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Cis-Double Bond Formation by Thioesterase and Transfer by Ketosynthase in FR901464 Biosynthesis

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Supporting Information

ABSTRACT: Modular polyketide synthases (PKSs) are well known to use ketosynthase (KS)-driven carbon−carbon bond formation, dehydratase-mediated dehydration to form double bonds, and product release by thioesterase (TE), all of which are regarded as the "canonical" roles for most polyketide biosyntheses. FR901464 is biosynthesized by a complex acyltransferase-less PKS system involving a nonterminal TE domain and several mutated KS domains. Here we demonstrate that this TE catalyzes the dehydration of the polyketide intermediate to yield a *cis*-double bond and a mutated KS transfers the nascent polyketide chain with only a *cis*-double bond to the downstream acyl carrier protein. These findings not only provide new insights into different enzymatic functions of PKS domains but also suggest an alternative strategy for *cis*-double bond formation during the polyketide assembly line.

Many therapeutic drugs, including amphotericin (with antifungal properties), avermectin (antiparasitic), epothilone (anticancer), erythromycin (antibacterial), and rapamycin (immunosuppressant), are well known as polyketide natural products and biosynthesized by polyketide synthases (PKSs) through a modular assembly line type pathway.[1] Generally, an acyltransferase (AT) domain selects the specific substrate and transfers it onto the thiol group of the 4′-phosphopantheinyl arm attached to the acyl carrier protein (ACP) domain for the next carbon−carbon bond formation, catalyzed by a β-ketoacyl synthase (KS) domain.[2,3] The newly generated β-ketoacyl intermediate can be steroselectively reduced by ketoreductase (KR) into a hydroxyl group, which sometimes is further dehydrated to yield a double bond by dehydratase (DH).[4] A thioesterase (TE) domain is usually responsible for polyketide chain release.[4,5] Although some exceptions were observed, the AT-catalyzed acyl transfer, KS-driven C−C bond formation, DH-catalyzed dehydration to form a double bond, and TE-mediated product release are well accepted steps and regarded as the "canonical" mechanism for most modular PKSs.

FR901464 (Figure 1A), a polyketide compound produced by *Pseudomonas* sp. no. 2663, represents a new family of potent anticancer natural products, the spliceosome inhibitors.[6] So, FR901464 and its natural and artificial analogues provide a new opportunity to develop novel anticancer drugs with different modes of action.[7] Biosynthetic studies have revealed that FR901464 is biosynthesized by AT-less PKSs hybridized with a nonribosomal peptide synthetase (NRPS) and an isoprenoid-like β-branching system.[8] A Baeyer−Villiger (BV) monooxygenase domain was recently demonstrated to catalyze the BV oxidation of an ACP-tethered intermediate, which further triggers the polyketide chain release.[9] However, several questions remain to be solved. First, the extension module-1 (M-1) of FR9C is organized into KS$_{(1-1)}$-KR-ACP$_{(1-1)}$-KS*$_{(1-2)}$-ACP$_{(1-2)}$-ACP$_{(1-3)}$-TE-KS*$_{(1-3)}$-ACP$_{(1-4)}$, and the second and third KS domains are likely inactive for C−C bond formation because the conserved motif C-H-H is mutated into C-A-H or C-N-H. Moreover, the KS*$_{(1-2)}$-ACP$_{(1-2)}$-ACP$_{(1-3)}$-TE-KS*$_{(1-3)}$-ACP$_{(1-4)}$ domain organization does not correspond with the structure of final product. Additionally, the molecular basis of how the *cis*-double bond ($C_{2'}$−$C_{3'}$) is generated remains obscure, despite the fact that the biosynthetic pathway has been proposed (Figure 1A). Herein, we report *in vitro* biochemical evidence to answer these questions and reveal that the TE functions in an unprecedented role to yield a *cis*-double bond during polyketide assembly and the KS* acts as a gatekeeper to transfer the resultant polyketide intermediate to the downstream ACP (Figure 1B).

