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Nongenetic Reprogramming of a Fungal Highly Reducing Polyketide Synthase

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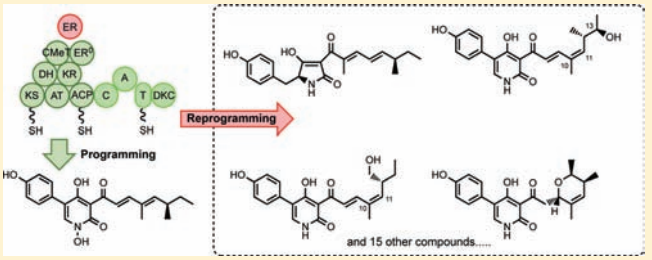
Supporting Information

ABSTRACT: The biosynthesis of the fungal metabolite tenellin from *Beauveria bassiana* CBS110.25 was investigated in the presence of the epigenetic modifiers 5-azacytidine and suberoyl bis-hydroxamic acid and under conditions where individual genes from the tenellin biosynthetic gene cluster were silenced. Numerous new compounds were synthesized, indicating that the normal predominant biosynthesis of tenellin is just one outcome out of a diverse array of possible products. The structures of the products reveal key clues about the programming selectivities of the tenellin polyketide synthase.



INTRODUCTION

Fungal polyketides are a diverse group of often bioactive secondary metabolites. Their biosynthesis has been investigated at genetic[1] and enzymological levels,[2] but understanding lags behind that for bacterial polyketide synthases. All fungal polyketide synthases (PKS) discovered to-date are classified as Type I iterative systems, consisting of multifunctional multidomain proteins broadly analogous to single modules of bacterial modular PKS, but where individual active sites catalyze multiple reactions on the growing substrate during biosynthesis. Fungal PKS are further classified into broad classes which reflect their domain structure and chemistry: nonreducing (nr) PKS generally produce poly-β-keto intermediates which cyclize to aromatic products; partially reducing (pr) PKS catalyze limited reductions of the β-carbonyl; and highly reducing (hr) PKS generally produce compounds arising from a complex interplay of chain extension, *C*-methylation, keto reduction, dehydration, and enoyl reduction. Fungal PKS are evidently programmed, and high-fidelity enactment of the program means that byproducts are rarely observed. Evidence suggests that the product−template (PT) domain of nr-PKS may control chain length (i.e., the number of extension iterations), and stabilization and cyclization program elements of the highly reactive nascent polyketide, but there is no equivalent domain in the pr- and hr-PKS classes.[3]

Polyketide biosynthesis in fungi is generally followed by a series of (often oxidative) tailoring reactions which rapidly diversify the carbon skeleton produced by the initial PKS. An example of this is the biosynthesis of the 2-pyridone tenellin (**1**) by the entomopathogenic fungus *Beauveria bassiana* CBS110.25 (teleomorph *Cordyceps bassiana*, Scheme 1).[4−6] In this case, acetate is extended four times by a hr-PKS, programmed *C*-methylations occur after the first and second extension, and a cycle of full reduction occurs after the first extension (Figure 1). Subsequent programmed extensions and reductions occur until a β-ketopentaketide is finally intercepted by tyrosine bound to a non-ribosomal peptide synthetase (NRPS) fused to the tenellin hr-PKS (Scheme 1), which acts as an efficient off-loading mechanism. Programming in these systems is required to control chain length, methylation pattern, and reduction steps.

The product of the first stage of biosynthesis is pretenellin A (**2**), which is the substrate for oxidative ring expansion to form the pyridone pretenellin B (**3**) and subsequent *N*-hydroxylation to form **1**.[7−9] We have recently shown that the program of the tenellin PKS is influenced by the presence or absence of a *trans*-acting enoyl reductase (ER) encoded by *tenC*.[10] A similar effect is displayed during the biosynthesis of other fungal polyketides such as lovastatin.[11] Sequence comparison of the integral ER of TENS suggests it is inactive, and, as expected, heterologous expression of *tenS* alone produces polyunsaturated compounds including prototenellins A−C (**4**−**6**, Figure 2).

Importantly, **4**−**6** show evidence of reprogramming of the PKS; for example, **4** necessitates methylation reprogramming, while the polyketide moiety of **5** is shorter and less methylated than that of tenellin **1**. Co-expression of *tenS* with *tenC* in the heterologous host *Aspergillus oryzae*, however, leads to the production of pretenellin A (**2**) as the sole product where all programmed steps have occurred faithfully.[6] It is thus clear that the presence or absence of the ER encoded by *tenC* can affect the program of the hr-PKS.

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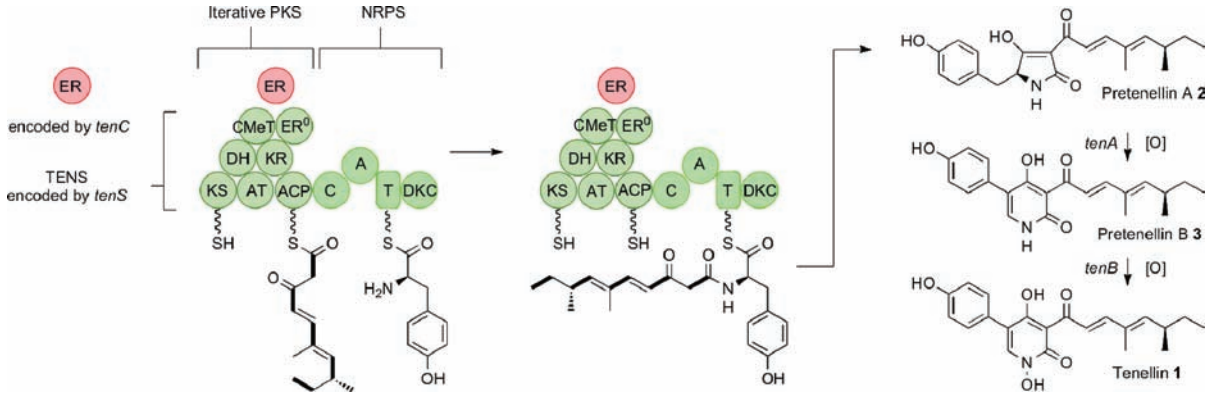
Scheme 1. Biosynthesis of Tenellin (1) in *B. bassiana*



