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Heterologous expression of highly reducing polyketide synthase involved in betaenone biosynthesis†

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A unique highly reducing polyketide synthase (HR-PKS) with a reductase domain was identified in a betaenone biosynthetic gene cluster. Successful heterologous expression and characterization of the HR-PKS and *trans*-acting enoyl reductase (ER) provide insights into the core structure formation with a decalin scaffold and allow reconstitution of the betaenone biosynthetic machinery.

Fungal polyketides are structurally diverse secondary metabolites exhibiting a wide range of biological activities.[1] Representative examples are the Ras farnesylation inhibitor phomoidride[2] and the cholesterol-lowering agents lovastatin[3] and zaragozic acid.[4] Assembly of the carbon skeleta is catalyzed by type I polyketide synthases (PKSs).[5,6] Unlike the bacterial multi-modular type I PKSs, fungal type I PKSs have a single modular architecture and iteratively use a single set of active sites through multiple catalytic cycles.[5,6] According to the domain organization, fungal type I PKSs are classified into non-reducing (NR), partially reducing (PR), and highly reducing (HR) PKSs. Among them, HR-PKSs contain three fundamental chain extension domains, β-ketoacyl synthase (KS), acyltransferase (AT), and the acyl carrier protein (ACP), and additional β-keto-processing domains, ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER). Branched methyl groups on the polyketide chain are introduced by the catalysis of methyltransferase (MT). Intriguingly, most HR-PKSs lack a chain-release domain, and limited information is available on the chain-release mechanism. This is one of the bottlenecks in the analysis of the function of HR-PKSs and the reconstitution of the biosynthetic machinery.

Betaenones A–C (**1**–**3**) are phytotoxic polyketides isolated from *Phoma betae Fr.*, the causal fungus of leaf spot disease in sugar beets (Scheme 1).[7] Among betaenones, **3** exhibits the highest phytotoxic activity causing wilting of the host plant.[7] The unique structural feature of **1** is the highly substituted tricycle[6.2.2.0]dodecane skeleton derived from an intramolecular aldol reaction of **3**, which harbors a β-ketoaldehyde side chain attached to a decalin scaffold. Feeding experiments with isotopically labeled precursors suggested that the carbon skeleton is constructed in a common polyketide pathway catalyzed by HR-PKS.[8] Treatment with a cytochrome P450 inhibitor, ancymidol, enabled us to isolate probetaenone I (**4**).[9] Incorporation of isotopically labeled **4** into **2** showed that **4** is a biosynthetic intermediate in betaenone B biosynthesis.[10] Taking together these results, the biosynthetic pathway of **1** was proposed as shown in Scheme 1. Of particular interest in the skeletal construction are the plausible direct reductive cleavage of the linear polyketide chain to give the β-ketoaldehyde moiety found in dehydroprobetaenone I (**5**), a putative biosynthetic intermediate in the production of **1**, and [4+2] cycloaddition to construct the *trans*-decalin skeleton. In this communication, we report the identification of a betaenone biosynthetic gene cluster and the heterologous expression of HR-PKS in *Aspergillus oryzae* to elucidate the characteristic chain-release mechanism and decalin scaffold formation. The successful functional characterization of HR-PKS allows us to reconstitute the biosynthetic machinery of betaenone B.

Isolation of probetaenone I and betaenone C as biosynthetic intermediates showed an involvement of the reductase (R) domain of HR-PKS, a typical domain that catalyzes the reductive release of the aromatic polyketide chain in the NR–PKS system, and oxidative modification enzymes. The draft genome sequence of *P. betae* revealed that the genome contains six HR-PKS genes, of which two HR-PKSs have a terminal reductase (R) domain. Of these two HR-PKS-encoding open reading frames, we assumed that the HR-PKS gene in the gene cluster also containing a cytochrome P450 gene is responsible for betaenone biosynthesis. This putative biosynthetic gene cluster (*bet*) encoded four genes, as shown in Fig. 1 and Table S1 (ESI†). Bet1 exhibits similarity to HR-PKS and is composed in a KS-AT-DH-MT-ER^0-KR-ACP-R domain order, which is identical in domain organization to those of typical HR-PKSs, except for the C-terminal R domain and inactive ER domain (see below). Bet2 is a cytochrome P450, a typical oxidation enzyme that