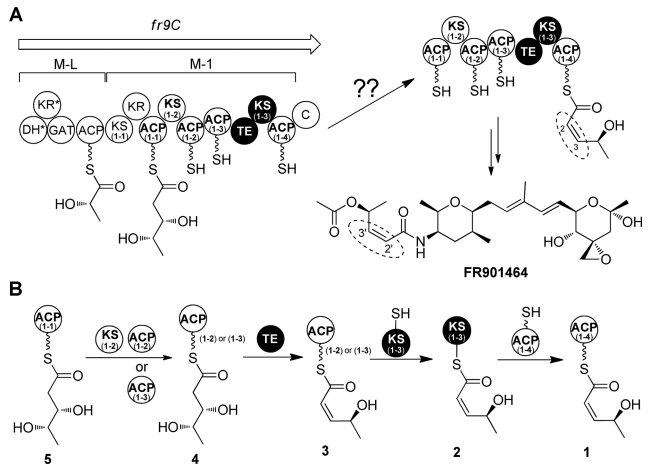

Figure 1. Biosynthesis of *cis*-double bond in FR901464. (A) Domain organization of modules involved in the unsolved double bond formation. (B) Proposed biosynthetic pathway of *cis*-double bond moiety in FR901464.

Comparative analysis of FR901464 and thailanstatins (analogues of FR901464) biosynthetic gene clusters well

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established the connection of product structure with its biosynthetic genes,[8,10] so the possibility of post-PKS tailoring modifications to generate a *cis*-double bond, such as the $\Delta_{2,3}$ double bond in phoslactomycin or fostriecin and the $\Delta_{11,12}$ double bond in tirandamycin,[11] could be ruled out. Normally, an A-type KR domain and DH domain are required for *cis*-double bond formation.[12] However, the only KR domain in module-1 is B-type for producing the D-hydroxyl group, and the expected DH is missing. The lack of a DH domain in the PKS assembly line is not unprecedented, and similar phenomena were observed in leinamycin and chivosazol biosynthesis.[13] Especially in the latter process, a "stuttering mechanism" was proposed to explain the strange relationship between the absence of DH and *cis*-double bond following the epothilone biosynthesis, in which the *cis*-double bond was generated by the DH of the next module.[14] We therefore reasoned that a similar mechanism might be used in FR901464. According to this model, polyketide chain transfer from ACP in module-1 to the ACP of the next PKS module is one of the necessary steps. Given the fact that M-1 contains the "redundant" KS*$_{(1-2)}$-ACP$_{(1-2)}$-ACP$_{(1-3)}$-TE-KS*$_{(1-3)}$-ACP$_{(1-4)}$ domain, the ACP$_{(1-3)}$ to ACP$_{(1-4)}$ acyl chain transfer should be involved and is most likely mediated by KS*$_{(1-3)}$.

To validate this hypothesis, we heterologously expressed and purified His-tagged ACP$_{(1-2)}$, ACP$_{(1-3)}$, KS*$_{(1-3)}$, and ACP$_{(1-4)}$ from *E. coli* BL21 (DE3) (Figure S1). The substrate thioester **4x** and possible products **3x** (with *cis*-double bond) and **3y** (with *trans*-double bond) were also synthesized following the established routes (Figures S2 and S3). The ACP-tethered intermediate **4** was generated by 4′-phosphopantetheinyl transferase (Sfp) using **4x** and apo-ACP$_{(1-3)}$ as substrates (Figure 2A). Unfortunately, **4** is easy to hydrolyze and extremely unstable in the assay conditions, which prevented us from biochemically characterizing the subsequent reaction performed by KS*$_{(1-3)}$. Similarly, we enzymatically synthesized the ACP-bound intermediates **3-2**, **3-3**, **3c-2**, and **3c-3** (Figures 2B-III, V and S5), which were further used as substrates to explore the KS*$_{(1-3)}$-mediated acyl transfer reaction. An obvious new product, **1**, was observed by high-performance liquid chromatography (HPLC) analysis (Figure 2B-IV, VI) and further confirmed by liquid chromatography−mass spectroscopy (LC-MS) data (Figures 2C and S9, and Table S3). However, no comparable conversion was observed when the assay used **3c-2** or **3c-3** as substrate (Figure S5). These results showed that KS*$_{(1-3)}$ could catalyze the only *cis*-double-bonded intermediate transfer from upstream ACP$_{(1-2)}$ or ACP$_{(1-3)}$ to downstream ACP$_{(1-4)}$, which also hinted that the *cis*-double bond should be formed before transfer. Thus, the "stuttering mechanism" similar to epothilone biosynthesis could be ruled out.

