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Fungal Polyketide Synthase Product Chain-Length Control by Partnering Thiohydrolase

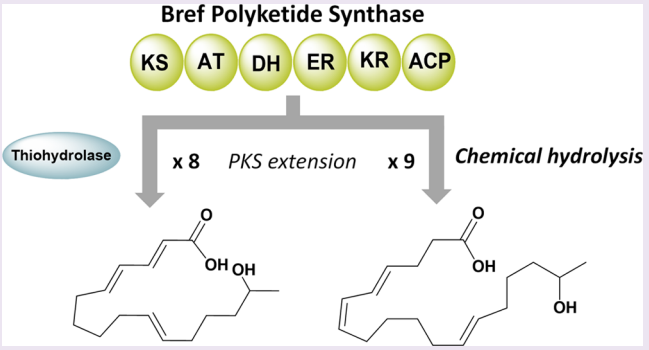
Angelica O. Zabala,[†] Yit-Heng Chooi,[§] Moon Seok Choi,[†] Hsiao-Ching Lin,[†] and Yi Tang*[,†,‡]

[†]Department of Chemical and Biomolecular Engineering and [‡]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States

[§]Research School of Biology, Australian National University, Canberra, ACT 0200, Australia

Supporting Information

ABSTRACT: Fungal highly reducing polyketide synthases (HRPKSs) are an enigmatic group of multidomain enzymes that catalyze the biosynthesis of structurally diverse compounds. This variety stems from their intrinsic programming rules, which permutate the use of tailoring domains and determine the overall number of iterative cycles. From genome sequencing and mining of the producing strain *Eupenicillium brefeldianum* ATCC 58665, we identified an HRPKS involved in the biosynthesis of an important protein transport-inhibitor Brefeldin A (BFA), followed by reconstitution of its activity in *Saccharomyces cerevisiae* and in vitro. Bref-PKS demonstrated an NADPH-dependent reductive tailoring specificity that led to the synthesis of four different octaketide products with varying degrees of reduction. Furthermore, contrary to what is expected from the structure of BFA, Bref-PKS is found to be a nonaketide synthase in the absence of an associated thiohydrolase Bref-TH. Such chain-length control by the partner thiohydrolase was found to be present in other HRPKS systems and highlights the importance of including tailoring enzyme activities in predicting fungal HRPKS functions and their products.



Fungal polyketides constitute an important group of natural products that includes statins, antibiotics, and anticancer agents.[1,2] The biosynthesis of polyketide in fungi is performed by Type I iterative polyketide synthases (IPKSs), which are multidomain megasynthases.[3] The highly reducing IPKSs (HRPKSs) are a large subgroup of the IPKSs that are associated with the biosynthesis of highly reduced compounds, such as lovastatin and fumonisin. The minimal PKS domains of HRPKSs, which consist of ketosynthase (KS), malonyl-CoA:acyl carrier protein transacylase (MAT), and acyl carrier protein (ACP), catalyze the selection of malonyl building blocks and the repeated Claisen-like chain extension steps. HRPKSs also consist of a set of tailoring domains, including ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and the C_α-methyltransferase (MT). Although the domain organization of HRPKSs bears strong resemblance to that of mammalian fatty acid synthases (FASs), the HRPKSs operate in a much more sophisticated fashion.[4] Most notably, the HRPKSs use the single set of tailoring domains in different permutations during every extension cycle, which results in the high degree of variability at individual α- and β-positions in the products. Product chain-length control also varies between different HRPKSs, which results in polyketides that have a wide range of sizes. Additionally, HRPKSs differ from FASs in that there is no dedicated and fused thioesterase (TE) domain at the C-terminus of the megasynthase and instead rely on the *in trans* interaction with discrete TE or acyltransferase-like enzymes for product release.[2] Our current understanding of these unique features of HRPKSs has remained at an early stage, thereby limiting our ability to link reduced polyketides to corresponding HRPKSs and to predict product structures from the vast number of HRPKSs identified from fungal genome sequencing efforts.

In order to better understand these enigmatic features of HRPKSs, it is important to work with a suitable model system for biochemical analysis. First, the model HRPKS should be a standalone enzyme that generates a product of substantial chain length that could be detected and analyzed (i.e., UV-active). This circumvents the dependence on downstream enzymes (e.g., other PKSs) for further modifications that convolutes product analysis. Second, the HRPKS should be programmed to synthesize a product with variable degrees of β-reduction within each extension cycle to allow investigation of the permutative tailoring rules. Lastly, it is ideal to work with an HRPKS that is involved in the biosynthesis of a bioactive polyketide product to aid the re-engineering of the HRPKS for analogue generation.

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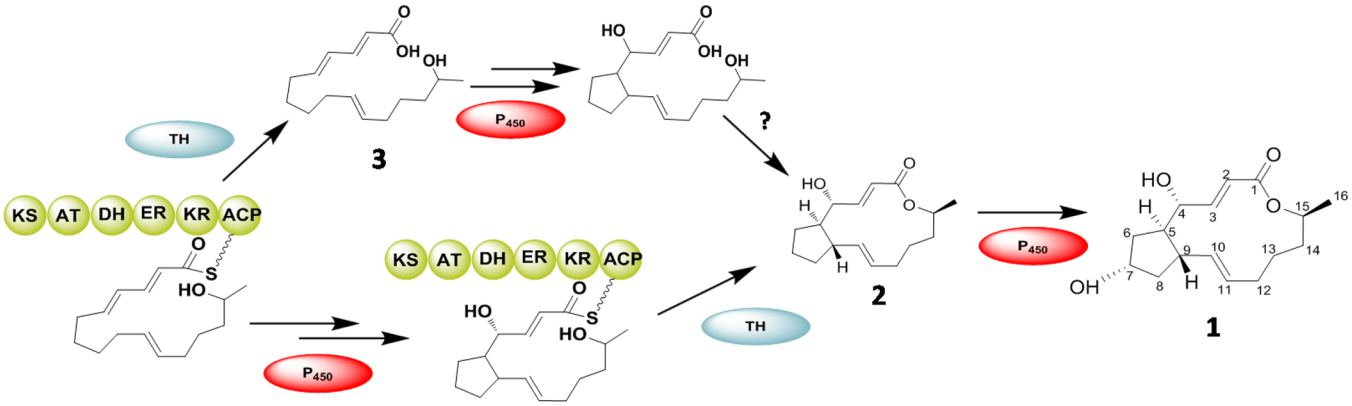

Figure 1. Putative biosynthethic pathway for **1**. The HRPKS is proposed to synthesize the precisely reduced octaketide precursor, which could then be directly offloaded by the thiohydrolase enzyme followed by a P450-mediated formation of the cyclopentane ring and macrocyclization to afford the 7-deoxy BFA **2** (top scheme). Alternatively, the first ring annulation can also occur on the ACP-tethered intermediate before the thiohydrolase release and lactonization (bottom scheme). The C7-hydroxylation is believed to be the final step in the process to obtain the final structure of **1**.