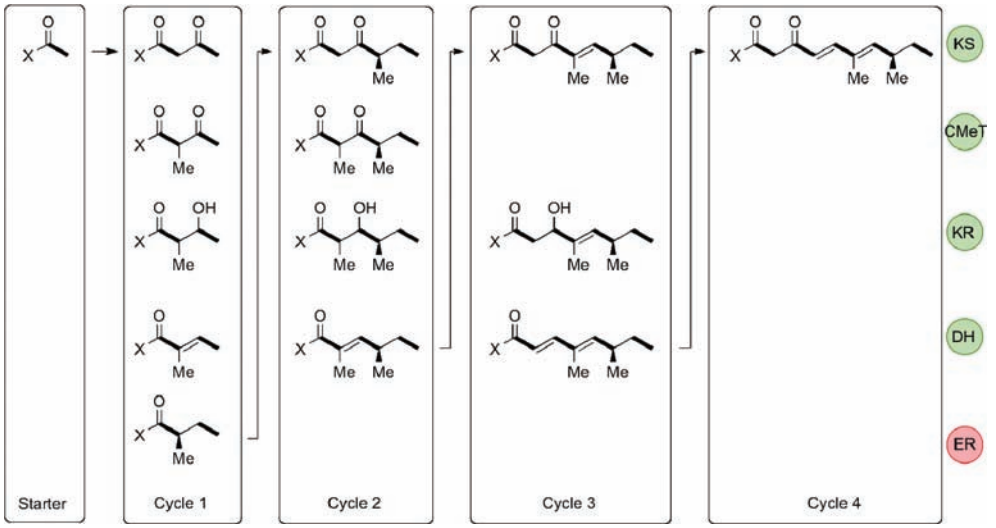


Figure 1. Programming evident during the biosynthesis of the tenellin pentaketide.

We reasoned that this behavior might represent a mechanism by which *B. bassiana* could control the program of the *tenS* PKS, potentially through differential gene expression. This would be possible in eukaryotic organisms where each structural gene is controlled by its own promoter, and thus expression of individual genes within a cluster could be differentially enhanced or repressed. This in turn could lead to differences in protein stoichiometry which might affect programming.

Molecular tools are available to study such processes; chromatin modifying chemicals such as 5-azacytidine (5AC)[12] and suberoyl bis-hydroxamic acid (SBHA),[13] for example, have been shown to affect gene expression in fungi and in some cases can activate previously silent biosynthetic gene clusters.[14] Additionally, RNA silencing methods such as RNA interference (RNAi) are able to reduce targeted gene expression.[15] We thus set out to discover whether epigenetic[16] and silencing methods could reveal new elements of programming during tenellin biosynthesis.

RESULTS AND DISCUSSION

We began by undertaking a thorough LCMS examination of the compounds produced by *B. bassiana* CBS110.25 under standard fermentation conditions (TPM media,[17] 25 °C, 200 rpm shaking, 10 days).

Tenellin (**1**, 60 mg·L^{-1})[18] and prototenellin D (**7**, 34 mg·L^{-1})[9] were the major metabolites; 15-hydroxytenellin (**8**)[9] was observed at lower concentration (13 mg·L^{-1}), and pretenellin A (**2**)[6] was another minor component (12 mg·L^{-1}), while pyridovericin (15-hydroxypretenellin B, **9**)[19] was observed at trace levels (<1 mg·L^{-1}). These known compounds **1**, **2**, **7**, **8**, and **9** were identified by LCMS and NMR and quantified by comparison with standards. Sufficient prototenellin D (**7**) was collected to allow reaction of the tyrosine hydroxyl with (*S*)-camphanyl chloride and the growth of crystals. The X-ray crystal structure revealed the absolute configuration of the three stereocenters of **7** as shown in Figure 2. Since **7** probably arises directly by hydroxylation of pretenellin A (**2**), it is reasonable to assume the same absolute configurations of the two stereocenters in **2** and thus the *R*-configuration of the pendant aliphatic methyl group of tenellin and its analogues. The structure also indicates that pretenellin A (**2**) is constructed from L-tyrosine.

Compounds **1** and **2** arise as the expected final and intermediate products of expression of the tenellin gene cluster. However, pyridovericin (**9**) and 15-hydroxytenellin (**8**) likely arise via cytochrome P450 mono-oxygenation of **3** and **1**, respectively, while prototenellin D (**7**) probably arises by benzylic oxidation of pretenellin A (**2**). We have previously shown[9] that **7** is not an