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† Electronic supplementary information (ESI) available: Experimental procedures, LC-MS data, multiple sequence alignment, phylogenetic analysis, conformational search of **5**, and ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/c4cc09512j

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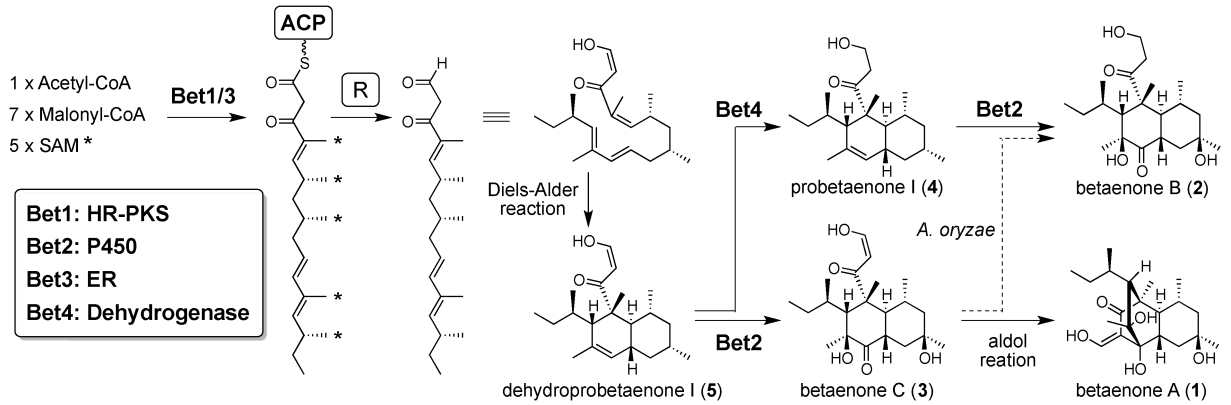

Scheme 1 Proposed biosynthetic pathway of betaenone A. Methyl groups derived from SAM are marked with an asterisk.

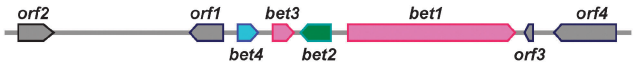

Fig. 1 Genetic organization of the betaenone biosynthetic gene cluster.

activates an inert C–H bond. Bet3 exhibits similarity to ER and might act as a functional alternative to the inactive ER domain of Bet1. Bet4 exhibits homology to short-chain dehydrogenase, possibly involved in a modification reaction (Table S1, ESI†). Two genes, *orf1* and *orf2*, are located upstream of *bet4* and encode a putative FAD-dependent oxidase and dehydrogenase, respectively (Table S1, ESI†).

To characterize the function of individual biosynthetic enzymes, we applied a heterologous expression system in *A. oryzae*, a promising method to elucidate the biosynthetic machinery of fungal metabolites. Representative examples are found in the total biosynthesis of tenellin,[11] aphidicolin,[12] paxilline,[13] aspyridone,[14] aflatrem,[15] and anditomin (11 + 1 genes).[16] For skeletal construction of **1** in *A. oryzae*, *bet1* and *bet3* genes were subcloned into fungal expression vectors, pUARA2 (*argB* marker) and pUSA2 (*sC* marker),[15] to construct pUARA2-*bet1* and pUSA2-*bet3*, which were then introduced into *A. oryzae* NSAR1 to construct both a *bet1* single transformant and a *bet1/3* double transformant (Fig. S1(A), ESI†). The metabolite profile of each transformant cultured on a solid medium was examined by LC-MS (Fig. 2(A)–(C)). One new metabolite (188 mg per kg of rice) was observed only in the extracts obtained from *bet1/3* transformants (Fig. 2(C) and Fig. S2(A), ESI†). Based on HR-MS data (*m/z* 319.2682 $[M + H]^+$), the molecular formula of the new metabolite was determined as $C_{21}H_{34}O_2$, which is consistent with that of dehydroprobetaenone I (**5**). In addition, the ^{1}H-NMR spectrum of the new metabolite was similar to that of **4** except for characteristic signals of an enol form of the β-ketoaldehyde moiety at 15.5 (s), 7.39 (m), and 5.41 (d, J = 4.9 Hz) ppm (see ESI†), suggesting production of **5** in the *bet1/3* transformant. Treatment of the metabolite with $NaBH_4$ resulted in reduction of the aldehyde moiety (Scheme S1, ESI†). The ^{1}H-NMR spectrum of the product completely agreed with that of **4**,[9] allowing us to determine the structure of the observed metabolite as **5**. These results showed that the collaborative action of Bet1 and Bet3 plays a key role in the skeletal construction of **5** in betaenone biosynthesis.