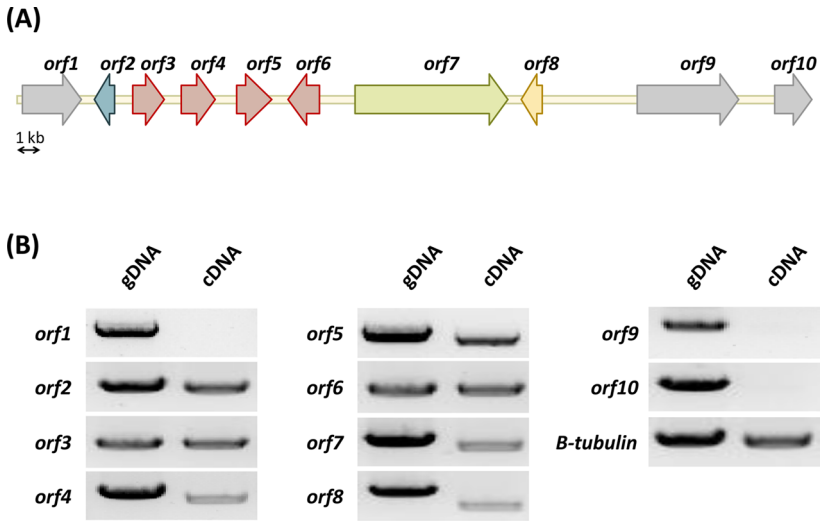

Figure 2. Transcriptional analysis of genes in Contig_286 determines the putative boundary of the *bref* cluster. (A) Arrangement of genes in Contig_286. (B) RT-PCR analysis on the annotated genes within the contig. The template mRNA was extracted from a Day2 BFA-producing culture of *E. brefeldianum* in the optimized production media, MEM.

Several HRPKSs involved in the biosynthesis of pharmaceutically important polyketides have been investigated, such as LovB and Hpm8 that are responsible for the biosynthesis of lovastatin and hypothemycin, respectively. In the LovB system, however, a yet unresolved Diels−Alder cyclization step embedded among the chain extension steps has complicated analysis of the HR-PKS alone.[5,6] On the other hand, HRPKSs from resorcylic acid lactone (RAL) pathways (such as Hpm8) and from HRPKS-nonribosomal peptide synthetase (NRPS) hybrids require downstream enzymes/domains for product transfer and further modification, which represents an added level of complexity in deconvoluting HRPKS functions and products.[7−9]

In this study, we chose the HRPKS responsible for brefeldin A (BFA) as a model system (Figure 1). BFA (**1**) is a protein-transport inhibitor isolated from several species of filamentous fungi.[10−14] It is used to study protein transport among eukaryotes but has also been found to have antiviral, antifungal, and antitumor properties.[15] The polyketide origin of this 16-membered macrolactone was previously established through feeding studies with labeled acetate.[16−18] The proposed biosynthesis of BFA involves formation of an acyclic polyketide chain that is differentially tailored throughout the backbone (Figure 1). The presence of the terminal hydroxyl group, along with the strategically positioned double bonds, is proposed to enable cyclization of the acyclic precursor into the fused, bicyclic structure seen in BFA. We hypothesized that a single HRPKS should be sufficient to generate the entire carbon backbone without the need for an additional PKS. If the acyclic product indeed contains conjugated double bonds as proposed, the biosynthetic product should be readily identifiable and isolated. Collectively, the BFA HRPKS appears to fit the mold as a good model HR-PKS for biochemical analysis. Here, we first identified the HRPKS most likely responsible for BFA biosynthesis from *Eupenicillium brefeldianum* ATCC 58665. We further demonstrate that using a product-based assay, important programming rules of HRPKSs were elucidated, including NADPH concentration-dependent extent of reduction and the control of HRPKS product chain length by an associated discrete thiohydrolase (TH). These insights further improve our understanding of how HRPKSs function.

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RESULTS AND DISCUSSION

Identification of the Brefeldin A PKS by Bioinformatic and Transcription Analysis. The genomic DNA of the BFA-producing strain *E. brefeldianum* ATCC 58665 was sequenced using Roche (454) GS FLX Titanium series and Illumina HiSeq 2000. The resulting GS FLX Titanium reads were first assembled using GS de novo assembler; the output contigs in FASTA format were then combined with the supplementary HiSeq 2000 reads in a hybrid assembly using the Geneious Assembler embedded in the Geneious software suite.[19] The hybrid assembly generated 708 scaffolds consisting of nearly 36 Mbases of nonredundant reads that roughly reflect the *E. brefeldianum* genome size. A local BLAST database was created using the scaffolds. Using the KS domain of the nonaketide synthase LovB as a query sequence, 24 putative PKSs were identified: 11 HRPKSs; eight nonreducing PKSs (NRPKSs); two partially reducing PKSs (PRPKS); and two HRPKS-NRPS hybrids (Supplementary Figure S1). The lack of C_α-methylation in BFA excluded the MT-containing HRPKSs, narrowing down the search to five HRPKS-containing gene clusters. Subsequently, RT-PCR was performed on the mRNA of the BFA-producing culture to determine the transcription of the HRPKS genes, of which only Contig_286 PKS was highly transcribed at the time point that coincided with BFA production (Figure 2 and Supplementary Figure S2), indicating the high likelihood of this HRPKS being involved in BFA biosynthesis.

The HRPKS of Contig_286 (*orf7*) contains the following domains linearly juxtaposed from N- to C-terminus: KS, MAT, DH, ER, KR, and ACP. Using the maximum likelihood statistical method on MEGA Version 5, a phylogenetic tree with HRPKSs of known natural products was constructed.[20] Contig_286 HRPKS cladded with those that biosynthesize relatively longer (>C10) acyclic polyketides such as the polyene portion of fumagillin and fumonisin (Supplementary Figure S3). The closest homologue is an uncharacterized HRPKS from *Trichoderma virens* Gv29-8, TRIVIDRAFT_151590 (59% identity, 73% similarity). Within the 37,663 bp of Contig_286, nine other open-reading frames (ORFs) were identified using Softberry prediction (Table 1). These include an α–β hydrolase (*orf2*), which interestingly has the closest homologue, TRIVIDRAFT_53350 (60%, 77%), encoded immediately upstream of the TRIVIDRAFT_151590 PKS gene in *T. virens* Gv29-8. Hence, this may be the partnering enzyme involved in the release of the ACP-tethered polyketide thioester product via either hydrolysis to yield an acyclic product (as a thiohydrolase) or macrocyclization to yield a cyclized product (Figure 1). The neighboring genes are consistent with that of a possible BFA biosynthetic gene cluster, including four genes encoding P450 monooxygenases (*orfs 3*–*6*). Previous feeding studies have shown that the C4 and C7 hydroxylation in BFA resulted from oxidative tailoring, while the cyclopentane ring formation was similarly proposed to be P450-mediated.[17,21] Hence the collection of P450s here may be responsible for these transformations on the polyketide product. To analyze if these genes are co-transcribed with the HRPKS gene in this cluster, a transcriptional analysis was performed using gene-specific primers (Figure 2B). All P450s and TH encoding genes were highly transcribed in the producing culture, along with *orf8* of unknown function. These genes were putatively assigned to comprise the *bref* cluster, with the HRPKS and TH renamed to Bref-PKS and Bref-TH, respectively.