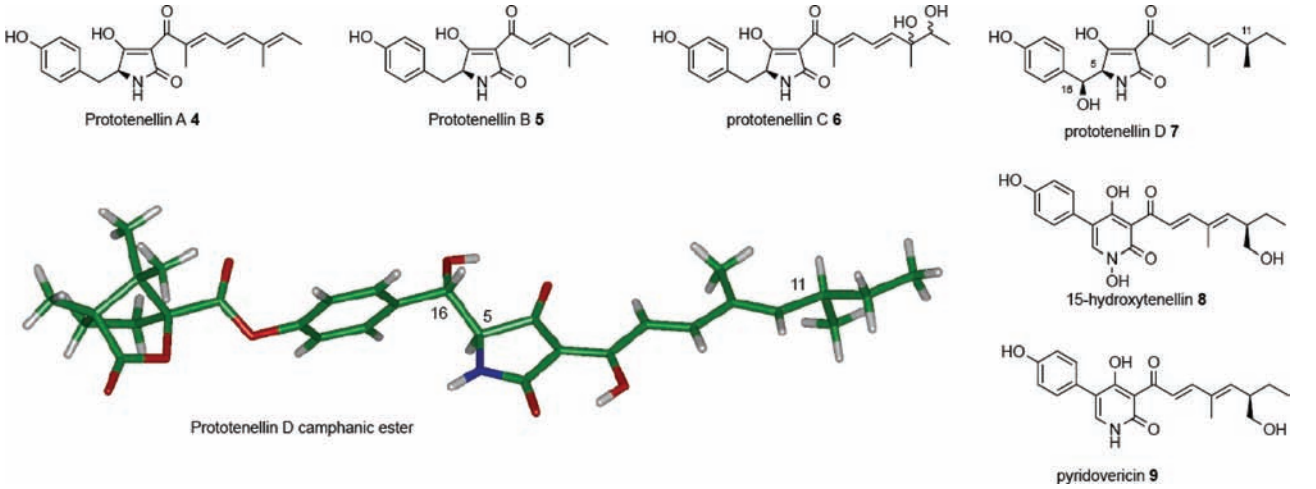


Figure 2. Known compounds from *Beauveria bassiana* CBS110.25 and related organisms. Note that in the solid state the camphanic ester adopts a different tautomeric form compared to **7** in solution.

Table 1. Summary of Compounds Produced[a]

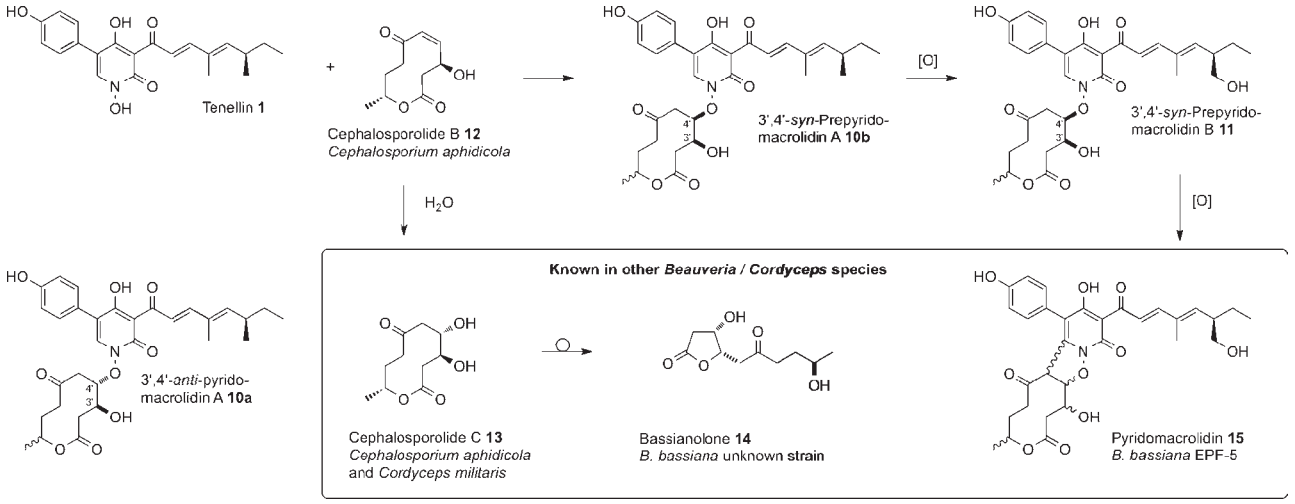
		WT			*tenA* RNAi			*tenB* RNAi			*tenC* RNAi
compound	new (X) /ref		+5AC	+SB		+5AC	+SB		+5AC	+SB	
tenellin (**1**)	4	60	55	86					13		21
pretenellin A (**2**)	6	12	11	9	121	85	101		17		16
pretenellin B (**3**)	9							57	25	32	
*prototenellin A (**4**)*	6		7								*16*
*prototenellin B (**5**)*	6										*8*
prototenellin D (**7**)	9	34	89	77		7	10	22	64	52	80
15-hydroxytenellin (**8**)	9	13	8								
pyridovericin (**9**)	19	<1		<1				22	39	27	
3′,4′-*anti*-prepyridomacrolidin A (**10a**)	X		9								
3′,4′-*syn*-prepyridomacrolidin A (**10b**)	X		85	19					8		
prepyridomacrolidin B (**11**)	X		30								
*prototenellin E (**16**)*	X		*19*	*11*		*8*	*14*		*16*	*3*	*5*
syn-12-hydroxypretenellin A (**17**)	X					24	6				
anti-12-hydroxypretenellin A (**18**)	X					36	8				
14-hydroxypretenellin A (**19**)	X					29	7				
syn-13-hydroxypretenellin B (**20**)	X								12	8	
anti-13-hydroxypretenellin B (**21**)	X							9	16	17	
*(10,11-Z)-pyridovericin (**22**)*	X								*11*		
*(10,11-Z)-syn-13-hydroxypretenellin B (**23**)*	X								*8*		
*(10,11-Z)-anti-13-hydroxypretenellin B (**24**)*	X								*13*		
*prototenellin F (**25**)*	X								*9*		
anti-13,15-dihydroxypretenellin B)**26**)	X								10	7	
total		119	313	202	121	189	146	112	261	146	146
% reprogrammed		0	8	5	0	4	10	0	22	2	20

[a] Reprogrammed compounds are shown in italics. Values given are concentrations in $mg \cdot L^{-1}$. Quantification was done by calibrated HPLC; see Supporting Information for details. 5AC = 5-azacytidine, SB = suberoyl bis-hydroxamic acid.

intermediate on the tenellin biosynthetic pathway. The endogenous genes encoding these monooxygenase steps are currently undiscovered.

