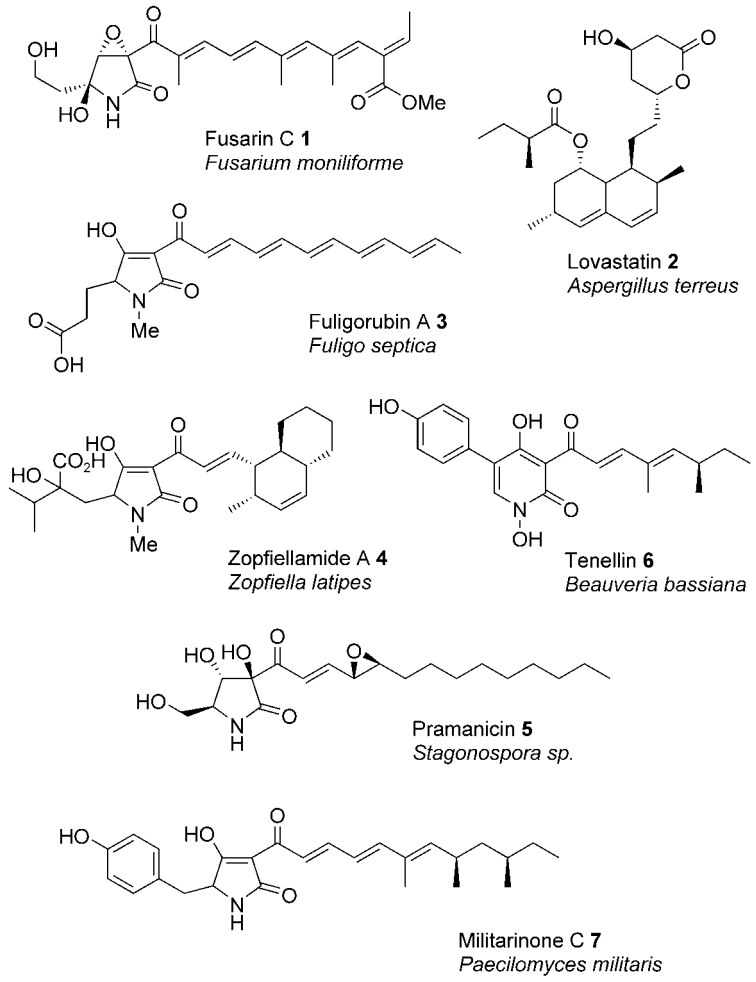


Figure 4. *Structure and location of the* fusA *gene. Approximate domain boundaries, nearby ORFs, approximate position of introns (grey bars) and alignment between* F. moniliforme *26 kb gDNA and* F. venenatum *4 kb cDNA clones are shown.*



fungus.[14] 2-Pyridones, such as tenellin (**6**) from an entomophagogenic fungus,[15] are also related because evidence suggests that they derive by ring expansion of tetramic acids such as the known militarinone C (**7**) from *Paecilomyces militaris*.[16]

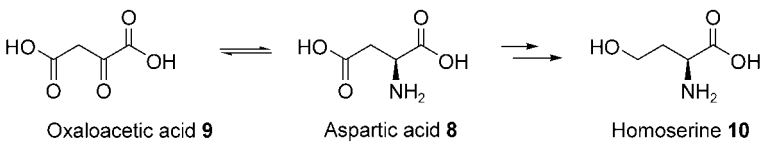
Feeding experiments with labelled acetate and amino acids have shown that pramanicin (**5**) is derived by condensation of an octaketide with L-serine.[14] Experimental results are limited, but other acyl tetramic acids also appear to be derived by fusion of polyketide and amino acid precursors.[17,18] Feeding experiments have shown that the pyrrolidone ring of fusarin C (**1**) is derived in a similar way—condensation between a polyketide and a Krebs cycle, or related, intermediate. In this case a likely candidate would be either aspartic acid (**8**, derived in one step from oxaloacetate (**9**), Scheme 2) or homoserine (**10**), itself derived by reduction of aspartate (**8**). Thus, while the biosynthetic studies involving isotopic feeding experiments have divulged the origin of the carbon and nitrogen atoms, the mechanism for ring formation has remained elusive.