Given the fact that the chain transfer occurred only for the *cis*-double bond intermediate, we inferred that the dehydration of **4** was performed by one domain in module-1 and hypothesized the TE domain as the most probable candidate. To prove this hypothesis, we expressed and purified His-tagged TE domain from *E. coli* BL21 (DE3) (Figure S1) to perform a series of biochemical experiments. The intermediate **4** was used as substrate, TE was added to the essay for 15 min, and the reaction was then detected by HPLC and HR-MS. We found that the amount of substrate decreased and holo-ACP increased simultaneously, but the prospective dehydration product was not detected. This illustrated that not dehydration, but hydrolysis of thioester, occurred in this essay because of the

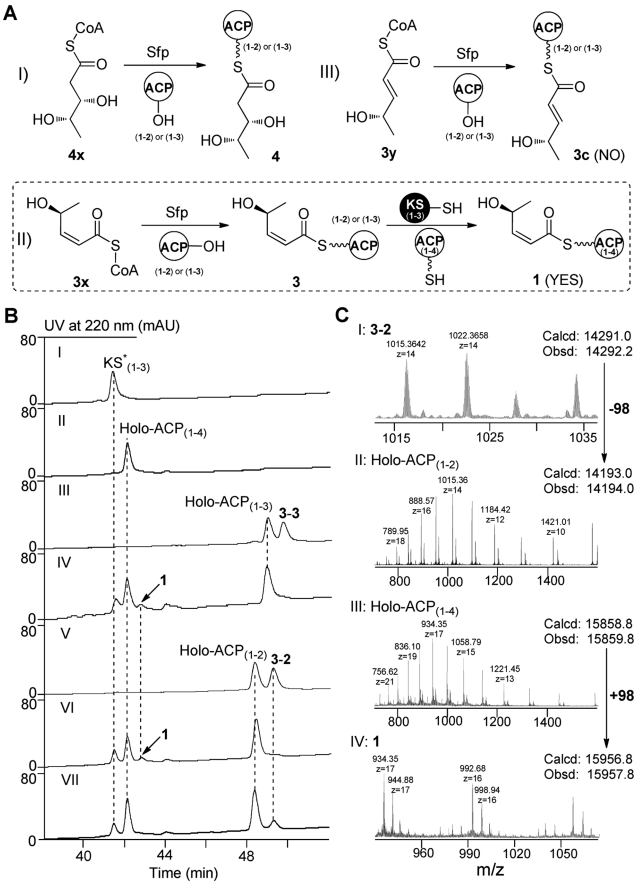

Figure 2. In vitro assays of the polyketide chain transfer catalyzed by KS*$_{(1-3)}$. (A) Biochemical reaction: (I) **4** is not stable enough for the assay; (II) full reaction in this assay; (III) **3c** is not the substrate of KS*$_{(1-3)}$. (B) HPLC analysis: (I) KS*$_{(1-3)}$; (II) Holo-ACP$_{(1-4)}$; (III) generation of **3-3** using **3x** and apo-ACP$_{(1-3)}$ by Sfp; (IV) full assay of **3-3** with KS*$_{(1-3)}$; (V) generation of **3-2** using **3x** and apo-ACP$_{(1-2)}$ by Sfp; (VI) full assay of **3-2** with KS*$_{(1-3)}$; (VII) assay of **3-2** with KS*$_{(1-3)}$ mutant (the active site Cys was mutated into Ala). (C) MS analysis of the reaction catalyzed by KS*$_{(1-3)}$ using **3-2** as substrate: (I) substrate **3-2**; (II) generation of holo-ACP$_{1-2}$; (III) holo-ACP$_{(1-4)}$; (IV) the product, **1**.