We next examined the effect of epigenetic modification by growing *B. bassiana* CBS110.25 in the presence of the DNA methyltransferase inhibitor 5-azacytidine (5AC) and the histone deacetylase inhibitor suberoyl bis-hydroxamic acid (SBHA, Table 1).[14] In the presence of 5AC (100 μM) the fungus produced approximately the same titers of **1**, **2**, and **8** as previously. However, the overall titer of tenellin-related compounds increased nearly

Scheme 2. Proposed Biosynthesis of Pyridomacrolidin Analogues



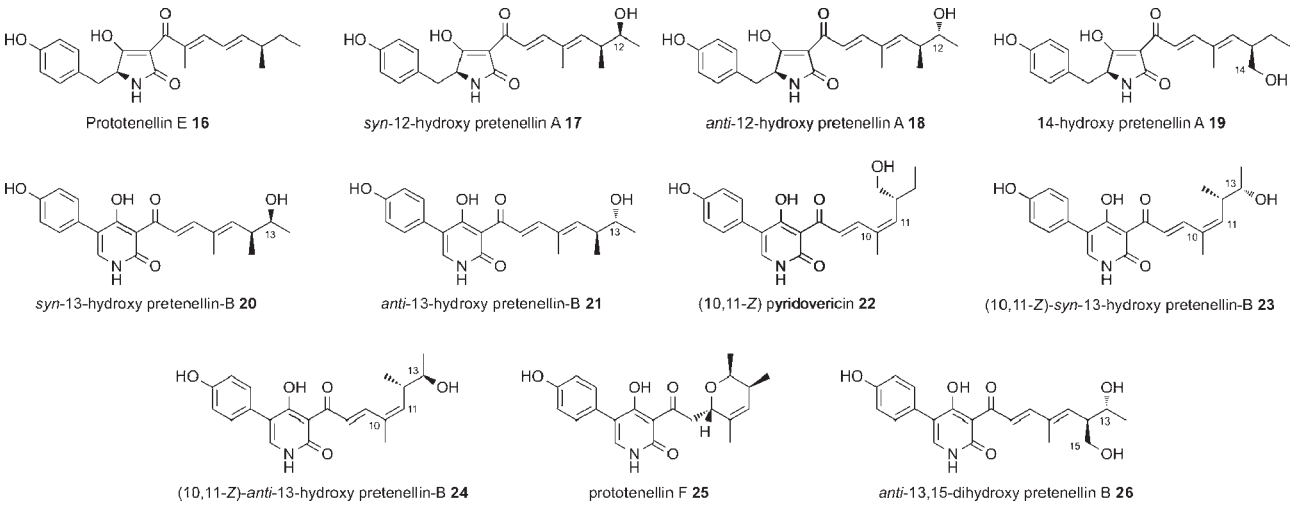


Figure 3. New compounds isolated from dual RNAi and epigenetic modification.

3-fold compared to the unmodified *B. bassiana* fermentation (Table 1) and qRTPCR studies (see below) showed a substantial increase in tenellin pathway mRNAs that would account for this. prototenellin D (**7**) was also produced but at nearly three times (89 mg·L^{-1}) its previous concentration.

Several new compounds were also observed in this fermentation. These were isolated and purified, and their structures were determined by full NMR analysis (see Supporting Information for details). Thus, 3′,4′-*anti*-prepyridomacrolidin A (**10a**, 9 mg·L^{-1}), its 3′,4′-*syn* diastereomer **10b** (85 mg·L^{-1}), and prepyridomacrolidin B (**11**, 30 mg·L^{-1}) evidently arose by condensation of tenellin with a decanolide such as cephalosporolide B (**12**),[20] which is related to the known *B. bassiana* compound bassianolone **14**[21] via the known *Cordyceps* compound cephalosporolide C (**13**,[20,22] Scheme 2).

Oxidative ring-closing of **11** could also give rise to pyridomacrolidin **15**,[19] which is a known product of *B. bassiana* EPF-5; however, this compound and **12**, **13**, and **14** were not observed directly in any of our experiments. It is also apparent that *B. bassiana* grown in the presence of 5AC is capable of producing *reprogrammed* compounds. Thus, prototenellin A (**4**, 7 mg·L^{-1}) and a new compound, the dihydro analogue, prototenellin E (**16**, 19 mg·L^{-1}; see Figure 3), were produced. Both compounds feature a different methylation pattern compared to tenellin and must arise via reprogramming of methylation during polyketide biosynthesis.

The fermentation was then repeated using SBHA (100 μM) as the epigentic modifier. This gave very similar results, with the main differences being the absence of prototenellin A (**4**) and the production of **10b** as the only pyridomacrolidin, in much reduced yield (Table 1). Again, the tenellin pathway was highly upregulated, but SBHA appeared to be much less efficient at switching on the cryptic decanolide pathway.

The construction and use of RNAi vectors for silencing *tenA* and *tenB* expression has been described previously.[9] These vectors carry the *tenA* and *tenB* coding regions inserted in antisense orientation for expression from the strong constitutive *Aspergillus nidulans gpdA* promoter (P_{gpdA}). In *B. bassiana* transformants they appear to cause complete knockdown of their respective encoded proteins, such that *B. bassiana* transformed with

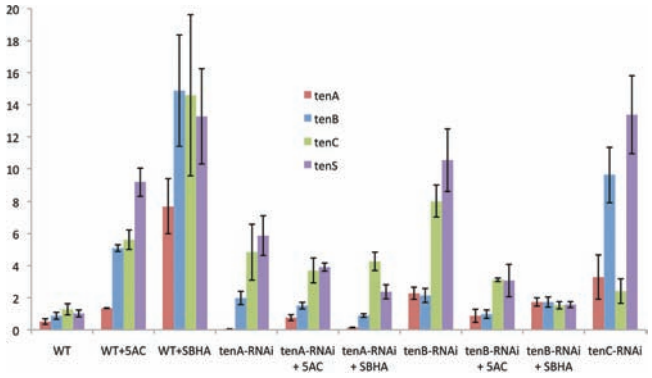


Figure 4. Relative concentrations of mRNA extracted from WT and gene-silenced transformants of *B. bassiana* cultured under the indicated conditions. All concentrations are shown relative to the concentration of *tenS* in the unmodified fermentation of WT *B. bassiana*.

the P_{gpdA}·anti-*tenA* has the same phenotype as a *tenA* knockout strain, i.e., production of the tetramic acid pretenellin A (**2**) and no production of pyridones.[9] Likewise, the P_{gpdA}·anti-*tenB*-silenced strain produces the pyridone pretenellin B (**3**) but no *N*-hydroxypyridones (Table 1).

