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Late Stage Oxidations during the Biosynthesis of the 2-Pyridone Tenellin in the Entomopathogenic Fungus *Beauveria bassiana*

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Abstract: Late stage oxidations during the biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana* were investigated by a combination of gene knockout, antisense RNA, and gene coexpression studies. Open reading frames (ORF) 3 and 4 of the tenellin biosynthetic gene cluster were previously shown to encode a *trans*-acting enoyl reductase and a hybrid polyketide synthase nonribosomal peptide synthetase (PKS-NRPS), respectively, which together synthesize the acyltetramic acid pretenellin-A. In this work, we have shown that ORF1 encodes a cytochrome P450 oxidase, which catalyzes an unprecedented oxidative ring expansion of pretenellin-A to form the 2-pyridone core of tenellin and related metabolites, and that this enzyme does not catalyze the formation of a hydroxylated precursor. Similar genes appear to be associated with PKS-NRPS genes in other fungi. ORF2 encodes an unusual cytochrome P450 monooxygenase required for the selective *N*-hydroxylation of the 2-pyridone which is incapable of *N*-hydroxylation of acyltetramic acids.

Introduction

Tenellin **1** is a 2-pyridone produced by the entomopathogenic fungus *Beauveria bassiana* (teleomorph *Cordyceps bassiana*).[1] It is a member of a large class of related compounds including other structurally complex 2-pyridones and acyltetramic acids isolated from fungi. Many of these compounds possess interesting biological activities, for example: fischerin **2**[2] from *Neosartorya fischeri* which is toxic to mammals; leporin-B **3**[3] (*Aspergillus leporis*) which is toxic to insects; PF1140 **4** a broad spectrum antifungal antibiotic from *Eupenicillium sp*. PF1140;[4] and sambutoxin **5**[5] and related compounds[6] which are hemorrhagic mycotoxins from *Fusarium oxysporum*. We have investigated the biosynthesis of tenellin **1** and shown that a *ca* 12 kb open reading frame (ORF), known as *tenS*, encoding a hybrid polyketide synthase nonribosomal peptide synthetase (PKS-NRPS) is involved in its construction.[7] Similar PKS-NRPS genes have been shown to control the construction of fusarin-C **6**,[8] equisetin[9] and more recently aspyridone-A **7**,[10] pseurotin-A[11] and the cytochalasins.[12] Heterologous expression of *tenS* in the fungal host *Aspergillus oryzae*, however, led to the production of a series of compounds (**8** - **10**) which cannot be precursors of tenellin **1** because they differ in the structures of the PKS derived side chain. Coexpression of *tenS* with ORF3, encoding a free-standing enoyl reductase (Figure 1), on the other hand, led to the production of pretenellin-A **11** (Scheme 1), with the correctly elaborated side chain, which can be regarded as the likely precursor of tenellin **1**.[13]

Two oxidative chemical transformations, ring expansion and *N*-hydroxylation, are required to convert pretenellin-A **11** to tenellin **1** (Scheme 1). ORFs 1 and 2 of the putative tenellin biosynthetic gene cluster encode cytochrome P450 oxidases and these appear to be potential candidates for the catalysis of the two required steps. We decided to probe the chemical steps

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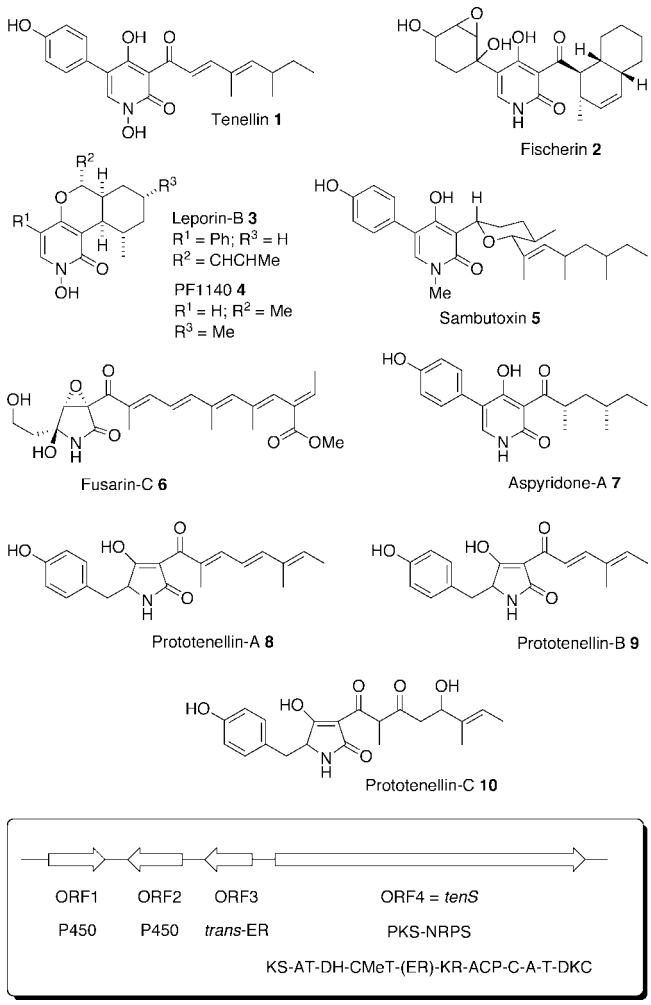

Figure 1. Compounds related to tenellin **1** and the *tenS* gene cluster (NCBI accession number AM409327) in *Beauveria bassiana*.

catalyzed by the ORF1 and ORF2 proteins through a combination of gene ablation and coexpression experiments.

Results

Metabolites of *B. bassiana.* The wild-type (WT) *B. bassiana* strain was grown in liquid culture for 10 days prior to extraction with organic solvent. The extract was initially examined by liquid chromatography mass spectrometry (LCMS) and several major compounds were isolated and their structures elucidated (see Supporting Information). As expected, tenellin **1** was observed as the major metabolite (Table 1). Pretenellin-A **11**, which we have previously identified as the product of heterologous expression of *tenS* and ORF3,[13] was also observed as a minor metabolite in the WT strain. A third compound with *m*/*z* 16 units greater than pretenellin-A **11** was also observed by LCMS. This compound was isolated and its structure elucidated by ^{1}H, ^{13}C and correlation NMR spectroscopy and shown to be a 16-hydroxylated version of pretenellin-A. Although this compound could potentially be a precursor of tenellin, we also regarded it as a potential shunt compound (see below) and thus named it prototenellin-D **12**. Finally, a fourth compound with *m*/*z* 16 units greater than tenellin was isolated from the WT *B. bassiana* strain. This was identified as 15-hydroxy tenellin **13**.

Construction of Antisense RNA and Knockout Strains. We have previously used gene knockout (KO) strategies in *B. bassiana* through the insertion of an antibiotic resistance cassette into the target gene *via* homologous recombination.[7] However, in the case of the *tenS* KO we had to examine nearly 100 individual clones to find a genuine KO as the rate of ectopic integration of the selection marker in *B. bassiana* was high (>90%). In order to improve the chance of success we decided to also use an RNA silencing approach - specifically antisense RNA (aRNA) - as this avoids the requirement for homologous recombination.[14] RNA methods have been recently demonstrated as effective in linking sequenced genes to the biosynthesis of cytochalasins in *Penicillium expansum*[12] but have not previously been used in Beauveria species.

B. bassiana is sensitive to the herbicide glufosinate ammonium (basta) and we chose this selection marker for the construction of both aRNA and KO vectors. In order to construct KO vectors for ORF1 and ORF2, PCR was performed on *B. bassiana* gDNA to amplify 5′ and 3′ targeting fragments (*ca* 700 to 1200 bp) for each ORF. All the PCR products were initially cloned into pENTR/D-TOPO (Invitrogen), and the KO plasmids were constructed by inserting a *bar* cassette (*A. nidulans trp*C promoter driving a basta resistance gene) between the targeting fragments.