instability of **4** or dehydration product as mentioned above. Therefore, we chemically synthesized *N*-acetyl cysteamine thioester analogues (diastereoisomers **4a** and **4b**, Figures 3A and S4) of **4** as substrates for the dehydration assay. We also chemically synthesized **3a** and **3d** possessing different configurations of double bond as standard compounds of dehydration products (Figure S4). However, except for compound **3d**, the other three compounds **4a**, **4b**, and **3a** easily decomposed into a lactone and *N*-acetyl cysteamine via intramolecular addition and elimination reactions (Figure S6), so it was difficult to purify them further. After incubation of **4a** and **4b** with TE, HPLC and MS analysis indicated that the amount of **4a** decreased rapidly and a new small peak emerged, with a molecular weight consistent with the dehydrated product and retention time identical to that of standard **3a** (Figures 3B and S10). However, **3d** (*trans*-configuration) could not be detected in the same assay. These data proved that the TE domain could catalyze dehydration of **4a** to generate the *cis*-double bond product **3a**. Meanwhile, we noticed that only a trace of un-dehydrated **4b** (3-hydroxyl group, L-configuration) decreased in this assay, which indicated that **4a** (3-hydroxyl

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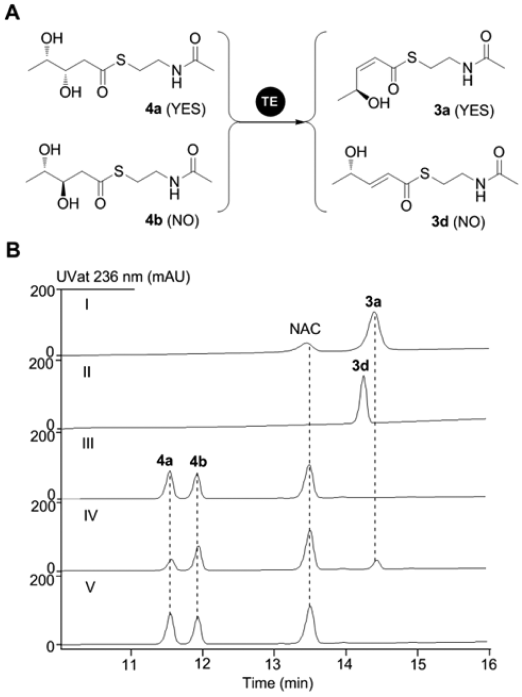

Figure 3. In vitro assays of dehydration catalyzed by TE. (A) Biochemical reaction. (B) HPLC analysis (UV at 236 nm): (I) standard **3a**; (II) standard **3d**; (III) substrates **4a** and **4b**; (IV) full assay of **4a** and **4b** with TE for 15 min; (V) assay of **4a** and **4b** with TE mutant for 1 h (the active-site Ser was mutated into Ala).

group, D-configuration) is more suited as a substrate for TE. These results also certified that the B-type KR domain in this extending module catalyzed β-keto reduction to generate D-hydroxyl configuration indirectly, and this is all consistent with the bioinformatics analysis of the PKS domain.[8]

KS usually acts as the most vital enzyme, catalyzing the C−C bond formation via elongation of the C2 unit in type I PKS. The conserved motif of KS is the C-H-H motif and the Cys residue functions in loading the polyketide chain and the two His residues catalyze the decarboxylation and condensation of extender units. While there are some exceptions, the one or two conserved His residues of KS sometimes are naturally mutated and the Cys is conserved. These KSs were suggested to transfer the polyketide chain between different domains, but this has never been biochemically proven.[8,15] Now biochemical evidence confirmed that KS*$_{(1\text{-}3)}$ catalyzes the transfer of polyketide chain from ACP$_{(1\text{-}2)}$ or ACP$_{(1\text{-}3)}$ to ACP$_{(1\text{-}4)}$. Furthermore, we could not detect transfer from ACP$_{(1\text{-}4)}$ to ACP$_{(1\text{-}3)}$ or ACP$_{(1\text{-}2)}$, which indicated that the transfer catalyzed by KS* is directive. We also mutated the conserved Cys to Ala, and the activity of the resultant mutant was completely abolished (Figure 2B-VII), which confirmed that the Cys residue is important for the transfer reaction. In addition, we demonstrated that only the *cis*-double bond intermediate, but not the *trans*-double bond intermediate, could be successfully transferred to the downstream ACP by KS*, indicating that this type of KS also acts as a gatekeeper for the next NRPS module in the biosynthetic machinery.