We next performed epigenetic modification of the silenced strains (Table 1) by growing them in the presence of 5AC and SBHA. Once again these experiments yielded new compounds. For example, the *tenA*-silenced strain of *B. bassiana* produced both *syn* and *anti* diastereomers of 12-hydroxypretenellin A (**17** and **18**) as well as 14-hydroxypretenellin A (**19**) in the presence of either SBHA or 5AC. As observed previously, the 5AC experiments produced higher titers.

Both sets of experiments also resulted in the production of the reprogrammed compound prototenellin E (**16**). Additionally, the *tenB*-silenced strains produced a range of reprogrammed olefin isomers in the presence of 5AC. For example, (10,11-*Z*)-pyridovericin (**22**, 11 $mg \cdot L^{-1}$) and both *syn* and *anti* diastereomers of (10,11-*Z*)-13-hydroxypretenellin B (**23** and **24**, 8 and 13 $mg \cdot L^{-1}$, respectively) were produced (Table 1, Figure 3). It is highly unlikely that these *Z*-isomers could be isolation artifacts, i.e., arising by an isomerization process, as they were not observed in parallel experiments using the *tenB*-silenced strain with SBHA where the analogous 10,11-*E* compounds **20** and **21** were isolated and characterized using identical methodology. In the presence of 5AC, the *tenB*-silenced strain also produced prototenellin F (**25**, 9 $mg \cdot L^{-1}$), in which the side chain had cyclized. Another hydroxylated compound was also observed; either epigenetic modifier in combination with RNAi silencing of *tenB* produced *anti*-13,15-dihydroxypretenellin B (**26**).

qRTPCR EXPERIMENTS

Mycelia were collected from all fermentations after 9 days and used to prepare total RNA. This was used as a template for quantitative reverse transcription PCR (qRTPCR)[23,24] for the four known tenellin biosynthetic genes. The *B. bassiana* β-tubulin gene was used as a control, and all results were normalized to its relative transcript concentration. The concentration of β-tubulin varied by no more than a factor of 2 across all these experiments (i.e., less than 1 ct unit), indicating very good biological replication. Furthermore, melting analysis for all qRTPCR products indicated high specificity of the primers used (efficiencies between 0.97 and 1.08; see Supporting Information).

The results of this analysis (Figure 4; see Supporting Information for full data) show significant variations in concentration of mRNA for the various tenellin biosynthetic genes under the different fermentation conditions. In the unmodified organism it appears that transcript concentration is roughly equal for the four genes within error (i.e., within 0.8 ct unit, or a factor of less than 2).

As expected, in the *tenA*-silenced strain, the *tenA* transcript was only detected at very low concentration. This correlates with the absence of production of any pyridones in this strain. However, unexpected changes were also observed, including an increase in the overall concentration of all mRNAs (*e.g. tenB* by a factor of 2, *tenC* by a factor of 3.5 and *tenS* by a factor of 5.5). Thus silencing of *tenA* appeared to affect the regulation of the other genes in the cluster.

Likewise, silencing of *tenB* led to similar results: an overall increase in the concentration of all tenellin biosynthetic transcripts, but a relative lowering of expression of *tenB* itself compared to the others, consistent with the inability of this strain to produce *N*-hydroxylated pyridones. The relatively low level of *tenA* transcript correlates with a lower proportion of pyridones produced by this strain (35%) than in unmodified *B. bassiana* (62%).

Addition of SBHA or 5AC to unmodified *B. bassiana* led to a dramatic up-regulation of transcription of all the *ten* genes. This varied between a roughly 1.5-fold uplift for *tenA* in the presence of 5AC to a 17-fold increase for *tenB* in the presence of SBHA. Again, ratios of specific mRNA varied. In the case of 5AC, *tenC* titers were only approximately half those of *tenS*, but SBHA produced the opposite effect, with *tenC* transcript slightly more abundant than *tenS*. As indicated above, the general up-regulation of the genes correlates with large increases in titer in these experiments. For example, *B. bassiana* + 5AC produced nearly 3-fold more metabolites, while growth in the presence of SBHA produced nearly double the metabolites of the unmodified fermentation.

Epigenetic modification plus silencing appears to have caused a combination of effects. A general uplift in overall transcript concentrations compared to unmodified *B. bassiana* is apparent, but accompanied by changes in ratios of the individual species with some notable effects. In the case of *tenB* silencing, for example, no *N*-hydroxylated species were produced in the absence of epigenetic modifiers, whereas tenellin **1** itself (13 $mg \cdot L^{-1}$) and the *N*-oxygenated macrolide **10b** (8 $mg \cdot L^{-1}$) were produced by the *tenB*-silenced strain in the presence of 5AC. This observation correlates with a small relative uplift in *tenB* transcript concentration in the presence of 5AC (Figure 4) and shows how the addition of epigenetic modifiers can counteract silencing.

Knowing that the presence or absence of the *tenC*-encoded *trans*-acting ER can dramatically affect programming,[9] we set out to test whether varied levels of this protein could directly influence programming. We therefore transformed *B. bassiana* with the silencing construct P_{gpdA}anti-*tenC*, which was created in the same way as the *tenA* and *tenB* silencing systems. Fermentation of *B. bassiana* transformants led to the production of a number of compounds, including tenellin (**1**) and prototenellin D (**7**). Reprogrammed compounds were also observed, including prototenellin A (**4**), prototenellin B (**5**), and prototenellin E (**16**).

The overall production of compounds is comparable with the other RNAi and wild-type (WT) experiments (146 $mg \cdot L^{-1}$), but the reprogrammed compounds make up 22% of the total. Transcript levels were again assessed by qRTPCR, revealing a

13-fold increase in *tenS* transcript, as well as an 11-fold increase in *tenB*. In fact, the concentration of *tenC* also increased (roughly 1.5-fold), but its concentration relative to *tenS* fell to below 20% from roughly parity in the WT strain.