Creation of an aRNA vector required selection of a suitable promoter. The *A. nidulans gpdA* (glyceraldehyde-3-phosphate dehydrogenase) promoter has previously been demonstrated to drive strong constitutive expression in *B. bassiana*[15,16] so we selected it to ensure high levels of aRNA production. The promoter was amplified by PCR and inserted into vector pCB1530, which contains a selectable *bar* cassette. To enable rapid cloning of diverse gene fragments we inserted a Gateway cloning cassette[17] "in reverse orientation" downstream of the *gpdA* promoter (i.e., with attR2 adjacent to the promoter), creating fungal expression vector pCBgpdA-GA. ORF1 and 2 were amplified by PCR and cloned directionally into pENTR/D-TOPO (Invitrogen). Gateway recombination was then used to insert the fragments in antisense orientation with respect to the *gpdA* promoter into pCBgpdA-GA.

The two linearized KO and two circular aRNA plasmids were then introduced into *B. bassiana* by PEG mediated protoplast transformation and selection for basta resistance. A total of 18 potential ΔORF1 KO and 17 ΔORF2 KO transformants were obtained in which the intended double crossovers would have resulted in gene KO. A total of 29 aRNA transformants were obtained for ORF1 and 39 aRNA transformants for ORF2.

Chemical Analysis of *B. bassiana* Transformants. All 18 ΔORF1 transformants were individually grown in production medium, extracted and the organic extracts analyzed by LCMS. Only one transformant failed to produce tenellin **1**, and produced pretenellin-A **11** and prototenellin-D **12** instead (Table 1). PCR analysis of this transformant indicated that the ORF1 deletion had occurred as intended. All other putative ΔORF1 transformants displayed the WT phenotype and produced tenellin **1** and co-occurring compounds. In these cases PCR analysis showed that ORF1 was still intact and that the KO plasmid must have integrated elsewhere in the genome. In the case of the ΔORF2

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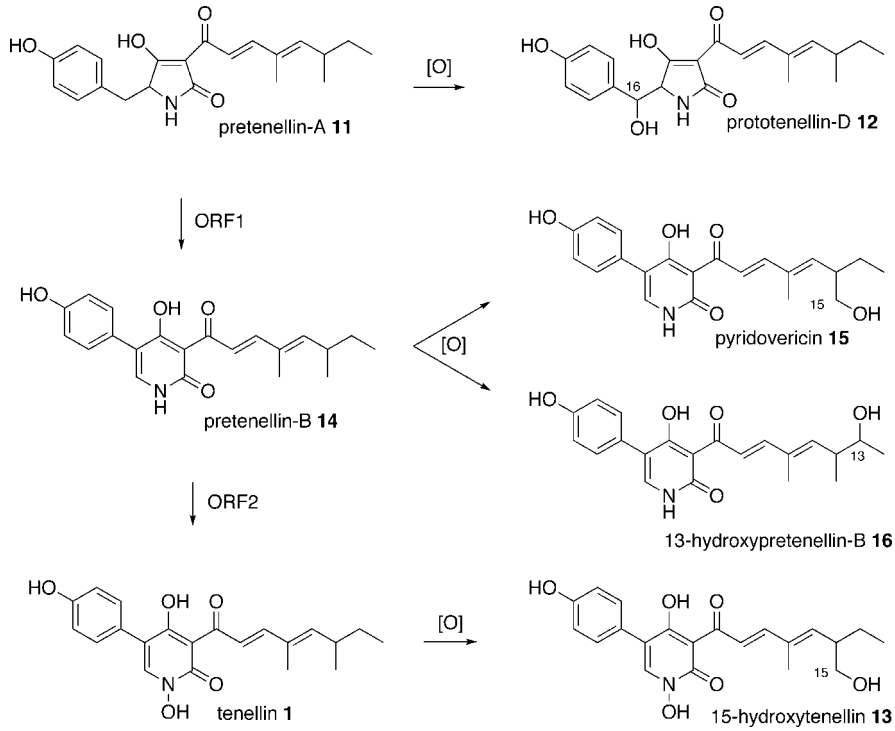
Scheme 1. Proposed Biosynthetic Relationships of Compounds Isolated in This Study[a]



[a] Note that different numbering systems are in use for the tetramic acid and 2-pyridone series.[1,13]

Table 1. Chemotypes of the *B. bassiana* and *A. oryzae* Strains Discussed in the Text[a]

Compound	No.	yield / mg·L^{-1}	RT / min	*m/z* [M]H$^+$	uv$_{max}$ / nm	*Chemotype (frequency)* WT -	ΔORF1 KO (1/18)	ORF1 aRNA1 (1/16)	ORF1 aRNA2 (3/16)	ORF2 aRNA1 (2/16)	ORF2 aRNA2 (2/16)	*A. oryzae* 134 (8/9)	*A. oryzae* 34[13] -
Tenellin	**1**	20.4	44.4	370	250, 343	✓	✗	✗	✗	✗	✗	✗	✗
Pretenellin-A	**11**	1.0	43.4	356	220, 358	✓	✓	✓	✓	✗	✗	✓	✓
Prototenellin-D	**12**	15.6	38.9	372	222, 359	✓	✓	✗	✓	✗	✓	✗	✗
Pretenellin-B	**14**	18.3	43.3	354	246, 339	✗	✗	✗	✗	✓	✓	✓	✗
Pyridovericin	**15**	10.8	33.1	370	246, 339	✗	✗	✗	✗	✓	✓	✗	✗
15-Hydroxytenellin	**13**	3.8	34.3	386	249, 341	✓	✗	✗	✗	✗	✗	✗	✗
13-Hydroxy-pretenellin-B	**16**	4.6	32.3	370	246, 339	✗	✗	✗	✗	✓	✓	✗	✗

[a] aRNA = antisense RNA; KO = knockout; *A. oryzae* 34 = *A. oryzae* + ORF3 + *tenS*; *A. oryzae* 134 = *A. oryzae* + ORF1 + ORF3 + *tenS*. Yields given are isolated purified yields from WT *B. bassiana*, except for **14**−**16** which were isolated from aRNA strains.

transformants, extracts of all 17 were analyzed and shown to display the WT phenotype.

A total of 16 ORF1 aRNA transformants were individually grown in production medium, extracted and the organic extracts examined by LCMS. One transformant produced only pretenellin-A **11**, three transformants produced both pretenellin-A **11** and prototenellin-D **12**, while all other transformants showed the WT phenotype (Table 1). None of the non-WT phenotypes produced pyridones in observable amounts. Of the 16 ORF2 aRNA transformants, two produced a new compound related to tenellin by being 16 *m/z* units lighter. This compound was isolated and shown to be a non-*N*-hydroxylated pyridone which we name pretenellin-B **14** (Scheme 1). A second compound with the same *m/z* as tenellin, but with a much shorter retention time, was also isolated from these two clones. This was purified and its structure was elucidated. It proved to be the known compound pyridovericin **15** which is hydroxylated at C-15,[18] but not at the pyridone nitrogen. These two transformants also produced the novel 13-hydroxypretenellin-B **16** in low concentration. Two further ORF2 aRNA transformants produced prototenellin-D **12** in addition to pretenellin-B **14** and the non-*N*-hydroxylated pyridones **15** and **16**. All other ORF2 aRNA transformants showed the WT phenotype.