We have shown that the 11.9 kb *fusA* gene in *F. moniliforme* and a homologous gene in *F. venenatum* are responsible for the early stages of fusarin biosynthesis. Fusarin synthase (FUSS), encoded by *fusA*, consists of a Type I iterative PKS fused to an NRPS. It appears likely that the iterative Type I PKS generates a tetramethylated polyunsaturated heptaketide **11** as its end product (Scheme 3). This is consistent with the active sites present in the PKS portion of FUSS. The likely inactivity of ER, supported by sequence analysis, corresponds with the polyene structure. This situation is similar to that found for the lovastatin (**2**) nonaketide synthase (LNKS). In the absence of an accessory protein (lovC), LNKS produces a monomethylated polyunsaturated *heptaketide*, which is very similar to the heptaketide proposed here as an intermediate in fusarin biosynthesis (and which is a likely unmethylated intermediate in fuligorubin A (**3**) biosynthesis). Similarly to FUSS, LNKS is proposed to possess an inactive ER domain: interaction with lovC somehow confers the ability of LNKS to regioselectively perform ER reactions and to extend the growing polyketide to a nonaketide. Interestingly LNKS also possesses a condensation domain in the same position as FUSS, but no other NRPS domains are present in LNKS.[19] This raises an intriguing question: could LNKS once have possessed a full NRPS module and been responsible for the biosynthesis of compounds similar to zopfiellamide A (**4**)?

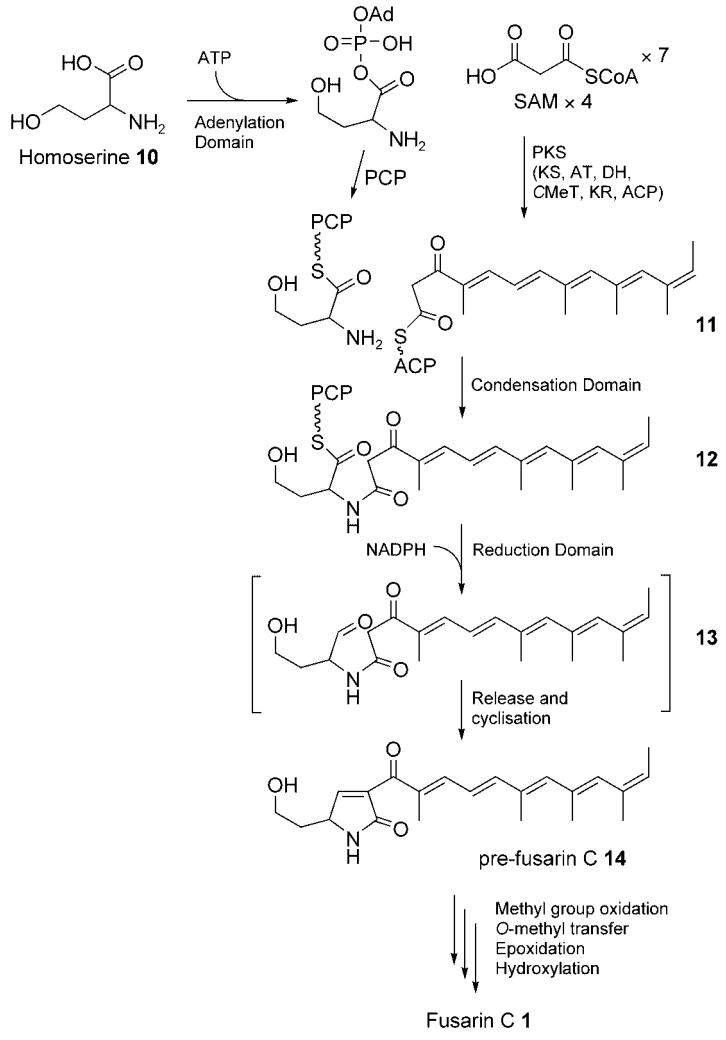
We propose that the fully elaborated polyketide is held by the ACP domain of the FUSS PKS as a thiolester. Homoserine (**10**) is then adenylated and transferred to the PCP where it is attached as another thiolester. At this stage the condensation domain catalyses nucleophilic attack by the homoserine nitrogen atom on the ACP-bound polyketide, thereby forming an intermediate amide **12**, and the polyketide is formally transferred to the NRPS portion of FUSS.