Prototenellins A and B were expected products as these are formed in the absence of the *trans*-ER encoded by *tenC*,[9] but the other compounds have been correctly reduced, showing that at least some *trans*-acting ER must be present, consistent with the detection of *tenC* by qRTPCR. An examination of relative quantities of reprogrammed compounds present in each experiment (Table 1) shows that the *tenC*-silencing and *tenB*-silencing +5AC experiments generated the highest proportions (20−22%) of these compounds. However, comparison of the transcript concentrations for these two experiments does not reveal a simple relationship.

CONCLUSIONS

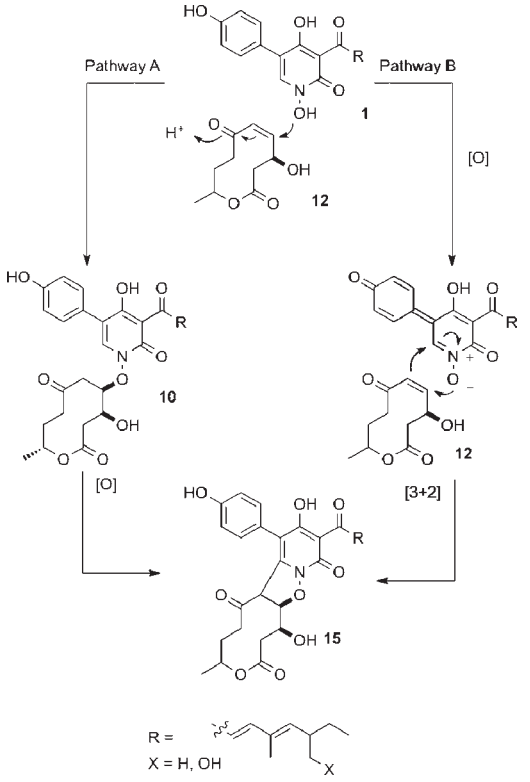
The biosynthesis of most natural products is often viewed as a highly efficient process, the efficiency largely arising because of the high selectivity and fidelity of each biosynthetic reaction. Under laboratory conditions it is often observed (and it is indeed highly desirable) that organisms produce either a single compound or a small family of closely related compounds. For example, four closely related compounds are produced during the biosynthesis of tenellin (**1**) in *B. bassiana* CBS110.25 under standard conditions. Pretenellin A (**2**) is a precursor of **1**, and this appears to undergo a shunt reaction when it is hydroxylated to form prototenellin D (**7**). Tenellin itself can also be hydroxylated at a different position and presumably by a different monooxygenase to produce 15-hydroxytenellin (**8**). All four compounds possess the same polyketide carbon skeleton, indicating the high fidelity of the tenellin polyketide synthase component of TENS. However, our previous expression studies with *tenS* showed that the TENS PKS is inherently capable of generating diverse polyketide structures,[6] an observation that appeared at odds with the apparently high fidelity of tenellin biosynthesis in the WT organism.

The experimental results described here indicate that *B. bassiana* is indeed capable of generating significant chemical diversity based around the biosynthesis of **1**. A total of 22 different compounds have been observed arising from gene silencing, attempted epigenetic modification, and a combination of the two. This diversity arises from four main sources.

First, as is the case with most filamentous fungi, oxidative enzymes such as cytochrome P450 monooxygenases are common and can diversify carbon skeletons by hydroxylation at unactivated positions.[25] *B. bassiana* CBS 110.25 appears capable of producing enzymes which hydroxylate at the aliphatic 12- and 14-positions, as well as the benzylic position of pretenellin A. It is possible that a single monooxygenase with low selectivity could be responsible for all the observed hydroxylations on the side chain, with a second enzyme responsible for the benzylic hydroxylation to form prototenellin D (**7**). A related effect has been observed by Vederas and co-workers during the biosynthesis of lovastatin: knockout of *lovA* involved in selective oxidation of a dihydrodecalin precursor led to the upregulation of a series of unrelated and nonselective oxidases.[26]

Second, the biosynthesis of new metabolites, such as the putative decanolide precursor cephalosporolide B (**12**), can be induced together with enzymes that link this to the tenellin skeleton (Scheme 2). *B. bassiana* CBS 110.25 has not previously been reported to produce compounds related to pyridomacrolidin (**15**).

Scheme 3. Proposed Biosynthesis of Pyridomacrolidin (15) via Prepyridomacrolidin (10)

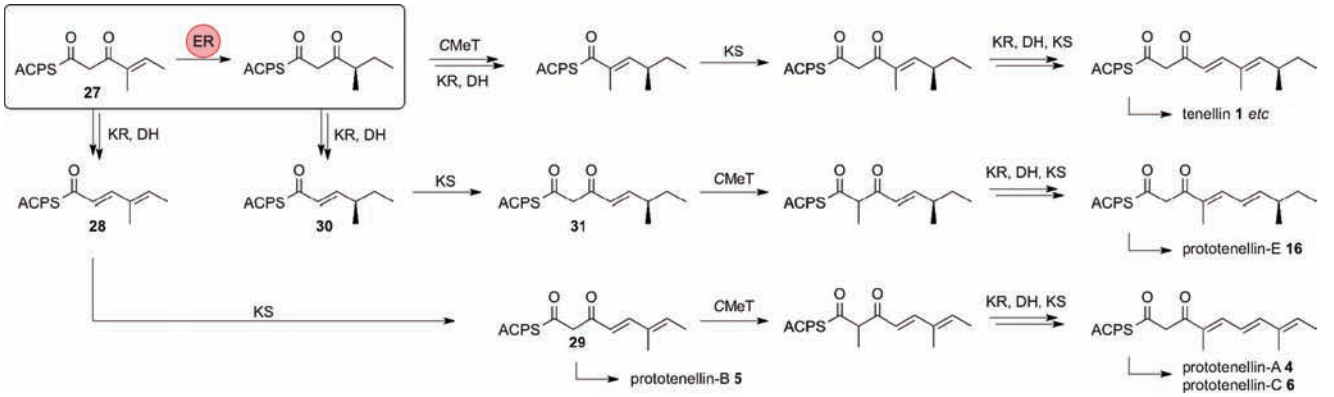


This compound is itself interesting—Baldwin and co-workers, for example, suggested that it might arise by an oxidation of the pyridone followed by a single-step [3+2] cycloaddition with a suitable decanolide (Pathway B, Scheme 3).[27] However, our results suggest that construction of the isoxazolidine ring of **15** probably arises by a stepwise addition−oxidative ring-forming sequence in which prepyridomacrolidin (**10**) is an intermediate (Pathway A, Scheme 3).