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Coexpression of ORF1, ORF3, and *tenS* in *Aspergillus oryzae*. In a further experiment ORF1 was cloned into a derivative of the expression vector pTAex3 in which a phleomycin resistance cassette replaced the *argB* selectable marker. This construct was then transformed into the pre-existing *Aspergillus oryzae* ORF3/*tenS* strain (*A. oryzae* 34) created previously[13] to give *A. oryzae* 134. Triple selection (*argB* auxotrophy for *tenS*, basta resistance for ORF3 and phleomycin resistance for ORF1) led to the isolation of 48 transformants. Nine of these were grown, extracted with organic solvent and the concentrated extract analyzed by LCMS. One clone produced no new metabolites, but in the other eight transformants pretenellin-B **14** was detected as the major metabolite, with traces of pretenellin-A **11**.

ORF1 and ORF2 Activity in *B. bassiana* Cell-Free Extracts. In order to further investigate whether pretenellin-A **11** and prototenellin-D **12** were precursors of tenellin **1** we prepared cell-free extracts (CFE)[19] of *B. bassiana* Δ*tenS* which had been grown under tenellin-production conditions for ten days. This extract is incapable of tenellin biosynthesis, but should contain the cytochrome P450 proteins encoded by ORFs 1 and 2, among others. Purified pretenellin-A **11** and prototenellin-D **12** (20 μg) were each incubated with the CFE at 25 °C for 30 min, then extracted with EtOAc. The EtOAc extracts were evaporated, dissolved in MeOH and examined by LCMS (Figure 2). In the case of pretenellin-A **11**, the 2-pyridones pyridovericin **15** and 15-hydroxy tenellin **13** were detected (Figure 2, panels A and B; see Table 1 for RT data). However, when prototenellin-D **12** was incubated with the CFE, no pyridones were detected (Figure 2, panels E and F). This CFE proved to be rather unstable, however, and ring-expanding activity was lost after a few minutes' incubation at 25 °C.

These experiments also show that in the CFE the oxidative enzymes appear to be present with differing relative activities than is observed in whole cells. In WT *B. bassiana* pretenellin-A **11** is converted to both tenellin **1** (*via* pretenellin-B **14**) and **12**, while 15-hydroxylation appears relatively slow. In the CFE, however, the 15-hydroxylase appears to be relatively more active, so that no tenellin **1** or pretenellin-B **14** are observed. The different oxidative enzymes are presumably represented in the CFE differently than in whole cells because of selective losses during preparation. This also explains why no prototenellin-D **12** was observed when pretenellin-A **11** was the substrate.

Discussion

Chemical Steps Revealed. Four of the ORF1 aRNA transformants and the single genuine ΔORF1 KO failed to produce pyridones with only tetramic acids being observed. We thus conclude that the putative cytochrome P450 oxidase encoded by ORF1 must catalyze the oxidative ring expansion required to convert the tetramic acid of pretenellin-A **11** to the pyridones of pretenellin-B **14**, tenellin **1** and their derivatives **13**, **15** and **16**. This result was confirmed by the triple heterologous coexpression of ORF1 + ORF3 + *tenS* in *A. oryzae* 134 which produced pretenellin-B **14** (Table 1).

The ORF2 aRNA transformants produced pyridones, but none were *N*-hydroxylated. This shows that the ORF2-encoded cytochrome P450 oxidase must be responsible for pyridone *N*-hydroxylation (Scheme 1). The ORF2-encoded oxidase appears selective for pyridone oxidation as no *N*-hydroxylated tetramic acids were produced in the ORF1 aRNA transformants or the ΔORF1 knockout.

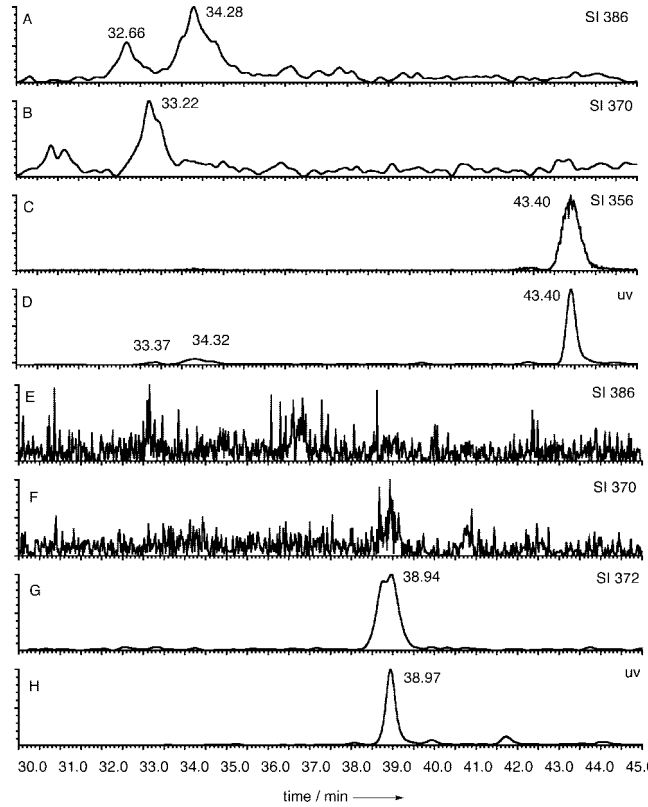

Figure 2. Cell-free conversion of pretenellin-A **11** to pyridones. (A) single ion monitoring at *m/z* 386 following incubation with pretenellin-A (20 μg); (B) single ion monitoring at *m/z* 370 following incubation with pretenellin-A (20 μg); (C) single ion monitoring at *m/z* 356 following incubation with pretenellin-A (20 μg); (D) diode array uv trace following incubation with pretenellin-A (20 μg); (E) single ion monitoring at *m/z* 386 following incubation with prototenellin-D (20 μg); (F) single ion monitoring at *m/z* 370 following incubation with prototenellin-D (20 μg); (G) single ion monitoring at *m/z* 372 following incubation with prototenellin-D (20 μg); (H) diode array uv trace following incubation with prototenellin-D (20 μg).

Interestingly, WT *B. bassiana* and several transformants (three of the ORF1 aRNA transformants, the ΔORF1 KO and two of the ORF2 aRNA transformants) produce the hydroxylated tetramic acid prototenellin-D **12** (Table 1). Three lines of evidence suggest that prototenellin-D **12** is in fact a shunt metabolite. First prototenellin-D **12** was produced in the ΔORF1 KO transformant where PCR analysis showed that ORF1 had been disrupted through insertion of a basta resistance cassette, thus, **12** cannot be a product of the ORF1-encoded enzyme. Second, pretenellin-A **11** was converted to 2-pyridones by the cell-free extract of *B. bassiana* Δ*tenS*. However, the same CFE could not convert prototenellin-D **12** to 2-pyridones, showing that **12** is not a precursor of the pyridones. Third, *A. oryzae* 134 transformants producing pretenellin-B **14** did not produce prototenellin-D **12** in detectable amounts, even though the ORF1 encoded oxidase is evidently present and active in these strains.