Table 1. Genes Annotated in Contig_286

gene	proposed function (domain organization)	sequence homologues (identities/positives)
orf1	F-box domain protein	*Aspergillus oryzae* RIB40 BAE55018 (26/42)
orf2/bref-TH	α–β hydrolase	*Trichoderma virens* Gv29-8 TRIVIDRAFT_53350 (60/77)
orf3	cytochrome p450	*Bipolaris maydis* C5 COCHEDRAFT_1151230 (42/60)
orf4	cytochrome p450	*Macrophomina phaseolina* MS6MPH_06433 (46/61)
orf5	cytochrome p450	*Aspergillus oryzae* CYP628C1 (52/70)
orf6	cytochrome p450	*Macrophomina phaseolina* MPH_06428 (46/64)
orf7/bref-PKS	HRPKS (KS-AT-DH-ER-KR-ACP)	*Trichoderma virens* Gv29-8 TRIVIDRAFT_151590 (59/73)
orf8	large tegument protein UL36	*Penicillium chrysogenum* Wisconsin 54-1255 Pc12g10520 (51/65)
orf9	WD-40	*Aspergillus terreus* NIH2624 ATEG_09923 (52/67)
orf10	kinesin	*Bipolaris victoriae* FI3 COCVIDRAFT_89854 (71/80)

Cloning and Expression of Bref-PKS and Bref-TH. The producing strain *E. brefeldianum* ATCC 58665 is a non-sporulating filamentous fungi, and attempts at genetic manipulation were futile due to the multinucleated protoplasts. To examine the activity and product of the Bref-PKS, the 7.1-kb intron-less gene was assembled from five cDNA fragments and placed under control of the ADH2 promoter by yeast recombination in *S. cerevisiae* BJ5464-NpgA (Supplementary Figure S4A).[5] Subsequently, BJ5464-NpgA harboring the Bref-PKS expression plasmid was cultured and grown to stationary phase for protein expression. The hexahistidine-tagged Bref-PKS was solubly expressed and was purified via nickel affinity chromatography at a yield of 2 mg/L (Supplementary Figure S4C). The intron-less gene encoding Bref-TH was similarly constructed from cDNA and was subsequently expressed in *E. coli* BL21(DE3) via an IPTG-inducible T7 promoter and purified using nickel affinity chromatography (Supplementary Figure S4B and C).

We first assayed the activities of the minimal PKS domains (KS, MAT, and ACP) by incubating Bref-PKS with 2 mM malonyl-CoA in PBS buffer, pH 7.4. In the absence of the reducing cofactor NADPH, the reductive domains are expected to be inactive and should yield an unreduced polyketide product. After 16 h, the reaction was either extracted directly or first treated with 1 M NaOH followed by extraction for product analysis. In the absence of base hydrolysis, no product was recovered. With NaOH treatment, we observed the production of 4-hydroxy-6-methyl-2*H*-pyran-2-one (TKL) (Supplementary Figure S5), which forms through the spontaneous cyclization and release of the unreduced triketide.[22] This result indicates that the minimal PKS components were active and product release from the PKSs requires additional factors.

Reconstitution of Bref-PKS Activities. Most HRPKSs do not have a dedicated domain for product offloading, relying instead on an *in trans* acyltransferase or TH to release the polyketide from the ACP tether.[2] Such partnering enzyme plays an important role in terminating the chain elongation and consequently in determining the length of the final product. To investigate the influence of the *in trans* Bref-TH on HRPKS

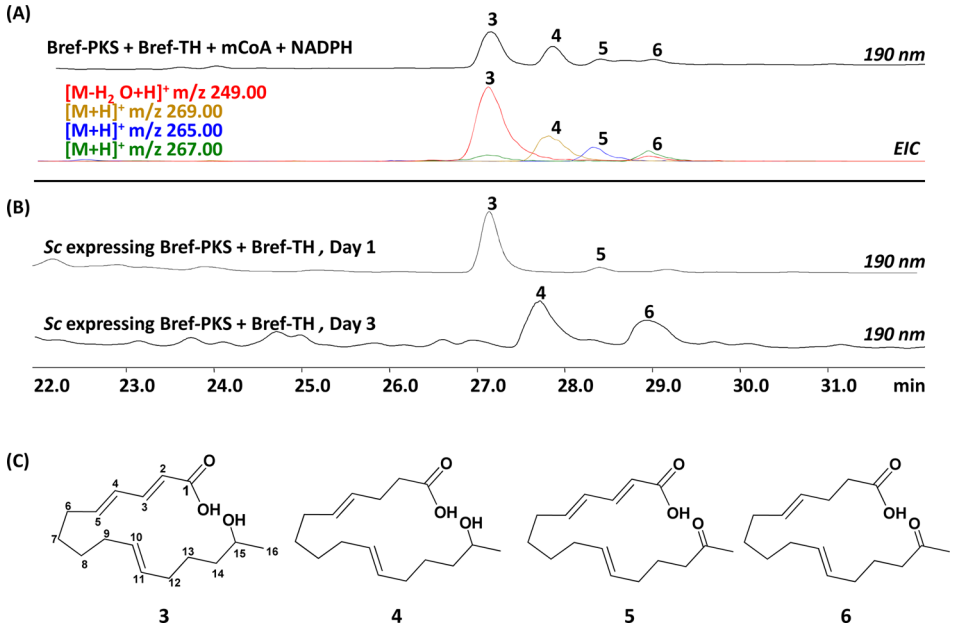

Figure 3. In vitro products of Bref-PKS and Bref-TH are acyclic octaketides with variable degrees of β-reduction. (A) HPLC and EIC trace of the in vitro reaction between Bref-PKS and Bref-TH. (B) Production of compounds **3**–**6** from the *S. cerevisiae*-NpgA strain co-expressing Bref-PKS and Bref-TH. Notice the change in the production profile between days 1 and 3. The compounds were purified according to their peak production period. (C) Elucidated structures of compounds **3**–**6** from the corresponding NMR spectra.

function, the purified enzyme was added to the Bref-PKS in vitro reaction at an equimolar ratio (20 μM) with 2 mM malonyl-CoA and 10 mM NADPH. The reaction was left at RT for 16 h and was extracted with ethyl acetate and analyzed by LC–MS. Four relatively hydrophobic compounds **3**–**6** emerged at the end of the gradient (5%–95% acetonitrile in H_2O, 30 min), with **3** being the dominant product (Figure 3A). The corresponding masses for compounds **3**, **4**, **5**, and **6** are 264, 268, 264 and 266, respectively (Supplementary Figure S6). Compounds **3** and **5** displayed λ_{max} of 260 nm, indicating the presence of a slightly conjugated structure, while compounds **4** and **6** did not have significant absorption above 220 nm. The masses of these compounds are consistent with that of an octaketide that has undergone several reductive modifications (for reference, the molecular weight of palmitic acid is 256).

In order to isolate sufficient amounts of **3**–**6** for structure elucidation, Bref-PKS and Bref-TH were co-expressed under the ADH2 promoter in the yeast host. Compounds **3** and **5** were harvested after 36 h of inoculation (1 and 0.5 mg/L final yield, respectively), while **4** and **6** were extracted after 72 h (2 and 4 mg/L final yield, respectively), depending on their highest production period (Figure 3B). Both **3** and **5** disappeared at the 72 h time point, suggesting the possible conversion to **4** and **6**, respectively. Each compound was purified to homogeneity and subjected to full NMR spectroscopy to elucidate their structures.