Sequence analysis and examination of crystal structures of NRPS adenylation domains, by Marahiel and Townsend (among others) with their respective co-workers,[20,21] has led to a model of amino acid selection by the adenylation domain. Although homoserine (**10**) has not, so far, been recognised as a substrate for other known adenylation domains, we applied this model to the adenylation domain from FUSS to look for any correlations that would be consistent with homoserine (**10**), or aspartic acid (**8**), as the likely substrate. However, low sequence homology between this fungal NRPS domain and the bacterial domains examined by others precluded any firm conclusions.

The final biosynthetic step involves reduction of the PCP-bound thiolester by the NRPS reductive domain. Similar domains are known, for example, in fungal lysine biosynthesis, where α-aminoadipate re-



Scheme 2. *Biosynthetic interconversion of the Krebs cycle intermediate oxaloacetate (**9**) with aspartic acid (**8**) and homoserine (**10**).*



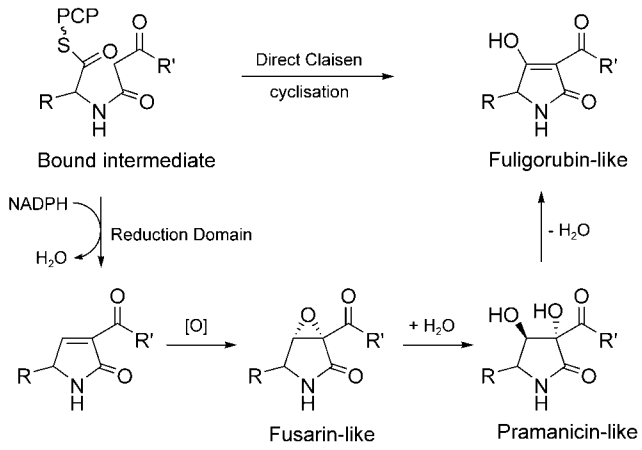
Scheme 3. *Proposed biosynthesis of pre-fusarin C (**14**). ATP = adenosine 5′-triphosphate, Ad = adenosine, CoA = coenzyme A, KS = β-keto-acyl synthase.*

ductase utilises an adenylation/reduction strategy to provide α-aminoadipate semialdehyde.[22,23] The reaction is also reminiscent of that catalysed by aspartate semialdehyde dehydrogenase in bacteria, where aspartyl phosphate is the substrate for transthiolesterification and reduction.[24] Similar reductase domains in the myxochelin synthase catalyse two reductions to yield an alcohol.[25] In this case, the outcome of the reduction would be a free aldehyde, **13**, and such compounds are chemically susceptible to intramolecular aldol reactions. We have no evidence for a catalysed cyclisation, although this cannot be ruled out. This reductive mechanism obviates the requirement for a thiolesterase domain, which is present in other NRPS modules.[26]

Fused PKS–NRPS systems are known, but FUSS is the first reported fused PKS–NRPS from fungi. The other known fused PKS–NRPS systems have been isolated from bacteria and feature modular Type I PKS components, for example, that involved in pederin biosynthesis in an unculturable symbiont (presumed to be *Pseudomonas* sp.) of *Paederus* beetles.[27,28] Other examples include the synthase involved in melithiazol biosynthesis in the myxobacterium *Melittangium lichenicola Me 146*.[29] FUSS is also unique in that it is an iterative Type I PKS that provides the polyketide moiety. However, the same domain architecture is present in FUSS at the fusion site as in the bacterial examples: the PKS ACP is joined to the NRPS condensation domain.