The simplest explanation for the production of prototenellin-D **12** is that adventitious benzylic hydroxylation of pretenellin-A **11** is catalyzed by a *B. bassiana* oxidative enzyme encoded elsewhere in the genome. Likewise, the presence of 15-hydroxylated compounds suggests the presence of an additional cytochrome P450 enzyme in *B. bassiana* selective for hydroxy-

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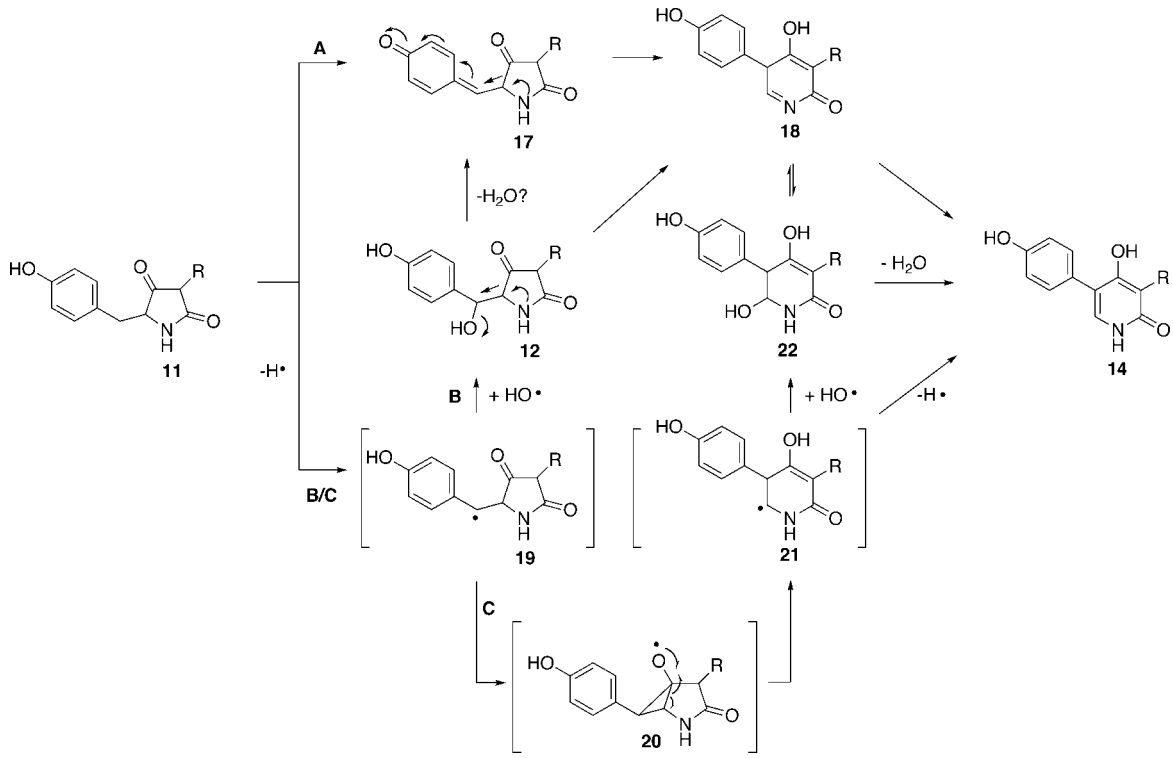
Scheme 2. Possible Chemical Routes and Mechanisms for the Oxidative Ring Expansion of Tetramic Acids to 2-Pyridones



lation of the polyketide side chain. Similarly selective cytochrome P450 enzymes are present during the biosynthesis of related compounds such as fusarin-C **6** in *Fusarium sp.,* which must also be oxidized on its polyketide side chain.[8] 13-hydroxy pretenellin-B **16** may be the product of the same enzyme, possibly with loose substrate specificity, or of a third P450.

Oxidative Ring Expansion. Three chemical mechanisms have been suggested for the ring expansion of tetramic acids such as **11** to 2-pyridones such as **14**.[7,13] The first proposed mechanism (route **A**, Scheme 2) involves oxidation of the tetramic acid **11** to a quinomethide **17**, followed by a ring expansion to **18** and tautomerisation to **14**. A variation of this route could be considered *via* two electron cation formation at the benzylic carbon; such an intermediate could be represented by O-protonated **17**. A similar two electron oxidation may be involved in the related rearrangement of littorine during tropane alkaloid biosynthesis.[20] The second possible mechanism (route **B**, Scheme 2) involves hydrogen atom abstraction at the benzylic position of the tetramic acid forming intermediate **19**, followed by addition of a hydroxyl radical to form the benzylic alcohol **12**. This could be followed by a concerted displacement of hydroxyl during the ring expansion giving imine **18** in common with pathway **A**, or a dehydration to quinomethide **17**. Such mechanisms have been suggested in the case of aspyridone **7**,[10] for example, and cytochrome P450 monooxidases are known to carry out hydroxylation reactions by this hydrogen atom abstraction and rebound hydroxylation mechanism. Oikawa and co-workers have recently shown that serine is the precursor of the fungal 2-pyridone PF1140 **4**, and similarly suggested a hydroxyl-displacement mechanism.[21] However, since serine is apparently the direct precursor of **4** this would not have to be an *oxidative* ring expansion as is the case during tenellin biosynthesis.

The third potential mechanism (route **C**, Scheme 2) again relies on hydrogen atom abstraction at the benzylic position giving intermediate **19**, but this is followed by radical-induced ring expansion *via* short-lived intermediate **20** to give putative radical **21**. This could be hydroxylated to give **22**, followed by elimination of water to give **14**, or loss of another hydrogen atom could potentially give the pyridone **14** directly. Similar radical-induced ring expansion mechanisms are known in synthetic chemistry.[22,23]

Mechanism **A** appears unlikely as it could not operate in the case of fungal 2-pyridones such as leporin-B **3** which lacks a *para*-hydroxyl. If mechanism **B** were operating then prototenellin-D **12** would be regarded as a true intermediate to tenellin **1**, but this appears not to be the case. Thus mechanism **C** appears the most likely in the case of the aromatic substituted 2-pyridones such as tenellin **1**, but further *in vitro* investigations will be required to confirm this hypothesis.

Occurrence of Ring Expanding Enzymes. 2-Pyridones are known secondary metabolites in a number of filamentous fungi and are very likely to be synthesized by ring expansion of acyl tetramic acid precursors. Blast-P analysis of the sequence of the ORF1-encoded ring expandase against the genomes of known filamentous fungi reveals at least eight other potential ring expanding enzymes (Table 2). The *Aspergillus nidulans* protein AN8411 (= *apdE*) is already implicated in the biosynthesis of aspyridone **7** together with another cytochrome P450 (AN8408, = *apdB*).[10] Our results suggest that the *apdE*-encoded protein (47.7% identity) is the ring expandase, while the *apdB*-encoded protein (16.6% identity) is probably responsible for a different oxidative step.

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Table 2. Comparison of Protein Sequences of Putative Ring Expanding Cytochrome P450 Enzymes with *B. bassiana* ORF1

organism	P450 protein	associated with PKS-NRPS	% identity	% similarity
A. nidulans	AN8411 (= *apdE*)	AN8412 (= *apdA*)	47.7	61.8
A. nidulans	AN8408 (= *apdB*)	AN8412 (= *apdA*)	16.6	29.8
N. fischeri	NFIA_001540	NFIA_001530	46.9	60.0
A. flavus	AFL2G_07514	AFL2G_07507	41.3	57.3
A. oryzae	AO090001000288	AO090001000277	27.8	39.5
F. oxysporum	FOXG_14594.2	FOXG_14587.2	53.3	67.8
F. oxysporum	FOXG_14589.2	FOXG_14587.2	43.0	59.4
F. oxysporum	FOXG_03951.2	FOXG_03945.2	40.7	54.6
F. verticillioides	FVEG_11928.3	FVEG_11932.3	40.3	54.4

A number of other filamentous fungi possess similar cytochrome P450 enzymes which are located close to hybrid PKS-NRPS genes (Table 2). It is therefore tempting to speculate that these genes are also involved in 2-pyridone biosynthesis. *N. fischeri* is known to produce the 2-pyridone fischerin **2** and it is likely that NFIA_001540 and the adjacent PKS-NRPS NFIA_001530 are involved in its biosynthesis. Likewise, *F. oxysporum* produces sambutoxin **5** and either the FOXG_14587 or FOXG_03945 PKS-NRPS encoding loci are likely to be involved in its biosynthesis. However, 2-pyridones are not known metabolites of *A. flavus*, *A. oryzae* or *F. verticillioides*, so it is likely, as in the case of aspyridone-A **14**, that these gene clusters are 'silent' or only expressed under very specific growth conditions. Other organisms are known to produce both tetramic acids and related 2-pyridones. For example militarinones B, C and D (**23**−**25**, Figure 3) co-occur in the entomopathogenic fungus *Paecilomyces militaris*.[24] Previously the benzylic alcohol **23** has been proposed as a precursor to the 2-pyridone **25**. However, our results suggest that **24** is likely to be the direct precursor of **25** and that **23** is a shunt metabolite.