From the NMR spectra (Supplementary Table S1 and Figure S15), **3** is determined to be a linear 16-carbon carboxylic acid (δ_{C1} 171.30) (Figure 3C). The ^{1}H NMR spectrum revealed three olefinic groups, one oxygenated methine, seven methylene groups and one methyl group. Based on COSY correlations, two of the double bonds (C2–C3 and C4–C5) form a diene that by HMBC correlations, was further conjugated to carbonyl C1. This conjugated dienoic acid accounts for the higher λ_{max} 260 nm of **3** compared to BFA (λ_{max}= 220 nm). Additionally, the large coupling constants of the connected protons (J_{HH} 15.3) indicate *trans* configurations of the double bonds. The remaining olefinic carbons are found to be at the C10–C11 position from detailed analysis of the COSY and HMBC correlations. The position of the C–OH group at C15 was confirmed by the direct correlation of the doublet C16 methyl protons to the oxygenated methine group. To determine whether this compound is a macrolactone or an acyclic molecule, we compared the carbon chemical shift of C15 to the corresponding carbons in BFA and in an uncyclized BFA analogue. The C15 chemical shift of **3** (δ_{C15} 68.5) accorded with the uncyclized form (δ_C 69.3),[14] as opposed to the slightly downfield shift in the lactone (δ_C 71.8).[23]

The structure of **4** (m/z = 267 [M + H]$^+$) was similarly elucidated from the 1D- and 2D- NMR spectra and from comparison to **3**. Instead of the olefinic protons (δ_H 5.78, 7.22) at the C2–C3 position, the C2–C3 carbons in **4** were found to have methylene protons (δ_H 2.27, 2.31) that were connected to the C4–C5 double bond in COSY (Supplementary Table S1 and Figure S16). This is in accordance with the loss of conjugation in the molecule (λ_{max} 220 nm) relative to **3**. The rest of the NMR signals of **4** are consistent with **3**. Interestingly, **5** (m/z = 265 [M + H] $^+$, λ_{max} 260 nm) and **6** (m/z = 267 [M + H] $^+$, λ_{max} 220 nm) were found to be structurally related to **3** and **4**, respectively, differing by only the absence of the C15 hydroxyl group (δ_{C15} 68.5, δ_{H15} 3.71). Instead, an aliphatic carbonyl signal (δ_{C15} 212) was observed in their respective ^{13}C NMR spectra, which was validated by HMBC correlations and by the appearance of C16 methyl group as a singlet (Supplementary Table S1 and Figures S14 and S15).

It is noteworthy that several important structural features of **3** are consistent with that of BFA including the C16 backbone. Additionally, the C2–C3 and C10–C11 olefinic groups in **3** are retained in BFA. The terminal hydroxyl group that is important for macrocyclization to form BFA is also present. On the other hand, while not observed in BFA, the C4–C5 double bond in **3** appears to be well-positioned for formation of the cyclopentane

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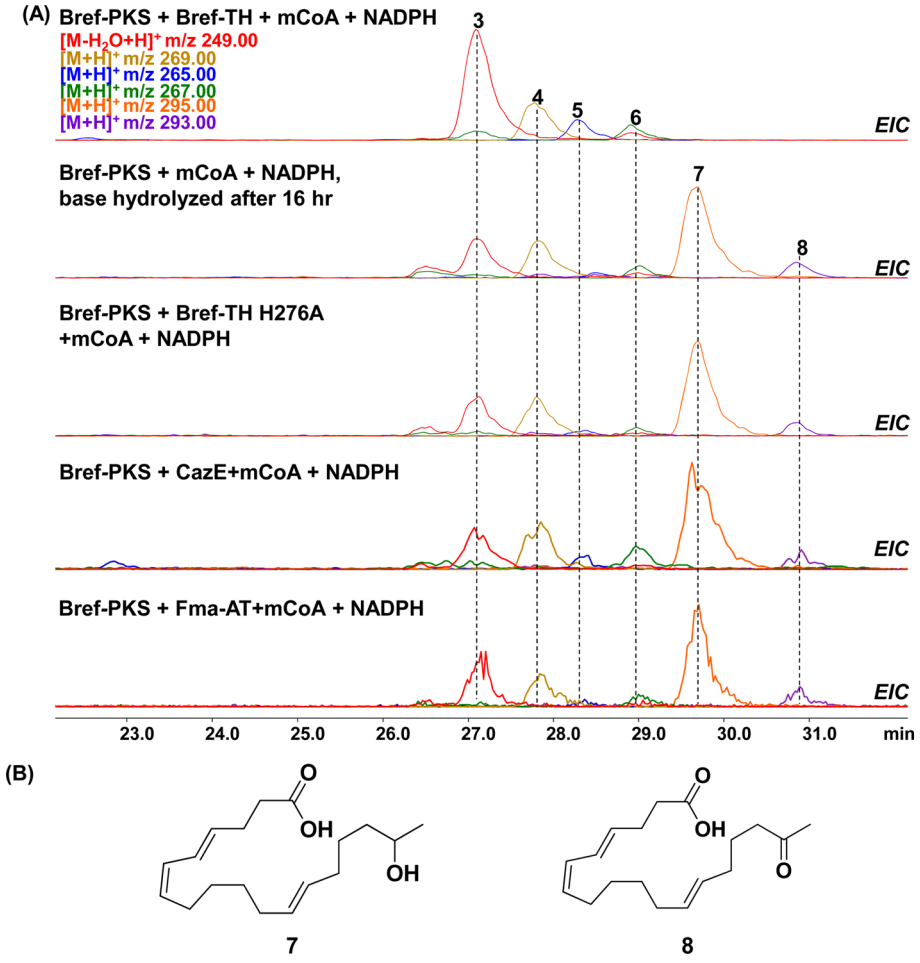

Figure 4. In vitro reactions with Bref-PKS demonstrate the TH-controlled PKS chain length release. (A) EIC spectra of the in vitro reactions showed the variation in the product profiles of Bref-PKS with Bref-TH; Bref-PKS with base hydrolysis; Bref-PKS with Bref-TH H276A; and Bref-PKS with other *in trans* releasing enzymes CazE and Fma-AT. The reactions consist of 20 μM Bref-PKS, 2 mM mCoA, and 10 mM NADPH with either 20 μM concentration of the releasing enzyme or base hydrolysis (1 M NaOH at 65 °C for 10 min). (B) Proposed structures of **7** and **8**.

ring in **1**, presumably mediated by a P450 oxygenase encoded in the *bref* cluster (Figure 1 and Supplementary Figure S14). The structural parallels, both in size and sites of unsaturation/hydroxylation, between **3** and BFA therefore strongly indicate Bref-PKS is indeed the HRPKS involved in BFA biosynthesis.

The discovery of compounds **3**–**6** containing varying degrees of β-reduction by Bref-PKS is unexpected. In generation of the ketones **5** and **6**, it appears the KR domain is prone to skipping ketoreduction of the diketide intermediate, which results in the C15 hydroxyl group. To further investigate the effect of NADPH concentration on the reductive programming rules of Bref-PKS, we performed in vitro assays by varying the NADPH concentrations from 0.5 to 10 mM. While **3** remained as the dominant product at NADPH concentrations higher than 2 mM, compound **5** was found to be the dominant product at lower but still physiologically relevant concentration (<2 mM) (Supplementary Figure S9). Hence, the KR domain is highly sensitive to availability of reducing cofactors, albeit only at the first ketoreduction step during Bref-PKS function. Interestingly, the C15 ketone observed in **5** is also found in the 7-dehydrobrefeldin A acid analogue that contains the cyclopentane ring but is not macrocyclized.[14] Therefore, the KR domain may indeed be imprecisely programmed to act at the C15 ketone in the native producer as well. Such NADPH-dependent tailoring can add another degree of complexity to HRPKSs in generating diversity among fungal polyketides.

The other octaketide products of Bref-PKS, **4** and **6**, lack the α–β double bond (C2–C3) observed in BFA and in **3** and **5**. Both were minimally produced in vitro but were the end products in the yeast in vivo culture after more extended fermentation (Figure 3B). The accumulation of **4** and **6** in yeast coincided with the disappearance of **3** and **5**, which strongly suggests the enoylreduction of **3** and **5** by endogenous yeast enzymes. This was further confirmed by the bioconversion experiments with purified **3** and **5**, in which both compounds were fully converted into **4** and **6**, respectively, by untransformed *S. cerevisiae* BJ5464-NpgA within 24 h of addition to the culture (Supplementary Figure S7). However, the production of **4** and **6** in the in vitro assay also suggests that the ER domains of Bref-PKS can partially reduce the enoyl during the last iteration. No aberrant enoylreduction of the C4–C5 and C10–C11 positions were observed among the products, therefore pointing to a "specific overreduction" of the ER domain at the last iteration.