Based on this analysis we therefore propose that FUSS catalyses the synthesis of pre-fusarin C (**14**) in which the polyketide has been fully elaborated and fused to homoserine aldehyde to form the unmodified 1,5-dihydropyrrol-2-one ring. Further reactions are required to form fusarin. These reactions involve oxidation of the C20 methyl group to a carboxylate moiety and formation of a methyl ester. Further reactions involve epoxidation of the ring and either hydroxylation α to the nitrogen atom or oxidation to an imine and addition of water to form the cyclic hemiaminal. These reactions would most likely be catalysed by oxidoreductases (at least three required) and an *O*-methyl transferase. However, no ORFs with these putative activities have been found, so far, in the immediate vicinity of *fusA*. The identified ORFs which are present are presumably involved in transport and export of fusarin C.

It seems likely that similar synthases are responsible for the biosynthesis of other acyl tetramic acids and compounds derived from them, such as the 2-pyridones. In the case of compounds such as pramanicin (**5**) this would involve the selection of serine as the amino acid component, manufacture of an octaketide and reductive ring formation similar to that catalysed by FUSS, followed by ring epoxidation and hydrolysis to form the *trans*-diol. In the case of other compounds such as fuligorubin A (**3**) and zopfelliamide A (**4**) the ring could be formed similarly, and epoxidation, hydrolysis and subsequent elimination of water would afford the observed ring structure (Scheme 4). Alternatively these compounds could be derived by nonreduc-



Scheme 4. *Alternative biosynthetic routes to observed natural product structures.*

tive chain release involving Claisen cyclisation to form the acyltetramic acid directly. Fuligorubin A (**3**) and zopfelliamide A (**4**) also require *N*-methylation and this is likely to be catalysed prior to ring formation by NRPS *N*-methylation domains, such as those present in the cyclosporin NRPS.[30]

Further chromosome walking is being carried out to find the "missing" fusarin C oxidative ORFs and to investigate ring-forming mechanisms in other fungal tetramic acids.

Experimental Section

General growth conditions: *F. venenatum* and *F. moniliforme* were grown and maintained on malt extract agar (MEA) plates containing MEA (Oxoid, 25.0 g) and agar technical (Oxoid, 7.5 g) in water (1000 mL). The plates were incubated at 28 °C until the colonies reached the edge of the plates (about 9 days). Liquid cultures were grown in a liquid medium containing glucose (20.0 g) and nutrient broth no. 2 (Oxoid, 30.0 g) in water (1000 mL) and were incubated at 25 °C and 200 rpm.

Fusarin C production time course: Spores collected from an MEA agar plate were evenly inoculated into 10 flasks, each containing 50 mL of producing medium, which consisted of glycerol (10.0 g), $(NH_4)_2HPO_4$ (1.0 g), KH_2PO_4 (3.5 g), $MgSO_4 \cdot 7H_2O$ (2.0 g) and NaCl (5.0 g) in water (1000 mL). Glucose (40.0 g) dissolved in water (100 mL) was autoclaved separately and combined before use. All the flasks were wrapped with aluminium foil and incubated at 25 °C and 200 rpm.

One flask was taken each day for fusarin extraction. Ethyl acetate (50 mL) was added to the flask. The flask was shaken at 150 rpm for 15 min. After separation from the aqueous phase, the organic phase was evaporated and the residue was resuspended in acetonitrile (1 mL). The samples were wrapped with aluminium foil and stored at −20 °C before LC-MS analysis.