***N*-Hydroxylation.** ORF2 is responsible for the hydroxylation of the 2-pyridone nitrogen of pretenellin-B **14**. *N*-hydroxylation can be achieved biosynthetically in a number of ways, chiefly *via* FAD-dependent monooxygenases, for example during the biosynthesis of δ-*N*-hydroxyornithine in fungal species,[25] and ϵ-*N*-hydroxylysine in mycobacteria.[26] However, Rieske-iron monooxygenases are also known to be involved during pyrrolnitrin biosynthesis in *Pseudomonas fluorescens* Pf5,[27] for example, and a nonheme diiron *N*-monooxygenase from the aureothin biosynthetic cluster in *Streptomyces thioluteus* has recently been shown to produce *p*-nitrobenzoic acid from *p*-aminobenzoic acid.[28] Cytochrome P450 monooxygenases are relatively rarely involved in *N*-hydroxylation (possibly because *C*-hydroxylation is more energetically favorable),[29] although examples are known during the biosynthesis of the *N*-hydroxy imine of nocardicin in the bacterium *Nocardia uniformis*[30] and the nitrile of borrolidin in the bacterium *Streptomyces parvulus*

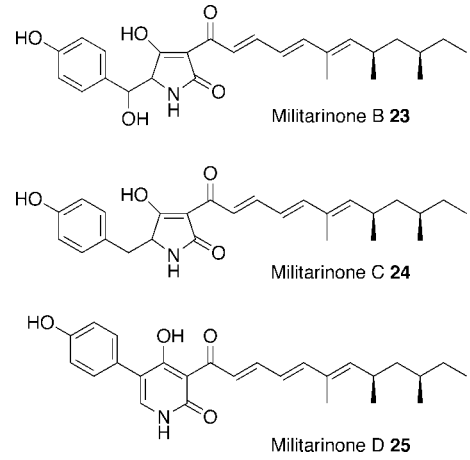

Figure 3. Related tetramic acids and 2-pyridones from *Paecilomyces militaris*.

Tü4055.[31] Blast-P searches using the peptide sequence of the ORF2-encoded protein reveal no especially close links to either of these bacterial P450 enymes. Blast-P searches of the available genomes of Aspergillus and Fusarium species reveal numerous cytochrome P450 homologues of ORF2, although these are rarely clustered with other biosynthetic genes. Thus ORF2 appears to fall into the relatively rare class of cytochrome P450 *N*-monooxygenases, and, as yet, it has no close relations in either bacteria or fungi.

Comparison of aRNA and KO Approaches in *B. bassiana.* Previous attempts to produce gene KO strains in *B. bassiana* revealed a low frequency of homologous recombination[7] and this observation was confirmed in the attempt to knock out ORF1 and ORF2. A combined total of 35 transformants yielded a single KO clone, i.e., a frequency of less than 3%. However the aRNA experiments were more successful with a silencing frequency of 25%. In the case of the KO experiments PCR showed that a very high frequency of ectopic integration accounted for the difficulty in retrieving KO clones. In the case of the aRNA experiments, PCR indicated that unsuccessful clones had probably undergone deletions or rearrangements of the inserted promoter and antisense regions.

The ORF1 KO showed the same phenotype as three of the ORF1 antisense RNA clones, however it is difficult to assess the significance of the variation in phenotype displayed by the ORF2 antisense RNA transformants because of the lack of KO transformants for comparison. Gene KO and antisense experiments might be expected to yield slightly different results. Gene KO leads to complete removal of the gene of interest, and therefore complete removal of the encoded catalyst, resulting in an irreversible block in the biosynthetic pathway. The effect of aRNA experiments can sometimes be different because the gene is still transcribed to mRNA. Two fates await the mRNA: it can be translated to protein which would be expected to be catalytically active; or it can be partially or totally blocked or degraded due to the presence of the aRNA. Thus in aRNA experiments one may expect a partial, or total, reduction in protein production depending of the effectiveness of the aRNA to direct destruction of the target RNA. This in turn leads to total, or partial reduction in protein production and catalytic activity.

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In the experiments described here, the *gpdA* promoter was used to drive production of the aRNA sequence. This promoter has previously been demonstrated to be a strong constitutive promoter in *B. bassiana* and it appears that the *gpdA* promoter is sufficiently strong to bring about post-transcriptional gene silencing through antisense production. This hypothesis is supported by the observation that 25% of the ORF1 aRNA transformants could not form pyridones at all, while 25% of the ORF2 aRNA transformants could not *N*-hydroxylate at all.

Conclusion

Our results show that the final stages of tenellin **1** biosynthesis involve a unique ring expansion from a tetramic acid to a 2-pyridone, catalyzed by a cytochrome P450 oxidase encoded by ORF1. This is followed by a second oxidative step in which the 2-pyridone nitrogen is hydroxylated by a rare cytochrome P450 monooxygenase encoded by ORF2. ORFs 1, 2 and 3, together with *tenS*, thus form the complete tenellin biosynthesis gene cluster and we thus name the genes *tenA*, *tenB*, *tenC* and *tenS*, respectively. Genes similar to *tenA*, encoding putative ring expanding enzymes, appear to be associated with PKS-NRPS genes in a number of fungi and are likely to be involved in 2-pyridone biosynthesis in these organisms. The chemical mechanism of the ring expansion catalyzed by the *tenA*-encoded cytochrome P450 enzyme remains to be elucidated, but results described here suggest a possible radical-induced process. *In vitro* studies, currently underway, should shed more light on this intriguing reaction.

Experimental Section

Fungal Strains and Culturing. *B. bassiana* strain 110.25 was obtained from CBS, Utrecht, Netherlands. *A. oryzae* M-2-3 was obtained from Professor I. Fujii, University of Tokyo. *B. bassiana* WT and silencing clones were grown on potato dextrose agar (PDA, Difco) for 7−10 days. Spores and mycelia were collected in 10 mL sterile distilled water, and 1 mL aliquots used to inoculate 100 mL tenellin production medium[7] in 500 mL Erlenmeyer flasks; cultures were incubated at 25 °C for 10 days with shaking at 150 rpm.

Construction of Knockout Plasmids pΔorf1 and pΔorf2. PCR reactions to amplify fragments of the tenellin biosynthetic gene cluster were performed with KOD DNA polymerase (Novagen). 5′ fragments of ORFs 1 and 2 were amplified with the primer pairs *orf1KOH1f*/*orf1KOH1r* and *orf2KOH1f*/*orf2KOH1r*, respectively. The products were cloned into pENTR/D-TOPO (Invitrogen). The resulting plasmids were cut with *Xba*I and *Kpn*I and a *bar* cassette was excised from plasmid pCB1546[32] with the same enzymes and ligated to them to give p*orf1*H1*bar* and p*orf2*H1*bar*. 3′ fragments of ORFs 1 and 2 were amplified with the primer pairs *orf1KOH2f*/*orf1KOH2r* and *orf2KOH2f*/*orf2KOH2r*, respectively, and cloned into pENTR/D-TOPO. The inserts were excised with *Xba*I and *Hin*dIII and inserted into p*orf1*H1*bar* and p*orf2*H1*bar* cut with the same enzymes to obtain pΔ*orf1* and pΔ*orf2*.