Bref-TH Controls the Programmed Release of the Octaketide Product from Bref-PKS. Having reconstituted the activities of the Bref-PKS, we then investigated the role of Bref-TH in chain termination and product release. The Bref-PKS assay was repeated without Bref-TH for 16 h, followed by

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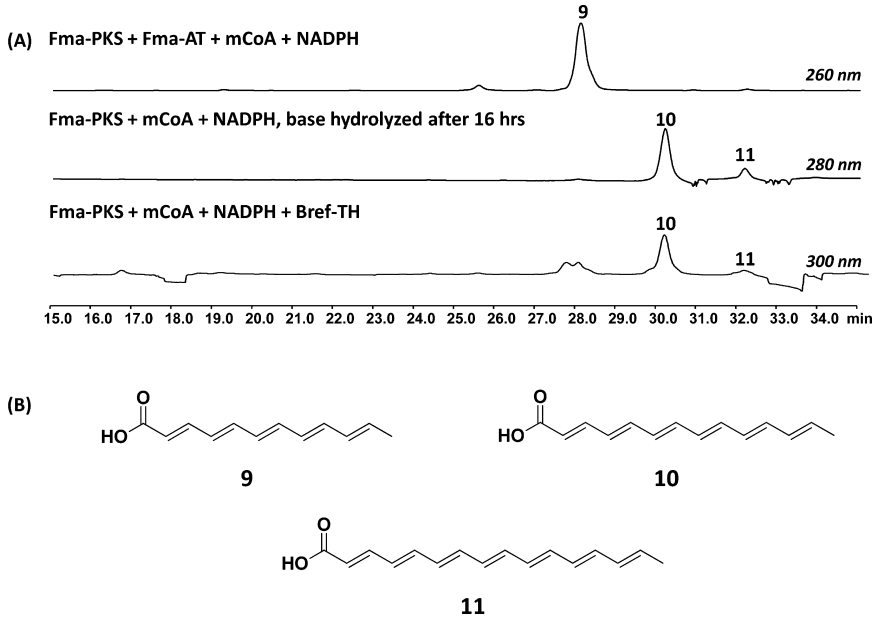

Figure 5. Fma-PKS produces longer polyenes in the absence of cognate Fma-AT. (A) HPLC profiles of Fma-PKS with Fma-AT; base hydrolysis; or Bref-TH. Fma-PKS produces a hexaketide polyene in the presence of the partner Fma-AT. In its absence or in the presence of the non-cognate Bref-TH, the PKS catalyzes 1 or 2 more extension steps to yield the heptaketide **10** and octaketide **11**. The reactions consist of 20 μM Fma-PKS, 2 mM mCoA, and 2 mM NADPH with either 20 μM of the releasing enzyme or base hydrolysis (1 M NaOH at 65 °C for 10 min). (B) Proposed structures of the polyene compounds produced in the in vitro assay.

either direct extraction with ethyl acetate or pretreatment with 1 M NaOH at 65 °C (base hydrolysis) before extraction. The amount of product turnover decreased significantly (10-fold) in the absence of Bref-TH, consistent with the proposed role of the hydrolase in facilitating chain release. When subjected to base hydrolysis or upon subsequent addition of equimolar amount of Bref-TH after 16 h, a higher level of product release was observed (Supplementary Figure S10). The octaketides **3**−**6** were recovered as previously, with the level of **4** now similar to that of **3**. The increased amount of ER over-reduction in the absence of Bref-TH may be due to stalling of the polyketide products on the Bref-PKS in the absence of TH-mediated release.

More importantly, the two new products **7** (m/z 295 $[M + H]^+$) and **8** (m/z 297 $[M + H]^+$) were isolated from the above Bref-PKS assay, with **7** now being the dominant product of all polyketide products (Figure 4A and Supplementary Figure S10). Both compounds displayed λ_{max} of 215 nm, which hints at the absence of conjugated dienoic acid moiety. The +28 mu increase in masses of **7** and **8** compared to that of **3** and **5** suggests the incorporation of a completely reduced ketide unit ($-CH_2-CH_2-$) as a result of an additional round of chain elongation and reduction by the Bref-PKS (Figure 4A and Supplementary Figure S10). Due to the single-turnover nature of the assay in the absence of the Bref-TH, compounds **7** and **8** could not be sufficiently obtained for structural elucidation. Expression of Bref-PKS alone in yeast did not lead to detectable amounts of **7** and **8** either. Therefore, to confirm compound **7** is indeed a nonaketide instead of octaketide, we performed the in vitro assay using 2-^{13}C-malonate and the MatB system, which generates the 2-^{13}C-malonyl-CoA in situ.[24] As expected, an increase of 9 mu in molecular weights was observed for both labeled **7** and **8**, confirming the incorporation of nine ketide units derived from malonate into the backbone (Supplementary Figure S8). Combining the UV and mass data (Supplementary Figures S6 and S8), we propose the structure of **7** and **8** as shown in Figure 4B, derived from an additional round of chain elongation from **3** and **5**, respectively, followed by full β-reduction. Selected ion monitoring of the mass of **7** and **8** in the Bref-PKS assay that contained Bref-TH yielded no trace of these two compounds. These results demonstrate that in the absence of the Bref-TH, the Bref-PKS functions primarily as a nonaketide synthase.

A protein family database search of Bref-TH indicated that the enzyme belongs to the Abhydrolase_6 family (E-value of 3.4 $\times 10^{-14}$). This diverse family is characterized by an α/β hydrolase fold and functions via a proposed catalytic triad.[25] Using Phyre2, we modeled the enzyme and identified the putative catalytic triad to be S116, D247, and H276 that lie within catalytic distances from each other (Supplementary Figure S11B).[26] Each residue was mutated to alanine, and the resulting recombinant proteins were expressed and purified from *E. coli* at comparable yields as wild-type for the in vitro reaction with Bref-PKS.[27] Adding either S116A or D247A mutant to Bref-PKS resulted in a similar product profile (**3**−**6**) as those seen with the wild type Bref-TH. Only the H276A mutant failed to catalyze release of polyketide products and yielded **7** as the major product upon base hydrolysis (Figure 4A). This suggests that only H276 is essential for the chain length-specific hydrolysis of the PKS product, possibly serving as the general base to facilitate the thiohydrolysis. We also probed the specificity of the hydrolase-PKS interactions by incubating Bref-PKS with CazE from the chaetoviridin pathway[22] and Fma-AT from the fumagillin pathway,[28] both known to participate in release of reduced polyketide products from the respective HPPKS partners. In each case, the non-cognate releasing enzymes did not lead to turnover of the octaketides, and the nonaketide products were instead detected using base hydrolysis (Figure 4A).

Chain-Length Control by the Releasing Enzyme Is Also Observed in Other HRPKS Systems. To examine whether chain-length regulation by the releasing enzyme is also

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observed in other HRPKSs, we assayed the Fma-PKS involved in the biosynthesis of the polyene portion of the meroterpenoid fumagillin. Fma-PKS was previously shown to produce the highly conjugated hexaketide pentaenoic acid **9** ([M + H$^+$] 191, λ_{max} 358 nm) in the presence of its releasing acyltransferase partner, Fma-AT.[28] When the Fma-PKS assay was performed in the absence of Fma-AT and subjected to base-hydrolysis followed by extraction, two new products were observed, **10** ([M + H]$^+$ 217) and **11** ([M + H]$^+$ 243), with λ_{max} of 378 and 398 nm, respectively. The periodic increases in both mass (+26 mu) and λ_{max} compared to **9** therefore strongly indicate **10** and **11** are heptaketide and octaketide polyenes, respectively, as shown in Figure 5. This is also verified by the in vitro labeling studies with 2-^{13}C-malonate that showed the corresponding mass shift of +7 and +8 for **10** and **11**, respectively (Supplementary Figure S12 and S13). When Bref-TH is used in the reaction with Fma-PKS, **10** and **11** were also dominantly produced relative to **9** (Figure 5A).