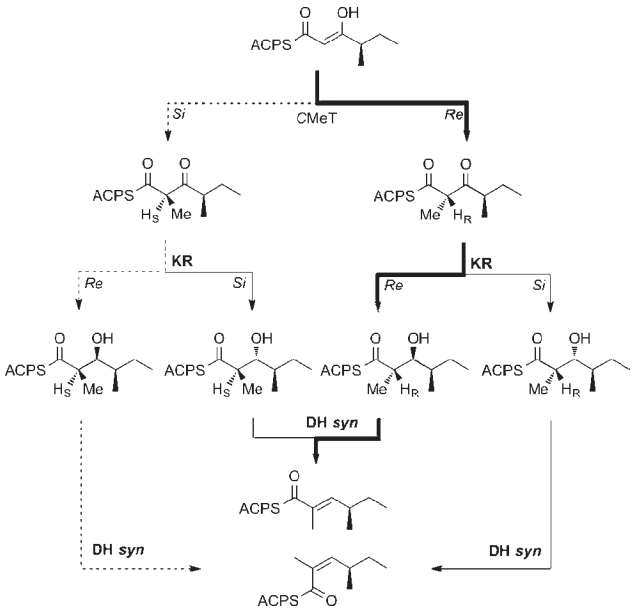
Third, and unexpectedly, diversity arises here by reprogramming of the polyketide synthase. Prototenellin A (**4**), the product of altered methylation and reduction, has previously only been observed in experiments where TENS functions in the absence of the *tenC*-encoded ER in a heterologous host.[6] However, **4** was observed in the native host *B. bassiana* for the first time when 5-AC was added to the medium or *tenC* was silenced. Prototenellin E (**16**) features the same methylation pattern as **4** but an unchanged reduction pattern, and this was also observed when the epigenetic modifiers SBHA or 5AC were added to the culture medium. The chain-shortened compound prototenellin B (**5**) was also observed when *tenC* was silenced.

The methylation-reprogrammed compounds reveal information about the selectivities of various catalytic domains of TENS (Scheme 4). In the absence of the *trans*-acting ER, it appears that the triketide β-keto intermediate **27** cannot be methylated; instead it is reduced and dehydrated to give **28** before a further extension gives substrate **29**, which can either act as a substrate for NRPS off-loading to give prototenellin B (**5**) or be further methylated, ketoreduced, and dehydrated before further chain extension and release as prototenellin A (**4**). Similarly, alkenic triketide **30** can be chain-extended to the corresponding

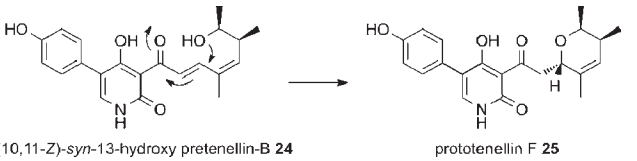
Scheme 4. Reprogramming of Methylation and Subsequent Steps



Scheme 5. Analysis of the Stereoselectivities of the CMeT, KR, and DH Reactions Catalyzed by TENS



Scheme 6. Formation of Prototenellin F (25)



tetraketide **31**, leading to prototenellin E (**16**). These observations suggest that the CMeT is unable to recognize a substrate with a methylated sp^2 carbon at the γ-position.

We also observed, for the first time, the presence of 10,11-*Z* compounds. These arose in combined silencing and epigenetic modification experiments. The possibility that these compounds were artifacts of the isolation (i.e., isomerization of 10,11-*E* compounds) can be ruled out because the corresponding 10,11-*E* compounds were isolated and characterized from different experiments where the *Z*-compounds were not present.

Fatty acid KR reactions are known to produce 3-*R*-3-hydroxyacyl intermediates by *Si* hydride addition to the β-ketothiolester.[28] Vederas and co-workers have shown that, for the highly reduced polyketide cladosporin, the KR gives a 3-*S* intermdiate via *Re* hydride addition (bold lines in Scheme 5).[29] All known DH enzymes operate with *syn* selectivity,[30] and it is likely that the TENS DH is no different, as the key histidine and aspartic acid active-site residues are conserved between mammalian FAS[31] and TENS DH domains (see Supporting Information). If *Re* and *syn* stereoselectivity operate in the case of the TENS KR and DH domains, then *C*-methylation would have to occur on the *Re* face of the molecule (Scheme 5, bold lines). Whatever the actual stereoselectivities of the TENS CMeT and KR steps, the *Z*-selectivity of olefin formation must arise from an alteration in either CMeT or KR selectivity (assuming DH remains *syn*-selective), but not both. Thus, if the CMeT acts with opposite selectivity and the KR is unchanged (dashed arrows, Scheme 5), or the CMeT is unchanged but the KR is reversed, a *Z*-olefin will arise.

Thus, all the observed reprogrammed compounds could arise by changes in the selectivity and reactivity of the CMeT domain alone. The mechanism for this observation is not revealed by these experiments, and such questions will require detailed *in vitro* investigations to resolve them.

The fourth way in which diversity can arise is the cumulation of altered chemical steps which can lead to compounds such as (10,11-*Z*)-*syn*-13-hydroxypretenellin B (**24**); this can undergo further potentially spontaneous reactions such as cyclization to form prototenellin F (**25**, Scheme 6).