Construction of Antisense RNA Plasmids, pAS-orf1 and pAS-orf2. The *gpdA* promoter was amplified by PCR using the primer pair *gpdApf*/*gpdApr* and pAN8−1[33] as template. The promoter was cloned into pCB1530[24] as an *Xba*I and *Xma*I fragment. The resulting plasmid was cut with *Sma*I and a Gateway destination cassette (Invitrogen) was ligated after the promoter. The plasmid pCB*gpdA*-GA, with the attR2 site of the Gateway cassette adjacent to the *gpdA* promoter, was used to prepare the antisense silencing plasmids; *orf1* and *orf2* were amplified from *B. bassiana* genomic DNA by the primer pairs *orf1ASf*/*orf1ASr* and *orf2ASf*/*orf2ASr*, respectively. The products were cloned into Gateway entry vector pENTR/D-TOPO and transferred into pCB*gpdA*-GA by *in vitro* recombination using Gateway LR Clonase enzyme mix II (Invitrogen).

Table 3. PCR Primers Used in This Work

primer	sequence 5′−3′ (added restriction site and CACC overhangs underlined)
orf1KOH1f	CACCTAGACAGCGTGAGCCTCCCAT
orf1KOH1r	TCTAGAAATGGTACCTCAGCACGGTCGGCATGCGTC
orf1KOH2f	CACCAAGCTTCGACAGCAGCGCATCAAAGATGGCCT
orf1KOH2r	ATCTAGATAAACGGCTCCGGGTCCC
orf2KOH1f	CACCATTAGCAATATGCCAAAGTC
orf2KOH1r	TCTAGAAATGGTACCTCACCCAAGTAATGAGAGGTA
orf2KOH2f	CACCAAGCTTGGATGAGATTTGTCACGGAAACGGTG
orf2KOH2r	ATCTAGATGATTTGTTGCCGATCCA
Δorf1analf	GCGGCTACCGGTGGATCTTT
Δorf1analr	GAGACCTCAGCTCTTCCTCGTC
gpdApf	TCTAGAGAATTCCCTTGTATCTCTAC
gpdApr	CCCGGGTGATGTCTGCTCAAGCGG
orf1ASf	CACCAAAATGCTGCCCCTCCTAGA
orfr1ASr	CTTGTAAAACGCAATCTTGA
orf2ASf	CACCTCCAGGCAATGTCCATGGTA
orf2ASr	AACTTTGCGCCTCTTGACCAGA
P4501Fwd	CACCATGCTGCCCCTCCTAGACAGCGT
P4501Rev	TTACTTGTAAAACGCAATCTTGA
P4502Fwd	CACCATGGCGCTTTTCCAGGCAATGTC
ORF1PromFwd	ATGTAGGTCAGGCGGGTTTCTACC
KOOrf1Rev	AATTCTGTGAGTCTGCGCTCG
Barorf1KOrev	CGTACGACAGAAGATGATATTGAAGGAGC
Orf1TermRev	CGCTGGCACCACACAAGTCACC
GPDrev	CACTTCATCGCAGCTTGACTAACAG
BarFwd	CCTCCACTAGCTCCAGCCAAGCCC
ORF2TermFwd	GACACTATGCATAGCCAGTCTG
ORF2PromRev	ACGCACTGACGTACTGTCGACG
BleHindF	CACCAAGCTTGAATTCCCTTGTATCTCTAC
BleR	GCCAGTGCCAAGCTCTAGAAAG

Construction of ORF1 Expression Plasmid, pBleAmyBO-RF1. Primers containing a *Hin*dIII site and *Xba*I site (BleHind-F and Ble-R) were used to amplify the *Ble*R cassette from pAN8−1.[25] *Ble*R was cloned into pENTR/D-TOPO and excised as a *Hin*dIII-*Xba*I fragment. pTAex3GS was digested with *Hin*dIII and *Xba*I to remove the *argB* selectable marker. The *Ble*R cassette and the pTAex3GS backbone were ligated together, to create pBleAmyBGS. ORF1 was amplified from *B. bassiana* DNA using the primer pair *P4501Fwd/P4501Rev* and cloned into pENTR/D-TOPO and then GATEWAY transferred into pBleAmyBGS, to produce the plasmid pBleAmyBORF1.

Fungal Transformation. *B. bassiana* protoplasts were prepared and transformed as described previously.[13] Plasmid DNA (10 μg) was used in transformations. The knockout plasmids, pΔ*orf1* and pΔ*orf2* were linearized by *Xba*I digestion prior to transformation. Transformants were maintained on PDA (Difco) plates containing 100 μg/ml basta (Aventis).

A. oryzae TenS+ORF3[13] (*A. oryzae* 34) was grown on DPY (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4$, 1.5% agar) plates for 10 days. Spores were collected into water (1 mL) and inoculated into 50 mL GN (2% glucose, 1% nutrient broth no. 2 (Laboratory M Ltd.)) and grown at 28 °C for 18 h at 200 rpm. Mycelium was collected by filtration and protoplasting performed using *Trichoderma harzianum* lysing enzymes (Sigma; 20 mg ml^{-1}) in NaCl (0.8 M) with gentle shaking at room temperature for 2 h. Protoplasts were centrifuged at 2500 *g* for 5 min and washed once with solution 1 (0.8 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5). Protoplasts were diluted to 3×10^7 ml^{-1} in solution 1 and 5 μg (10 μL) of pBleAmyBORF1 added to 100 μL aliquots. The protoplasts were incubated on ice for 2 min, 1 mL Solution 2 (60% PEG 6000, 0.8 M NaCl, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5) was then added and the mixtures incubated at RT for 20 min. CZDS soft top agar (7 mL, 3.5%

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Czapek-Dox broth, 0.7% agar, 1 M sorbitol) was added to the transformation mixtures, which were poured onto Czapek-Dox plates supplemented with sorbitol (1 M) and incubated at 28 °C. CZDS (7 mL) supplemented with phleomycin (Melford) and basta (both at 100 μg ml^{-1} to give final concentrations of 50 μg ml^{-1}) was overlaid 24 h post transformation, and plates incubated for a further 7 d. Single transformant colonies were transferred onto fresh Czapek-Dox plates supplemented with 50 μg ml^{-1} phleomycin and 50 μg ml^{-1} basta.

Genetic Analysis of *B. bassiana* Δorf1. Genomic DNA was isolated and amplified from *B. bassiana* Δ*orf1* transformants using the Extract-N-Amp Plant PCR kit (Sigma). The PCR was carried out using Δ*orf1analf and* Δ*orf1analr* primers, which give a 706 bp product for intact ORF1 and a 1598 bp product for the disrupted gene. PCR performed using the primer pair *ORF1PromFwd/BarORF1KORev* which bind within the ORF1 promoter and the bar resistance gene confirmed correct integration of the knockout cassette in Δ*orf1*−*6*. Knockout of the ORF1 gene was confirmed using the primer pair *ORF1PromFwd/KOOrf1Rev* which bind within the ORF1 promoter and the deleted region of the ORF1 gene.

Genetic Analysis of *B. bassiana* ORF1 and ORF2 aRNA Strains. PCR performed using the primer pair *ORF1PromFwd/ORF1TermRev* which bind within the ORF1 promoter and terminator respectively, resulted in the amplification of a 1.8 kb product confirming the presence of the intact ORF1 gene. Confirmation of the intact ORF1 silencing cassette was determined through amplification of a 2.1 kb product using the primer pair *GPDrev/BarFwd* which bind within the *gpd*A promoter and the basta resistance gene. PCR performed using ORF2TermFwd and ORF2PromRev primers which bind within the ORF2 terminator and promoter respectively, resulted in the amplification of a 1.8 kb product confirming the presence of the intact ORF2 gene. Confirmation of the intact ORF2 silencing cassette was determined through amplification of a 1.78 kb product using the primer pair *P4502Fwd/GPDrev* which bind within the *gpd*A promoter and the 5′ end of the ORF2 gene.