LC-MS analysis of fusarin C: Samples obtained from different days during the time-course experiment were analysed at the same time to limit any equipment inconsistency. LC-MS was carried out by using a Waters Platform II system comprising a Waters 600 HPLC coupled to a Platform LC mass spectrometer. Solvent A was 0.05% trifluoroacetic acid (TFA) in HPLC-grade water; Solvent B was 0.05% TFA in HPLC-grade acetonitrile. Solvents were filtered (0.4 μm) and degassed before use. The column was a Phenomenex Luna RPC_8 (150×2 mm) equipped with a Phenomenex Security Guard cartridge. Chromatograms were run at 0.5 mL min^{-1} over the following gradient: 0–1 min, 5% B; 1–13 min, gradient to 95% B; 13–16.5 min, 95% B; 16.5–17 min, gradient to 5% B; 17–21.5 min, 5% B. Products were detected by using a Waters 996 diode array detector at wavelengths between 200–400 nm. After passing through the diode array detector, the flow was split so that approximately 20% entered the mass spectrometer. Mass ions were detected in positive electrospray mode over a mass range of 150–600 Da.

RNA preparation and cDNA synthesis: Fungal total RNA was purified by using an RNeasy Plant miniprep kit (Qiagen) and reverse-transcribed by using SuperScript II (Invitrogen) and an oligo(dT) primer. The first-strand cDNA product was then used for PCR.

Total RNA was prepared on a large scale by using an RNA extraction kit (Amersham Pharmacia Biotech), and polyA$^+$ RNA was isolated from it with an mRNA purification kit (Amersham Pharmacia Biotech). Double-stranded cDNA was synthesised by using the SuperScript Choice system (Invitrogen) primed by a mixture of oligo(dT)$_{12-18}$ and random hexamers. This double-stranded cDNA was used for cDNA library construction.

Genomic DNA preparation: Fungal genomic DNA was prepared on a small scale by using a Plant genomic DNA miniprep kit (Sigma). This genomic DNA was used for PCR and Southern blotting analysis.

Genomic DNA for library construction was prepared as follows. Ground, frozen mycelium (5–10 g) was lysed with genomic DNA extraction buffer (10 mL, 10 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 8.0), 10 mM ethylenediaminetetraacetate (EDTA) and 0.5% sodium dodecylsulfate (SDS)) in a centrifuge tube. Aqueous extraction at 4 °C for 30 min was followed by phenol (phenol/chloroform/isoamyl alcohol (25:24:1)) extraction (4 °C, 20 min) on a horizontal cylindrical rotor. The aqueous phase was separated by centrifugation (7000 *g*, 15 min, 4 °C). The extraction was repeated until no protein was left between the two phases. Equal amounts of chloroform (chloroform/isoamyl alcohol (24:1)) were used to extract any trace of phenol and the phases were separated as before. Ribonuclease A (bovine pancreas) solution (10 μL, 20 mg mL^{-1}) was added to the aqueous solution and incubated at 37 °C for 30 min; this was followed by another round of phenol extraction and chloroform extraction as before. Genomic DNA was precipitated from the aqueous phase by adding isopropanol (0.7 volumes) and LiCl (0.1 volumes, 4 M). The precipitated genomic DNA was carefully separated from the liquid by turning the tube. After being washed with 70% ethanol (5 mL), the pellet was air dried and resuspended in TE (Tris EDTA) buffer. The genomic DNA was further purified by two rounds of equilibrium centrifugation in a continuous CsCl–ethidium bromide gradient (density 1.56 g mL^{-1}). After centrifugation (350000 *g*, 16 h, 15 °C), the DNA band was removed, diluted with TE buffer (2 volumes), ethanol precipitated and finally resuspended in TE buffer (200 μL, pH 7.5).

Library construction and screening: The vectors used for fungal cDNA and genomic library construction were Lambda-ZAPII (Stratagene) and λBlueStar (Novagen), respectively, and Gigapack III Gold (Stratagene) was used for packaging. To screen a library, approximately 250000 plaque-forming units were plated on each of two 230×230 mm (square) plates; resultant plaques were blotted onto nylon membranes (Zeta-Probe GT, Bio-RAD) for 4 min. The membranes were then treated sequentially with 0.5 M NaOH, 0.5 M NaOH+1.5 M NaCl, 1.5 M NaCl+0.5 M Tris/HCl (pH 7.5) and 2×standard saline citrate (SSC), before drying at 80 °C for 30–60 min.