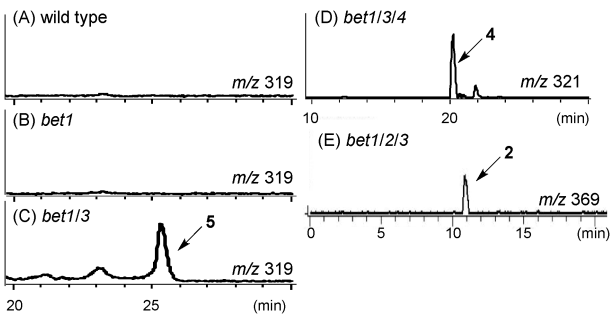

Fig. 2 LC-MS profiles of metabolites produced by transformants. (A) *A. oryzae* NSAR1. (B) *bet1* transformant. (C) *bet1/3* transformant. (D) *bet1/3/4* transformant. (E) *bet1/2/3* transformant.

After identifying the observed metabolite of the *bet1/3* transformant, we then focused on the subsequent reduction step. Since Bet4, which is homologous to short-chain dehydrogenase, is likely to be involved in the reduction of **5**, *bet4* was subcloned into the *Nhe*I site of the pAdeA2 vector (*adeA* marker; Fig. S3, ESI†) to construct pAdeA2-*bet4*. The *bet1/3* double transformant was then transformed with pAdeA2-*bet4* to create a *bet1/3/4* transformant (Fig. S1(B), ESI†). The triple transformant produced a new metabolite (17.4 mg kg^{-1}; Fig. 2(D) and Fig. S2(B), ESI†) having a retention time and the MS spectrum identical to those of authentic **4**,[17] demonstrating that Bet4 catalyzes reduction of **5** to **4** in betaenone biosynthesis.

Next, we turned our attention to the oxidative modification of the decalin scaffold. The plausible candidate *bet2*, a cytochrome P450 gene, was subcloned into the *NheI* site of the pAdeA2 vector to construct pAdeA2-*bet2*, which was used for transformation of the *bet1/3* double transformant, creating a *bet1/2/3* transformant (Fig. S1(C), ESI†). LC-MS analysis of the extracts showed production of a new metabolite (153 mg kg^{-1}) (Fig. 2(E) and Fig. S2(C), ESI†). The retention time and MS spectrum were identical to those of authentic betaenone B (Fig. S2(D), ESI†), thus revealing that this new metabolite **2** is betaenone B. The structure of **2** was further confirmed by ^{1}H-NMR analysis (see ESI†). These results suggested that Bet2