Discussion. In this work, we identified a gene cluster in *E. brefeldianum* that is most likely to be involved in the biosynthesis of the protein transporter inhibitor BFA. BFA is a fungal polyketide that is derived from a highly reduced polyketide synthesized by a HRPKS. The Bref-PKS along with a partnering Bref-TH were reconstituted in *S. cerevisiae* and in vitro. The reconstitution experiments showed that the dominant product of Bref-PKS in the presence of Bref-TH is an acyclic polyketide **3** that is of the same length as BFA and exhibited the expected β-reduction patterns for downstream conversion into BFA (Figure 1). Unexpectedly, Bref-PKS synthesized longer polyketide products in the absence of Bref-TH, implicating an important role of Bref-TH in controlling the chain length of the HRPKS. This phenomenon was also observed in the Fma-PKS and Fma-AT pair involved in fumagillin biosynthesis. The in vitro reconstitution studies reported here were crucial in enabling single turnover experiments (chemical hydrolysis) that were not observable under in vivo conditions.

Reduced polyketides synthesized by HRPKSs require accessory enzymes for product release. In most cases, this is completed by an assortment of enzymes belonging to the α–β hydrolase family. The protein–protein interaction between the HRPKS and the releasing enzyme, which facilitates product turnover and chain length control, is therefore an intricate part of the overall programming rule of these enzymes. When the releasing enzyme is an acyltransferase, the polyketide is either transferred to the hydroxyl group of a small molecule acceptor such as catalyzed by LovD in lovastatin biosynthesis[29] or the free thiol of the ACP domain of a partnering PKS catalyzed by the Starter-Unit:ACP acyltransferase (SAT) in dual PKS systems.[7,8,30] Thiohydrolases such as Bref-TH hydrolyzes the polyketide thioester to release the product. Other examples of TH include LovG that hydrolyses dihydromonacolin L from LovB.[31] Interestingly, the releasing α–β hydrolases are widely varied in sequences and also in catalytic mechanisms. Whereas LovD, LovG, and SAT domains utilize covalent catalysis via active site nucleophiles, enzymes such as Bref-TH and CazE apparently operate via noncovalent, general base catalysis similar to the trichothecene acyltransferase.[32] Each releasing enzyme is also highly specific for the partnering HRPKS ACP domain, as shown in our results. This exclusive protein–protein interaction likely arose during evolution to ensure minimal crosstalk between HRPKS gene clusters, as well as triggering allosteric structural changes required for catalysis.[33]

Using the Bref-PKS and Bref-TH pair (as well as the Fma pair), we showed that the releasing enzyme is important in ensuring the proper chain length control of HRPKSs. In the absence of the TH enzymes, both Bref-PKS and Fma-PKS synthesized longer products than what is reflected in the final product. This result can be rationalized in a competition model in which the polyketide chain can be either offloaded by the TH/AT at the correct size or can be recaptured by the KS domain for another round of elongation (Figure 6). In the absence of the TH, the polyketide chain remains attached to the ACP domain, which allows reentry into the KS domain. If the KS can accommodate a product of longer size, an additional extension step can take place as observed in products **7** and **10**. We did not detect any shorter polyketides in the in vitro assays,

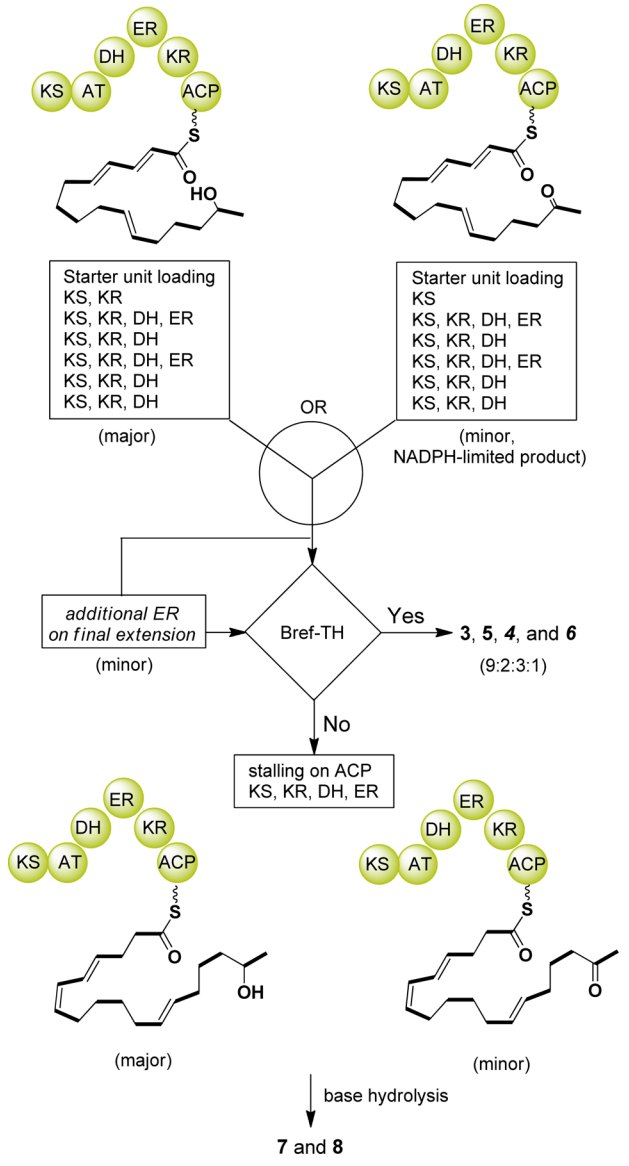

Figure 6. Summary of the programming rules exhibited by Bref-PKS. From the in vitro studies, we were able to fully reconstitute the complex programming of this model HRPKS. Bref-PKS uses different permutations of the reductive domains at each extension cycle and selectively offloads the correct octaketide products with the partner Bref-TH or the longer nonaketide products with base hydrolysis. Compounds **4** and **6** that resulted from additional enoyl reduction at the final extension are italicized.

indicating the high substrate specificities of the TH toward the correct acyl group. However, when equimolar amount of Bref-TH was added 16 h after initiation of the in vitro Bref-PKS reaction, the longer nonaketide products (**7** and **8**) were again observed at similar levels as the base-hydrolyzed reaction (Supplementary Figure S10). This indicates that Bref-TH is capable of hydrolyzing longer chain length but preferably hydrolyzes the correct octaketide chain in a timely manner when co-incubated with Bref-PKS. Interestingly, in both Bref-PKS and Fma-PKS, the extra ketide(s) that form as a result of excluding the TH in the reaction were completely processed by the available reduction domains. The recognition of the longer (and unnatural) substrates by these domains may similarly be due to stalling of the polyketide on the ACP domain, which led to the observed modification. Detailed kinetics studies using model substrates of varying length will provide insights into the substrate specificities of these tailoring domains.