Hybridisation was conducted at 55 °C overnight in 0.25 M sodium phosphate buffer (pH 7.2) containing 7% SDS with ^{32}P-labelled probes. Membranes were then subjected to 2×15 min washes at 65 °C with 2×SSC, and positive plaques were identified by autoradiography. Plaques were purified by successive rounds of plating and screening, and auto-excision was used to convert λ into plasmid clones. cDNA or genomic inserts were sequenced (Lark Technologies, Inc), and DNA sequences were analysed with Vector NTI software (Informax).

Construction of knockout plasmids: To construct *venpks*1–*hph* the *venpks*1 cDNA was transferred (as a 3.9 kb *Eco*RI fragment) from the original pBluescript vector to pGEM-T Easy (Promega), which does not contain any *Hin*dIII sites. A 1.7 kb *Hin*dIII fragment, corresponding to the *C*MeT encoding area of *venpks*1, was then replaced with the 4.2 kb hygromycin B resistance cassette (*hph*) from pCRII–pAN7–1 (pAn7–1 modified by exchanging the pUC18 vector for pCRII (Invitrogen) and insertion of a *Hin*dIII site at the 5′ end of the *hph* cassette).

*monpks*1–*hph* was constructed by replacing a 250 bp fragment of *monpks*1 (from a *Hin*dIII site close to the end of the 2.5 kb cDNA to the *Hin*dIII site of the pBluescript vector) with the *hph* cassette. To construct *monpks*2–*hph*, a 1.3 kb fragment (around the *C*MeT encoding area) was amplified by PCR with the primer pair 5′-ACTAGTCATGATGGCAGTTGG-3′ and 5′-ACTAGTCGCCCCGTGTACCTCAAGTCC-3′. The PCR product was first cloned and verified in pGEM-T Easy (Promega), then excised with *Spe*I and inserted into pCRII–pAN7–1 at the vector *Spe*I site.

PEG-mediated fungal transformation:[31] 20-hour-old fungal mycelium was converted into protoplasts by incubation with driselase (25 °C, 3 h) in osmotic buffer (1 M NaCl). Protoplasts prepared from cultures (50 mL) were resuspended in GTC buffer (400 μL, 1 M glucose, 10 mM Tris/HCl (pH 8.0), 50 mM $CaCl_2$). Knockout plasmid DNA (2 μg) was added to one half of the protoplast suspension, and sterile water (20 μL) was added to the other half as a control. Following addition of poly(ethylene glycol) (PEG) 4000 solution (50 μL, 60% (w/v) in GTC buffer) protoplasts were incubated in ice for 30 min, after which 60% PEG 4000 solution (1 mL) was added and incubation was continued at RT for 20 min. The PEG solution was diluted by adding GTC buffer (10 mL) and then removed following centrifugation (3000×*g*, 10 min). The protoplasts were resuspended with GTC buffer (200 μL), mixed with molten (42 °C) CD-G agar (5 mL, Czapek Dox agar containing 1 M glucose) and plated on Petri dishes containing CD-G agar (5 mL). After incubation for 3 h the protoplasts were overlaid with hygromycin B (100 or 200 $\mu g\,mL^{-1}$ in 10 mL molten CD-G agar) and incubation was continued until resistant colonies appeared.

Southern blot analysis: *F. venenatum* DNA was fully digested with *Bgl*II (overnight, 37 °C), and fractionated by electrophoresis on a 1.0% (w/v) agarose gel. The DNA was blotted onto a positively charged nylon membrane (Zeta-Probe GT, Bio-RAD) for 6 h with 0.4 M NaOH as a transfer buffer. After rinsing with 2×SSC the filter was dried at 80 °C for 30–60 min. Hybridisation with the ^{32}P-labelled *venpks*1 probe was performed as described above, except that the hybridisation temperature was 65 °C.

Data accession: Sequences for *fus*A from *F. moniliforme* have been deposited with GenBank (access code AY604568).

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