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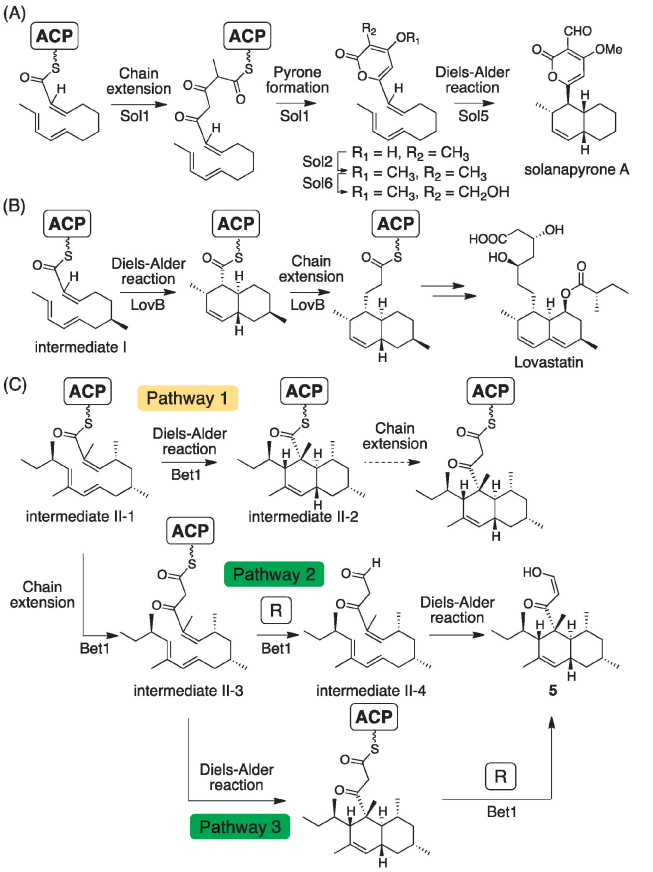
catalyzes multistep oxidations from **5** to **3** and that an enzyme existing in *A. oryzae* unexpectedly mediates the reduction of the β-ketoaldehyde moiety of **3** to give **2**. Introduction of *orf1* or *orf2* in the *bet1/2/3* transformant did not give a new metabolite, suggesting that functionally unassigned genes adjacent to the *bet* gene cluster can be involved in the aldol reaction of **3** to afford **1**. In the present transformation studies, the frequency rate (the ratio of functionally active clones to obtained clones) is summarized as follows: *bet1/3* (2/5), *bet1/3/4* (4/6), and *bet1/2/3* (5/5). Notably, in all cases, we obtained the functionally active clone in a single transformation, demonstrating that heterologous expression in *A. oryzae* is a reliable method to characterize the function of biosynthetic genes.

Currently, to our knowledge, polyketide chain-release mechanisms of HR-PKSs are mainly classified into the following three mechanisms (Table S2, ESI†): (1) spontaneous cleavage accompanying pyrone formation found in solanapyrone[18] and alternapyrone[19] biosyntheses, (2) polyketide chain transfer from HR-PKS to the starter unit:ACP transacylase (SAT) domain of NR–PKS in several HR-PKS–NR–PKS systems,[5,6] and (3) enzymatic cleavage of the polyketide chain catalyzed by thioesterase and acyltransferase reported in lovastatin biosynthesis.[20,21] Intriguingly, in these cases, no inherent chain-release domain is installed on the HR-PKS. By contrast, the production of **5** in the *bet1/3* transformant indicated that the C-terminal R domain of Bet1 catalyzes the reductive release of the polyketide chain, differentiating the function of Bet1 from that of other HR-PKSs. To reveal the function of the conserved SYK catalytic triad (S2705/Y2737/K2741)[22] found in the Bet1-R domain (Fig. S4, ESI†), mutational analysis was then conducted. In contrast to the *bet1/3* transformant, no detectable level of **5** was produced by double transformants with a *bet1* mutant gene (S2705A, Y2737F, and K2741A), suggesting the importance of the catalytic triad in reductive cleavage of the polyketide chain (Fig. S5(D)–(F), ESI†). Unfortunately, the chemical release of the PKS-bound mature polyketide chain by alkaline treatment[23] failed.

A previous synthetic study of betaenone,[17] in which a linear triene gave modified probetaenone I as the sole product suggested that existing stereogenic centers of the triene precursor control diastereoselectivity, and the role of the enzyme is acceleration of the reaction rate in the [4+2] cycloaddition possibly by stabilizing the transition state and activating the dienophile. Currently, enzymatic decalin scaffold formation by [4+2] cycloaddition is firmly established in the biosyntheses of the two fungal polyketides, solanapyrone[18,24] and lovastatin[23] (Scheme 2(A) and (B)). In the latter case, the HR-PKS LovB catalyzes a Diels–Alder reaction of intermediate I during the chain extension process (Scheme 2(B)). Our experimental evidence that the *bet1/3* transformant gave a [4+2] cycloadduct suggested that the HR-PKS Bet1 may mediate a Diels–Alder reaction of intermediate II-1 followed by additional chain extension of intermediate II-2 to afford the core structure of **5** (Scheme 2(C); pathway 1). However, the unusual observation that only the terminal carbonyl group of **5** was reduced by the small reducing agent $NaBH_4$ questioned this scenario. To examine the reactivity of the carbonyl group in **5**, we performed a conformational



Scheme 2 Proposed mechanism of decalin scaffold formation in (A) solanapyrone A, (B) lovastatin, and (C) betaenone biosyntheses.