These results support that the KS domain remains an important element in determining polyketide chain length. This is also evident in the phylogeny based classification of HRPKSs using KS domain sequences, which led to clading of the HRPKSs based on chain size (short, medium, and long, as shown in Supplementary Figure S3). Additionally, some HRPKS systems appear to maintain a high fidelity in producing the correct product chain length; for instance, both lovastatin diketide and nonaketide synthases always produce the correct chain length,[5,29] as well as the solanapyrone synthase that releases the product via pyrone formation.[34] However, as demonstrated here in Bref-PKS and Fma-PKS, these domains appear to also have some flexibility on the chain length programming and are insufficient to terminate at the correct chain length alone. In other words, KS domains are capable of synthesizing products of longer size without downstream enzyme control. This may rationalize an in vivo study involving the biosynthesis of fumonisins. Zhu et al. reported a successful complementation of the C_{16}-PKS ALT1 for the C_{18}-PKS FUM1-disruption strain to yield the C_{18} fumonisins.[35] In this case, offloading of the polyketide via a decarboxylative condensation with an alanine by Fum8 dictates the ultimate chain length in the final product.[36] Releasing enzyme control of chain length was also observed in the HRPKS-NRPS involved in the biosynthesis of preaspyridone, a precursor of the natural product aspyridone. In that example, when the fused NRPS module was excised from the megasynthetases, the PKS module was able to synthesize a polyketide product that is longer than that present in preaspyridone (pentaketide instead of tetraketide).[9] That is again most likely due to stalling of the polyketide chain on the PKS in the absence of a downstream domain (C domain in the NRPS) that offloads the correctly sized polyketide for subsequent modification. Additional factors that affect chain length control outside of KS were previously reported, such as the KR-dependent product size observed in PKS-NRPS domain swapping experiments.[37]

While it may appear that some HRPKSs become aberrant in their chain-length control in the absence of cognate releasing partners, it could be argued that these HRPKSs might have originated from ancestral PKS clusters that synthesize final products of longer chain length. In other words, this is Nature's alternative and quick way of generating chain length diversity without modifying the KS or other PKS components, which could be more difficult. In this way, the same HRPKS can be adapted to produce polyketide products of different chain lengths by partnering with releasing enzymes of different chain length specificities. Thus, Bref-PKS may have originated from an ancestral HRPKS cluster that produces a nonaketide product but was adapted to produce octaketide by coupling with the Bref-TH with shorter chain length specificity. This lesson from Nature has important implications for engineering of polyketide biosynthesis, as it suggests that we can attempt to alter the chain length specificity of the releasing enzyme instead of focusing on the HRPKS components to manipulate the chain length of the final product.

Implications of in Vitro Bref-PKS Data on the Biosynthesis of Brefeldin A. In light of the results of the reconstitution studies on Bref-PKS, we can improve our current understanding of the biosynthesis of BFA. From our data, we can conclude that Bref-PKS and Bref-TH are minimally required to produce the octaketide backbone of BFA. Additionally, Bref-PKS is capable of producing the correctly tailored intermediate for further modification to achieve the final structure of BFA. The C2−C3 and C10−C11 double bonds are identical to those in BFA, while the C4−C5 double bond is strategically placed in **3** to allow a proposed radical addition to the double bond leading to the intramolecular annulation that installs the cyclopentane ring (Supplementary Figure S14). The actual mechanism would require additional studies on the enzymes encoded by the genes in the *bref* cluster. We have also demonstrated that Bref-PKS synthesizes products with variable degrees of reduction, which explains the isolation of some BFA analogues, such as the unlactonized 7-dehydrobrefeldin A acid.[14]

The mechanism for macrolactonization of BFA is an intriguing aspect of the biosynthesis. Unlike the RAL systems, Bref-PKS does not partner with an NR-PKS that uses an *in cis* thioesterase for macrolactonization. However, we propose that the Bref-TH could similarly perform both offloading and lactonization, as it is the only candidate gene transcribed in the *bref* cluster. It is foreseeable that the mechanism would involve the C15-hydroxyl that has been observed in in vitro product **3**, which is the proper position for an intramolecular attack on the thioester bond. The catalytic His276 could deprotonate the C15-hydroxyl group and make it a suitable nucleophile for lactonization. Our observation that all of the products were linear, however, indicates the necessity of having the cyclopentane ring form first to "bend" the molecule and bring the nucleophile closer to the ACP to direct lactonization (bottom scheme in Figure 1). This is in contrast to what was previously hypothesized, where the macrolactonization precedes the cyclopentane ring formation.[17] Mabuni et al. originally proposed a mechanism involving a C4−C5 epoxide intermediate. However, this epoxide-opening mechanism requires an alkene at C9−C10, which is an unusual position in a polyketide chain.[17] Recently, Zhang et al. proposed an NADPH-dependent reductive cyclization catalyzed by the alcohol dehydrogenase IkaC for the formation of a five-membered ring within the macrolactam ikarugomycin.[38] However, we did not find a homologue of this enzyme in the *bref* cluster. In light of the structure of **3** produced by Bref-PKS, we propose a mechanism via a C9 radical intermediate catalyzed by a P450 enzyme (Supplementary Figure S14). Addition of the C9 radical to the C4−C5 alkene followed by an oxygen rebound will result in the cyclopentane ring and C4-hydroxyl of **1**. The actual cyclization mechanism will be the subject of further investigations, including determining the roles of the P450s in the cluster. Additionally, the role of Bref-TH in the lactonization of BFA should also be probed in the future.

As noted earlier, Bref-TH homologue is found in the vicinity of a Bref-PKS homologue in *Trichoderma virens*. Such HRPKS/*in trans*-TH pairs are also found in several other fungal genomes, including *Aspergillus nidulans* (AN7084.2/7083.2), *Botryotinia fuckeliana* (BcDW1_1087/1086), *Neofusicoccum parvum* (UCNRP2-2180/2181), and *Macrophomina phaeseolina* (MPH_06436/06434). These HRPKS/TH pairs may be responsible for production of similar linear reduced polyketide or macrolide compounds. Several non-RAL macrolides have been previously isolated from fungi such as putaminoxin,[39] pinolidoxin,[40] and balticolid;[41] the discovery and characterization of the Bref-PKS and Bref-TH therefore provide leads to the biosynthetic gene clusters that are responsible for production of such fungal macrolide scaffolds.

Conclusion. HRPKSs are still poorly understood enzymes that catalyze the synthesis of a wide array of compounds. In order to harness their biosynthetic potential, it is important to first improve our understanding of the underlying mechanism behind their activities by finding a model PKS to work with. In this paper, we report the heterologous expression and reconstitution of Bref-PKS involved in the biosynthesis of BFA to characterize the HRPKS programming rules in vitro. This system has the advantage of being a standalone enzyme with a sizable product and different reduction patterns at each extension. Using this HRPKS, we uncovered an NADPH-dependent reductive tailoring by the PKS in synthesizing products of mixed reduction. We also demonstrated that chain-length determination is not dictated by the KS domain alone but can be altered by the releasing enzyme. Such strategy could be Nature's way of generating chain length diversity without having to evolve the large multidomain megasynthases. The TH-mediated control is highly specific with respect to protein−protein interactions, as well as on acyl substrate recognition. This study further underscores the importance of including tailoring enzyme functions in relating and predicting fungal HRPKS and their products.

METHODS

Strain and Culture Conditions. *E. brefeldianum* ATCC 58665 was obtained from ATCC and maintained in GMM or SMM agar at 28 °C. For BFA production and for mRNA extraction, the strain was grown in liquid MEM media at RT with 250 rpm shaking.[42] *Saccharomyces cerevisiae* BJ5464 was used for protein expression of *bref*-PKS and subsequent in vivo production of **3**−**6**. This strain was maintained and cultured in YPD, while the transformants carrying the recombinant plasmids were grown in synthetic defined dropout media with appropriate supplements. *E. coli* TOPO 10 was used for the subcloning steps, and *E. coli* BL21(DE3) was used for expressing *bref*-TH.