In bacterial polyketide biosynthesis, the TE domain is always located at the end of the type-I PKS assembly line and catalyzes the hydrolysis or macrocyclization for product release.[5] Only in very rare cases, such as HSAF biosynthesis, was a TE domain found exhibiting both protease and peptide ligase activities.[16] Another exception was found in curacin biosynthesis: a decarboxylative elimination following hydrolysis to generate a terminal double bond was performed by a TE domain.[17] Recently, in some fungal PKSs, TE domains were also characterized as Claisen cyclase, deacetylase, and even transferase activity.[18] However, a TE domain located in the elongation module of type-I PKS is rarely observed, and catalyzing the dehydration of a D-hydroxyl group to form a *cis*-double bond is completely unprecedented. Structure-based mechanism studies revealed that the TE domain belongs to an α/β-hydrolase family and uses a S-H-D catalytic triad, in which the Ser is the key active site.[19] Additionally, it is widely accepted that the DH domain in PKS catalyzes the dehydration of a polyketide intermediate to produce a double bond between the α- and β-carbons. During this process, the catalytic Asp has been proposed to donate a proton to the β-hydroxyl group, and the catalytic His abstracts an α-proton to result in direct elimination of H_2O.[20] Though they play different roles in the PKS assembly line, one important fact is that both TE and DH possess a double hotdog fold and could be classified into the same hotdog superfamily.[21] Recently, additional hotdog enzymes involved in polyketide biosynthesis with other functions beyond normal TE and DH were also discovered, such as the product template domain from fungal iterative PKS,[22] DH-like domain for double bond migration (exemplified by Rhi-DH7),[23] branching domain in rhizoxin biosynthesis (Rhi-DB),[24] and pyran synthase (PS) domain for cyclic ethers formation.[25] Both sequence analysis and phylogenetic analysis (Figure 4) suggested that most are more homologous to the DH domain, and therefore could be considered as evolutional

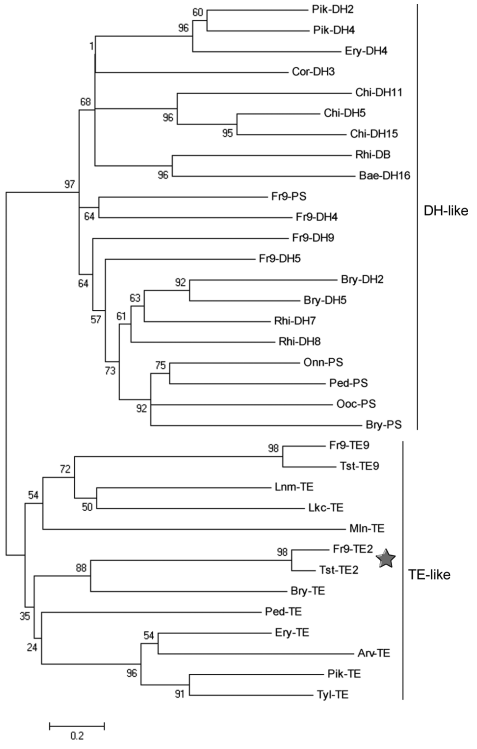

Figure 4. Phylogenetic analysis of typical hotdog enzymes including TE domain, DH and DH-like domain, branching domain (DB), and pyran synthase (PS) domain, constructed using MEGA v5.0 with 500 bootstrap replicates; scale 0.2 is the genetic distance. Detailed information for each domain is presented in the Supporting Information.

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variations of DH. In FR901464, the nonterminal TE (Fr9-TE2) and its homologue Tst-TE2 (77% identity), involved in thailanstatin biosynthesis, are closer to TE domains, which was supported by phylogenetic analysis (Figure 4). Moreover, mutation of the active site Ser to Ala completely abolished the double bond formation (Figure 3B-V), and changing His or Asp into Ala also decreased activity, which indicated that these residues are important to the biological activity. This is also similar to common TE domains. Currently, the enzymatic mechanism for double bond formation still needs to be further explored.

In conclusion, we have elucidated that a TE domain catalyzes the dehydration step for *cis*-double bond formation, followed by the resultant polyketide chain transfer to downstream ACP carried out by the KS domain in FR901464 biosynthesis. This exceptionally rare TE has shown DH function by formation of a *cis*-double bond as opposed to thioester hydrolysis or macrocyclization. These findings have further expanded our understanding of polyketide biosynthesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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