Screening of *B. bassiana* Δorf1 and Δorf2 Metabolites. *B. bassiana* transformants were grown on PDA plates for 3 weeks at 30 °C. Mycelium and spores were scraped from a 1 cm^2 area of the agar surface and gently shaken with 2 mL acetone at RT for 3 h. The acetone was collected and dried under N_2. The residue was dissolved in 170 μL MeOH, centrifuged for 5 min at 5 000 rpm, and the supernatant was analyzed by LCMS.

Screening of *A. oryzae* Metabolites. Eight day old cultures were pooled (2 × 50 mL in 250 mL Erlenmeyer flasks) and acidified to pH 3 with 37% HCl. The mixture was homogenized and extracted with ethyl acetate (2 × 500 mL), and concentrated *in vacuo*. The residue was dissolved in MeOH (1 mg ml^{-1}), centrifuged for 5 min at 5 000 rpm, and the supernatant was analyzed by LCMS.

Extraction and LCMS Analysis. Ten day old *B. Bassiana* cultures in tenellin production medium (100 mL in 500 mL Erlenmeyer flasks) were vacuum filtered. The mycelial mass was extracted with acetone (150 mL × 2). The acetone extract was concentrared to yield a brown aqueous residue which was further diluted with distilled water (250 mL) and extracted into ethylacetate (200 mL × 2). The organic layer was separated, dried with anhydrous $MgSO_4$ and concentrated *in vacuo* to yield a brown solid.

A solution of the respective extracts (10 mg ml^{-1}) was prepared in HPLC grade methanol and subjected to LCMS analysis. A Waters Platform LC system comprising a Waters 600 pump system, Waters 996 diode array detector (detecting between 210 and 400 nm), Macromass Platform LC mass spectrometer (detecting between 150 and 600 *m/z* units ESI$^+$) and Phenomenex Luna 5 μ C_{18} (2) (250 × 4.6 mm) reverse phase column equipped with a Phenomenex SecurityGuard precolumn (Luna C_5 cartridge) was used for all analyses. A gradient of water (+ 0.05% TFA, solvent A) and acetonitrile (+ 0.045% TFA, solvent B) was used. 50 μL of the extract solution (1−5 mg ml^{-1}) was injected using the following HPLC program: 0 min, 5% B; 5 min, 5% B; 45 min, 75% B; 46 min, 95% B; 50 min, 95% B; 55 min, 5% B; 60 min, 5%B).

Purification of Compounds. Compounds from WT *B. bassiana.* Crude extract (50 mg) obtained from *B. bassiana* WT was loaded onto a silica gel coated thin layer chromatography plate (20 cm × 20 cm). The plate was developed in chloroform/methanol/water (80:18:2) to yield eight bands baseline inclusive. A pale yellow band at R_f 0.38 corresponded to prototenellin-D **12**, while tenellin **1** appeared at R_f 0.47 as a bright yellow band. A faintly colored band was observed between prototenellin-D **12** and tenellin **1** bands. This band was a combination of 15-hydroxytenellin **13** and pretenellin-A **11**.

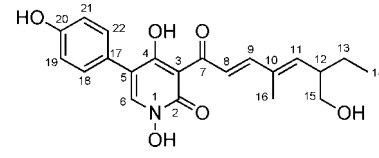
The bands were carefully scraped off and extracted with acetone/ethanol solvent system (9:1). The extracts were diluted with water and extracted further with ethyl acetate. The organic fractions were dried with anhydrous $MgSO_4$ and concentrated to yield residues of semi pure compounds.

Thirty mg/ml solutions of the semi pure compounds were subjected to HPLC preparative purification using a Phenomenex Luna 5 μ C_{18}(2) (250 × 4.6 mm) reverse phase column. A gradient of water (+ 0.01% TFA, solvent A) and acetonitrile (+ 0.01% TFA, solvent B) system was used. 50 to 75 μL was injected during each round of one hour HPLC program (0 min, 5% B; 5 min, 5% B; 45 min, 75% B; 46 min, 95% B; 50 min, 95% B; 55 min, 5% B; 60 min, 5%B). Eluants were collected using an automatic fraction collector (Pharmacia LKB-FRAC-100): 0.5 mL per 30 s. Tenellin **1** was collected from fractions 89−91; prototenellin-D **12** was obtained from fractions 78−80; 15-hydroxytenellin **13** eluted in fraction 69 while the minor pretenellin-A **11** was collected in fraction 87. The respective eluants were evaporated at reduced pressure to remove acetonitrile and then extracted from water into ethyl acetate which was concentrated *in vacuo* to generate the pure compounds: tenellin **1** 20.4 mg/L; prototenellin-D **12** 15.6 mg/L; 15-hydroxytenellin **13** 3.8 mg/L; and pretenellin-A **11** ≈ 1 mg/L.

Compounds from *B. bassiana* Transformants. Pretenellin-B **14**, pyridovericin **15** and 13-hydroxypretenellin-B **16** were isolated from an extract of an ORF2 aRNA clone (ORF2−9). Similar purification methods were adopted as described above. Eluants corresponding to the pure compounds were freeze-dried to yield: pretenellin-B **14** 18.3 mg/L; pyridovericin **15** 10.8 mg/L; and 13-hydroxypretenellin-B **16** 4.6 mg/L.

Characterization of Compounds. ^{1}H spectra were recorded on a Jeol ECP-400 or Varian VNMR spectrometer operating at 400 MHz. ^{13}C chemical shifts were deduced from HMQC, HMBC as well as 1D ^{13}C NMR experiments conducted at 100 or 125 MHz. See the Electronic Supporting Information for further details of structure elucidation. HRMS was measured at the University of Bristol, School of Chemistry HRMS facility. IR data were obtained using a Perkin-Elmer FTIR instrument. Melting point was determined using Electrothermal apparatus, while optical rotation was evaluated with an ADP 220 polarimeter.

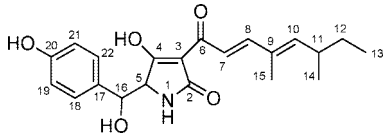
15-Hydroxytenellin 13. Yellow solid; mp 137−140 °C; IR (neat): ν_{max} 3749, 3226, 2929, 1644, 1609, 1514, 1428, 1367 and 1329 cm^{-1}; ^{1}H ($CDCl_3$, 400 MHz), δ = 8.01 (d, J = 15.3 Hz, 1H,



H-8), 7.87 (s, 1H, H-6), 7.69 (d, J = 15.3 Hz, 1H, H-9), 7.39 (d, J = 8.5 Hz, 2H, H-18, H-22), 6.93 (d, J = 8.5 Hz, 2H, H-19, H-21), 5.89 (d, J = 9.7 Hz, 1H, H-11), 3.69 (dd, J = 5.6, 10.5 Hz, 1H, H-15a), 3.57 (dd, J = 7.5, 10.5 Hz, 1H, H-15b), 2.72 (brm, 1H, H-12), 2.07 (s, 3H, H-16), 1.62 (m, 1H, H-13a), 1.30 (m, 1H, H-13b), 0.92 (t, J = 7.4 Hz, 3H, H-14); ^{13}C NMR ($CDCl_3$, 100 MHz), δ = 194.9 (C-7), 172.9 (C-4), 158.4 (C-2), 156.9 (C-20), 150.7 (C-9), 146.7 (C-11), 135.4 (C-10), 133.4 (C-6), 131.5 (C-18, C-22), 125.9 (C-17), 123.2 (C-8), 114.8 (C-19, C-21), 111.9 (C-5), 110.7 (C-3), 65.7 (C-15), 43.0 (C-12), 23.0 (C-13), 12.4 (C-

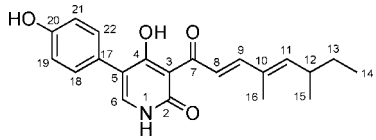
16), 10.1 (C-14), HRMS calcd for $C_{21}H_{23}NO_6Na$: 408.1417; found 408.1432 $[M]Na^+$.