None of the *B. bassiana* experiments described here have involved alteration of the genomic contexts of any of the *ten* genes—specifically, the genes themselves have remained unchanged. The metabolic changes have occurred as a result of changing levels of gene expression rather than changing the genes *per se*. Two important conclusions can be drawn from this observation. First, it is evident that *B. bassiana* can generate much chemical diversity from a limited gene set; second, it can do this rapidly without having to change the genes themselves. It appears that chemical diversity is in fact an inherent property of the biosynthesis of tenellin and that this property can be influenced by both internal (e.g., silencing) and external (e.g., epigenetic) stimuli, and such a mechanism could form the basis for the evolution of secondary metabolites. Organisms capable of producing only a single compound from a tightly programmed pathway would not be able to rapidly alter the compound as the

result of a new environmental challege. In contrast, the ability of a population of individuals to each produce a library of compounds through inherent plasticity of biosynthesis could allow rapid optimization of a diverse range of related compounds toward a "useful" compound. This is because the spectra of compounds produced by different individuals in the population would be diverse, and an environmental challenge could select individuals producing a higher proportion of a now more-desirable compound. Firn and Jones have argued for just such a mechanism during the evolution of secondary metabolism, but few good example are known to support their hypothesis.[32] Our results suggest that the tenellin PKS, and perhaps other fungal PKS, should be regarded as fundamentally diverse in their programmed ability to make compounds and that this diversity can be influenced by environmental factors.

We began by hypothesizing that differences in gene expression levels might be a mechanism by which *B. bassiana* could control the program of TENS. We measured gene expression levels during the production of compounds by the *ten* gene cluster under varied conditions. Gene expression levels certainly vary in the different experiments, and it is noteworthy that silencing *tenC* leads to one of the greatest changes in PKS programming. A similar, albeit low-level (1%), reprogramming of the lovastatin nonaketide synthase (LNKS) has been reported by Vederas and co-workers, who observed that heterologous coexpression of *lovB* (encoding LNKS which is homologous to TENS) with *lovC* (homologous to *tenC*) produced an unsaturated analogue of the normal product dihydromonacolin L.[33] This effect was attributed a mismatch in expression levels of *lovB* and *lovC*. However, in the tenellin system this effect appears not to be directly linked to transcript level because a high level of reprogramming was observed in the *tenB*-silencing + 5AC experiment in which *tenC* transcript levels were not significantly reduced. It should be noted that it is proteins which produce compounds through catalysis, and measurement of transcript levels serves only as a proxy for protein concentrations. Increased gene expression could lead to increased protein production, and increased protein concentration could conceivably affect protein−protein interactions and thus programming. However, in the absence of the ability to accurately measure *in vivo* protein concentrations in fungi, this must remain speculation. Furthermore, the epigenetic modifiers could cause wider-spread metabolic changes, including those affecting the availability of precursors (such as SAM, for example) which could also affect both titer and programming. More detailed *in vitro* experiments will be required to address this.

We also observed that silencing specific biosynthetic genes does not always lead to the expected results. This is clearly observed in the case of *tenC* silencing. Here the levels of *tenB* and *tenS* were *increased* very dramatically relative to WT levels, while *tenA* transcript concentration remained unchanged. *TenC* itself was increased, but by far less than the *tenS* and *tenB* transcripts. Once again the mechanisms for these effects are not clear. While silencing has been used widely in fungi to investigate its effects on single genes, we believe this is the first systematic examination of silencing within a biosynthetic gene cluster, and further studies in other clusters will have to be performed to discover if this effect is general.

The effect of epigenetic modifiers was to generally increase transcription of *tenS* genes. This is consistent with the effects of these compounds in other fungi, where treatment with 5AC and SBHA can lead to the activation of so-called silent biosynthetic gene clusters, e.g., decanolide.[14] In the case of *B. bassiana* CBS 110.25, we observed that both the total titer and the diversity of compounds produced increased. In fact, we observed cases where the up-regulation by the epigenetic modifiers appeared to overcome silencing; *tenB*-silenced *B. bassiana*, for example, does not make *N*-hydroxypyridones, but the combination of silencing plus epigentic modifiers led to an apparent increase in the *tenB* transcript and the production of tenellin (**1**) and the prepyridomacrolidins (**10**).

Fungi continue to be a highly valuable source of diverse secondary metabolites, but the approaches used to explore and exploit them as sources of bioactive compounds have undergone a paradigm shift. Current efforts focus on whole genome sequencing for the discovery of new biosynthetic gene clusters and the use of epigenetic modifiers to activate "silent" pathways.[14] Our results suggest that these strategies should be pursued with caution. First, it is evident that a gene cluster represents only the biosynthesis of a core metabolite; other modifying reactions can be performed by enzymes encoded by genes elsewhere in the genome. This has been nicely exemplified recently by Wang, Oakley, and co-workers, who have shown that prenyl transferases required for the biosynthesis of prenylated xanthones in *A. nidulans* are located distant from the core biosynthetic gene cluster.[34] Such genes may or may not be expressed under differing environmental conditions. Second, our investigations of the tenellin cluster indicate that polyketide biosynthesis is inherently diverse and can be reprogrammed, again in response to environmental stimuli. Third, it is clear that epigenetic modifiers do not affect gene expression in an even way, and that differential gene expression could again lead to reprogramming. Finally, it is clear that fungi can exert complex and as yet not understood mechanisms by which a fixed complement of genes can be rapidly exploited for the generation of a diverse library of related compounds.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for chemical and biological work, typical chromatograms, and multiple alignment of mFAS, TENS, and DMBS sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Note that numbering conventions are different for the tetramic acid and pyridone series of compounds.
(8) Naming conventions: We refer to genuine precursors with the name prefix *pre-*; these are compounds which lie on the biosynthetic route to the final compound. For example, pretenellin A (**2**) is a direct precursor of tenellin (**1**). Compound names with the prefix *proto-* refer to shunt or reprogrammed metabolites which cannot be precursors. For example, prototenellin A (**10**) cannot be a precursor of tenellin (**1**). In these cases A, B, C, etc. refer to the order in which the compounds were discovered.
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