Sequencing and Bioinformatic Analysis. The genomic DNA of *E. brefeldianum* was prepared from mycelium grown in stationary liquid culture.[43] Shotgun sequencing was performed at GenoSeq (UCLA Genotyping and Sequencing Core) with the GS FLX Titanium system (Roche) and at Ambry Genetics (Aliso Viejo, CA) using Illumina Hiseq 2000. The reads were assembled into contigs using SOAPdeNOVO.[44] The contigs were formatted to BLAST database format for local BLAST search using standalone BLAST software (v. 2.2.18). Gene predictions were performed using the FGENESH program (Softberry) and manually checked by comparing with homologous gene/proteins in the GenBank database. Functional domains in the translated protein sequences were predicted using Conserved Domain Search (NCBI). Modeling and alignment of the Bref-hydrolase was performed using Phyre2 server,[26] and the image was generated using Boxshade 3.21.

Expression Analysis by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The total RNA of *E. brefeldianum* ATCC 58665 was extracted from the culture grown in MEM media using the Ambion RNA extraction kit. The first strand cDNA was synthesized using the Oligo-dT primer and Improm-II reverse transcription system (Promega) according to the manufacturer's instructions. Desired cDNA was then amplified with GoTaq Green Master Mix (Promega) using gene-specific primers synthesized by Integrated DNA Technologies (Supplementary Table S2). gDNA template (previously prepared) was used for comparison using the same primer mix as the cDNA reaction.

Molecular Genetic Manipulation. Polymerase chain reactions for cloning were performed using Phusion high-fidelity DNA polymerase (New England Biolabs) or Platinum Pfx DNA polymerase (Invitrogen). PCR products were cloned into a PCR-Blunt vector (Invitrogen) for DNA sequencing and subcloning. Restriction enzymes (New England Biolabs) and T4 ligase (Invitrogen) were used, respectively, for the digestion and ligation of DNA fragments. All primers were ordered from IDT (Supplementary Table S2).

The intron-less transcript of *bref*-TH was obtained by amplification from the cDNA (as prepared previously) using primers containing the *EcoR*I and *Not*I restriction sites. The PCR product was digested with the corresponding enzymes and ligated to the linearized pHis8 vector.[45] The correct construct (designated pAZ93) was PCR-verified and sequenced to ensure intact ORF. pAZ93 was also used as the template for constructing the yeast plasmid (Trp3 marker) for the double transformation experiments with *bref*-PKS (pAZ112), as well as for constructing the *bref*-TH single and triple mutants using transfer PCR.[27]

To construct the intron-less transcript of *bref*-PKS, the 7.3-kb gene was divided into 5 equal-sized fragments; each fragment was amplified from the cDNA (as prepared previously) and was subcloned into PCR-blunt vector and sequence-verified with M13 primers to confirm absence of introns. After confirmation, the respective pieces were reamplified from the PCR-blunt subcloning constructs with primers that contain overlapping regions to the next segment of the gene. The pieces were transformed together with the linearized vector backbone derived from YEplac195 containing the ADH2 promoter and terminator with N-terminus FLAG-tag and C-terminus hexahistidine tag[46] into *S. cerevisiae* BJ5464-NpgA[47] using S. c. EasyComp Transformation Kit (Invitrogen) and selected on uracil-dropout semisynthetic media. The resulting transformants were screened by colony-PCR, and the plasmid in the correct transformant was rescued using the Zymoprep Yeast Plasmid Miniprep Kit (Zymo Research) and transformed into *E. coli* for propagation and sequencing verification. The resulting plasmid was designated pAZ94.

Protein Expression and in Vitro Reactions. The *bref*-TH-pHis8 construct (pAZ93) was transformed into BL21(DE3) via electroporation and cultured and induced with IPTG for expression. The His-tagged protein was purified using nickel-affinity chromatography. The mutant hydrolases were expressed and purified the same way as the wild type. Similarly, the *bref*-PKS construct (pAZ94) was retransformed into the yeast host for protein expression, as described elsewhere,[48] and subsequently purified using nickel-affinity chromatography and eluted using 250 mM imidazole.

Unless otherwise stated, the reactions involving Bref-PKS were set up on ice in mixtures containing 100 mM phosphate buffer, 0.5 to 10 mM NADPH, 2 mM malonyl-CoA, and Bref-PKS and Bref-TH enzymes (typically 20 μM) to a final volume of 100 μL. The reactions were left at RT overnight, extracted with 2 × 200 μL ethyl acetate with 0.1% acetic acid, and dried completely for analysis. In cases where base hydrolysis was performed, 10 μL of 1 M NaOH was added first, and the reaction was heated to 65 °C before extraction with the organic solvent.

For the ^{13}C labeling experiments, Bref-PKS was incubated with 20 μM MatB, 10 μM ATP, 10 mM NADPH, 100 mM [2-^{13}C]-malonate, 10 mM Mg_2Cl_2, 25 mM ATP, 10 mM CoA, 25 μM MatB, and 1 mM NADPH in 100 mM PBS buffer, pH 7.4 in 100 μL total volume. The reactions were performed at RT overnight and either directly extracted with ethyl acetate + 0.1% acetic acid or base-hydrolyzed using 1 M NaOH.

In Vivo Culturing and Extraction of Compounds. pAZ94 (*bref-PKS*, *ura3* marker) and pAZ112 (*bref-TH*, *trp3* marker) were cotransformed into *S. cerevisiae* BJ5464-NpgA[47] using S. c. EasyComp Transformation Kit (Invitrogen) and selected on uracil- and tryptophan-dropout semisynthetic media. The transformants were PCR-screened to confirm the presence of both plasmids, and the correct colony was grown on minimal media as seed culture for 3 days. Subsequently, the seed culture was diluted 1000× into 1 L of YPD and grown for at most 3 days at 28 °C with shaking at 250 rpm. The metabolites were monitored daily by sampling 500 μL of the culture and extracting with equal volume of ethyl acetate + 0.1% acetic acid for LC−MS analysis. For purification of compounds, **3** and **5** were harvested at the optimal production of 36 h, while **4** and **6** were extracted after 72 h. Twenty grams of Amberlite XAD02 polymeric adsorbent resin (Supelco) was added to the media after centrifugation of the cells to extract the organic compounds and was left overnight with shaking. The resin was collected by filtration, and the compounds were eluted using acetone. The solvent was dried, and the residue was partitioned with chloroform/water. The chloroform fraction was then dried completely and flash separated using the CombiFlash system. A C18 column was used with a water and acetonitrile solvent system. The fractions were analyzed, and those containing the desired compounds were pooled together for the final purification using semipreparative HPLC with Phenomenex Luna 5 μL 250 × 1000 mm C18 reverse phase column using an acetonitrile/water + 0.1% formic acid solvent system.

LC−MS Analysis. The dried samples were first dissolved in methanol and centrifuged for 8 min before being injected to the Shimadzu 2010 EV liquid chromatography mass spectrometer with positive and negative electrospray ionization and Phenomenex Luna 5 μL 2.0 × 10 mm C18 reverse-phase column. The samples were resolved on a linear gradient from 5% to 95% with a CH_3CN/H_2O + 0.05% formic acid solvent system for 30 min.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

Accession Codes

Sequence data from this article have been deposited to the GenBank Data Library with the accession code KJ728786.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yitang@ucla.edu.

Notes

The authors declare no competing financial interest.

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