Prototenellin-D 12. Pale yellow amorphous solid; mp 124−126 °C; $[\alpha]^{22}_D$ + 64.5 (c 0.50, MeOH); IR (neat): ν_{max} 3258, 2960,



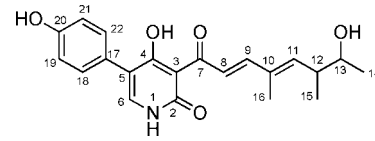
2926, 1605, 1557, 1515 and 1434 cm^{-1}. ^1H (400 MHz, CD_3OD,): δ = 7.46 (d, J = 15.6 Hz, 1H, H-8), 7.14 (d, J = 8.3 Hz, 2H, H-18, H-22), 7.00 (d, J = 15.6 Hz, 1H, H-7), 6.68 (d, J = 8.3 Hz, 2H, H-19, H-21), 5.86 (d, J = 9.5 Hz, 1H, H-10), 5.00 (d, J = 3.4 Hz, 1H, H-16), 4.25 (brs, 1H, H-5), 2.55 (brm, 1H, H-11), 1.87 (s 1H, H-15), 1.44 (m, 1H, H-12a), 1.33 (m, 1H, H-12b), 1.02 (d, J = 6.6 Hz, 3H, H-14), 0.88 (t, J = 7.3 Hz, 3H, H-13); ^{13}C NMR (100 MHz, CD_3OD), δ = 194.3 (C-4), 176.0 (C-2), 174.6 (C-6), 156.9 (C-20), 151.8 (C-10), 149.7 (C-8), 133.0 (C-9), 129.3 (C-17), 128.3 (C-18, C-22), 115.3 (C-7), 114.3 (C-19, C-21), 100.7 (C-3), 73.5 (C-16), 67.6 (C-5), 35.2 (C-11), 29.7 (C-12), 19.1 (C-14), 11.2 (C-15), 11.0 (C-13); HRMS calcd for $C_{21}H_{25}NO_5Na$: 394.1617; found 394.1624 $[M]Na^+$.

Pretenellin-B 14. Bright yellow powder; mp 206−208 °C; $[\alpha]^{22}_D$ −32 (c 0.50, MeOH); IR (neat): ν_{max} 3748, 2962, 2925, 1646, 1609,



1519, 1456 cm^{-1}; ^1H (400 MHz, DMSO-d_6,): δ = 17.5 (s, 1H, C4-*OH*), 11.6 (d, J = 6.4 Hz, 1H, *NH*), 9.49 (s, 1H, C20-*OH*), 8.01 (d, J = 15.6 Hz, 1H, H-8), 7.56 (d, J = 6.4 Hz, 1H, H-6), 7.51 (d, J = 15.6 Hz, 1H, H-9), 7.27 (d, J = 8.4 Hz, 2H, H-18, H-22), 6.78 (d, J = 8.4, 2H, H-19, H-21), 5.95 (d, J = 9.5 Hz, 1H, H-11), 2.50 (brm, 1H, H-12), 1.84 (s, 3H, H-16), 1.39 (m, 1H, H-13a), 1.28 (m, 1H, H-13b), 0.98 (d, J = 6.6 Hz, 3H, H-15), 0.83 (t, J = 7.7, 3H, H-14); ^{13}C NMR (125 MHz, DMSO-d_6), 194.2 (C-7), 177.4 (C-4), 162.2 (C-2), 157.2 (C-20), 151.2 (C-11), 149.9 (C-9), 141.1 (C-6), 133.1 (C-10), 130.5 (C-18, C-22), 123.9 (C-8), 123.6 (C-17), 115.4 (C-19, C-21), 113.2 (C-5), 106.3 (C-3), 35.1 (C-12), 29.9 (C-13), 20.4 (C-15), 12.9 (C-16), 12.3 (C-14); HRMS calcd for $C_{21}H_{23}NO_4Na$: 376.1519; found 376.1529 $[M]Na^+$.

13-Hydroxypretenellin-B 16. Yellow solid; mp 140−142 °C; IR (neat): ν_{max} 3243, 2967, 1645, 1609, 1518, 1453; ^1H (400 MHz, DMSO-d_6,): δ = 17.5 (s, 1H, C4-*OH*), 11.6 (brs, 1H, *NH*), 9.46 (s, 1H, C20-*OH*), 7.99 (d, J = 15.4 Hz, 1H, H-8), 7.55 (brs, 1H, H-6), 7.49 (d, J = 15.4 Hz, 1H, H-9), 7.25 (d, J = 8.5 Hz, 2H, H-18, H-22), 6.76 (d, J = 8.5, 2H, H-19, H-21), 6.02 (d, J = 10



Hz, 1H, H-11), 4.58 (d, J = 5.2 Hz, 1H, C13-*OH*), 3.45 (m, 1H, H-13), 2.51 (brm, 1H, H-12), 1.83 (s, 3H, H-16), 1.01 (d, J = 6.1 Hz, 3H, H-15), 0.97 (d, J = 6.7, 3H, H-14); ^{13}C NMR (125 MHz, DMSO-d_6), 194.2 (C-7), 177.4 (C-4), 162.2 (C-2), 157.2 (C-20), 150.0 (C-11), 148.8 (C-9), 141.2 (C-6), 133.3 (C-10), 130.5 (C-18, C-22), 123.9, (C-8) 123.6 (C-17), 115.5 (C-19, C-21), 113.2 (C-5), 106.4 (C-3), 70.0 (C-13), 41.5 (C-12), 21.8 (C-15), 16.5 (C-14), 13.1 (C-16); HRMS calcd for $C_{21}H_{23}NO_5Na$: 392.1468; found 392.1479 $[M]Na^+$.

Preparation and Use of Cell-Free Extract. A 10 day old culture of *B. bassiana* blocked mutant (*tenS* knockout strain,[7] 500 mL) grown in tenellin production medium was harvested by vacuum filtration. The extract was prepared using the method of Watanabe and Townsend.[19] The mycelia were thoroughly washed with distilled water, 35 g wet-weight of the mycelia were flash frozen in liquid nitrogen and pulverized using a pestle and mortar with continuous cooling using liquid nitrogen. The fine powdered mycelia were then resuspended into 70 mL buffer solution (50 mM potassium phosphate, pH 7.5; 30% glycerol; 2 mM DTT; 100 μM phenylmethylsuphonyl fluoride, 100 μM benzamidine hydrochloride and 1 mM EDTA). The mixture was stirred for 2 h at 4 °C, then centrifuged at 20 000 g for 20 min. The supernatant was decanted and used as cell-free extract (CFE).

Twenty microliters of 1 mg/mL solution of substrate in acetone (pretenellin A **11** or prototenellin D **12** as required) was added to 5 mL of CFE in a precooled 10 mL tube. The reactions were incubated at 25 °C for 30 min. The reactions were quenched by shaking with ethyl acetate (5 mL). The organic layer was carefully decanted after the mixture was allowed to settle and concentrated *in vacuo*. Samples were dissolved in 100 μL of HPLC methanol and subjected to LCMS analysis. Control experiments were conducted following the same protocols using a boiled CFE solution.

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Supporting Information Available: Supporting table and further experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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