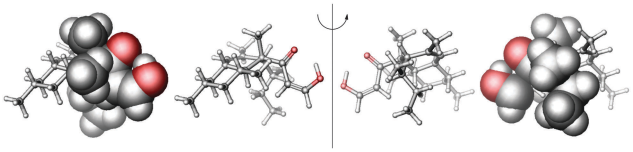


Fig. 3 Lowest energy conformer of **5**. Red and black balls show oxygen and carbon atoms.

search using MacroModel. All low-energy conformers obtained (<8 kJ mol^{-1}) were essentially the same as a decalin moiety and just differed in the orientation of secondary butyl and β-hydroxyenone groups (Fig. 3 and Fig. S6, ESI†). These results were supported by ^{1}H-NMR data. The highly congested carbonyl group in the conformers of **5** and probably in intermediate II-2 likely shows resistance to nucleophilic attack of $NaBH_4$ and enolate from the malonyl unit, respectively. In the previous report, we pointed out that prosolanapyrone synthase (PSS) and LovB/MlcA have a similar domain architecture and synthesize a common hexaketide precursor, but PSS did not catalyze the [4+2] cycloaddition, whereas LovB/MlcA did.[18] Based on these data, the [4+2] cycloaddition seems to occur after further chain elongation. This suggests two alternative triene intermediates II-3 and II-4 for the cycloaddition in betaenone biosynthesis (Scheme 2(C); pathway 2 and 3). Further experimental data are required to determine the actual biosynthetic pathway.

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Most HR-PKSs possess a functionally active ER domain (*cis*-ER) (Table S2, ESI†). By contrast, a prominent feature of the Bet1/3 system is that the *cis*-ER of Bet1 is inactive and Bet3 participates in the polyketide chain construction as a *trans*-acting ER (*trans*-ER). A similar collaborative action of *trans*-ER is frequently found in PKS-NRPS hybrids.[5,25] A common feature of Bet1 and PKS-NRPSs is that both PKSs produce a linear polyketide chain having a β-ketoacyl group. The exceptional case was reported for the LovB/C system (Table S2, ESI†), which produces polyketide with a β-hydroxy group.[23] Previous structural analysis of LovC provided sufficient data to propose the catalytic mechanism.[26] A phylogenetic analysis including 8 *trans*-ERs found in HR-PKS–PKS-NRPS systems and 22 ER domains (*cis*-ER) of HR-PKS–PKS-NRPSs suggested that ERs could be divided into 3 groups (Fig. S7, ESI†). *trans*-ERs, including Bet1 and LovC, form Clade I, while functionally active and inactive *cis*-ERs are classified into Clade II and Clade III, respectively. This classification is likely general because the uncharacterized HR-PKS system, comprising FSL1 (HR-PKS) and FSL5 (*trans*-ER) involved in fusarielin biosynthesis,[27] belongs to the same group as Bet1/3 and LovB/C. Multiple sequence alignment revealed a new ''fingerprint'' region to distinguish functionally active and inactive *cis*-ERs (Fig. S8, ESI†). In addition, a previously proposed point mutation in the NADPH-binding motif of *cis*-ERs[28] was also found in the case of Acrts2, which is classified into Clade II. The inactivation of the ER domain corresponds to the polyketide structure of ACR-toxin.[29] Notably, sequence and phylogenetic analyses of ERs revealed that Bet1 and LovB, having a C-terminal reductase domain and a condensation domain, respectively, are classified into the same clade with PKS-NRPS, suggesting an evolution of ancestral PKS-NRPS towards Bet1 and LovB by partial deletion of characteristic domains for NRPS.

In summary, we have identified and characterized the betaenone biosynthetic gene cluster. Through heterologous expression studies, we have established that Bet1 and Bet3 are key enzymes for skeletal construction in betaenone biosynthesis. To our knowledge, this is the first functional characterization of a fungal HR-PKS harboring an R domain, which catalyzes the reductive release of the polyketide chain. Based on the chemical reactivity and conformational search of dehydroprobetaenone I, we proposed the biosynthetic hypothesis that reductive cleavage of the linear polyketide chain followed by a Diels–Alder reaction gives the *trans*-decalin skeleton. Co-expression of *bet4* and *bet2* with *bet1/3* in *A. oryzae* also revealed subsequent reductive and oxidative modifications in betaenone biosynthesis.

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