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A Double-Hotdog with a New Trick: Structure and Mechanism of the *trans*-Acyltransferase Polyketide Synthase Enoyl-isomerase

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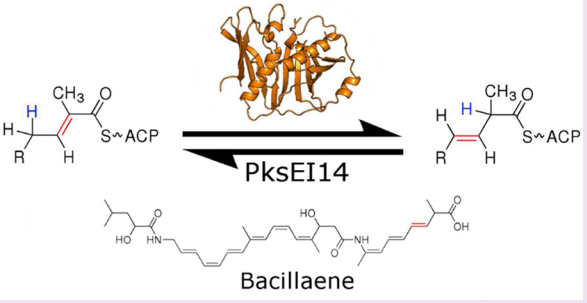
Supporting Information

ABSTRACT: Many polyketide natural products exhibit invaluable medicinal properties, yet much remains to be understood regarding the machinery responsible for their biosynthesis. The recently discovered *trans*-acyltransferase polyketide synthases employ processing enzymes that catalyze modifications unique from those of the classical *cis*-acyltransferase polyketide synthases. The enoyl-isomerase domains of these megasynthases shift double bonds and are well-represented by an enzyme that helps forge the triene system within the antibiotic produced by the prototypical bacillaene synthase. This first crystal structure of an enoyl-isomerase, at 1.73 Å resolution, not only revealed relationships between this class of enzymes and dehydratases but also guided an investigation into the mechanism of double bond migration. The catalytic histidine, positioned differently from that of dehydratases, was demonstrated to independently shuttle a proton between the γ- and α-positions of the intermediate. This unprecedented mechanism highlights the catalytic diversity of divergent enzymes within *trans*-acyltransferase polyketide synthases.



Polyketides are a structurally diverse class of natural products manufactured by a broad spectrum of bacteria, fungi, and plants. The clinical importance of such polyketides as the antibiotic erythromycin and the cholesterol-lowering agent lovastatin has led to the structural, functional, and mechanistic investigation of the enzymatic machinery responsible for their synthesis.[1] Type I polyketide synthases (PKSs) employ biosynthetic logic similar to the metazoan fatty acid synthase, which iteratively condenses and reduces acetate units into fatty acyl chains.[2,3] In contrast to fatty acid synthesis, PKSs operate in an assembly line fashion, where modules (sets of domains responsible for a single round of condensation and processing) are structured in a linear arrangement, each obtaining a ketide extender unit selected by an acyltransferase domain (AT), condensing that extender unit to a growing polyketide chain with the ketosynthase domain (KS) and processing the resulting β-ketoacyl chain depending on which processing domains are associated with the module. These domains include a ketoreductase (KR) that catalyzes stereoselective reduction of the β-carbonyl, a dehydratase (DH) that yields an α,β double bond through the elimination of water, and an enoylreductase (ER) that reduces the double bond to yield a completely saturated acyl intermediate.

Type I PKSs have been subdivided into two classes depending on whether ATs are encoded within modules (*cis*-AT PKSs) or discretely encoded as separate polypeptides that dock to the synthase (*trans*-AT PKSs).[4] While each of the domains from *cis*-AT PKSs have been structurally characterized, only recently has structural information become available for domains found within *trans*-AT PKS assembly lines.[1,5−7] The earliest discovered and archetypal *trans*-AT PKS is encoded within the *Bacillus subtilis* and *Bacillus amyloliquefaciens* genomes (PksX and Bae, respectively) and is responsible for the biosynthesis of the polyketide bacillaene, a polyene diamide that inhibits prokaryotic protein synthesis.[8] This PKS is illustrative of the unusual combinatorial logic in *trans*-AT PKS systems, which are commonly fused with nonribosomal peptide synthetases (NRPS) and employ processing domains such as methyltransferases, enoyl-CoA hydratases, pyran synthases, and β-branching domains (B), rarely or not observed in *cis*-AT PKSs.[4] The evolutionary acquisition of these additional processing domains has permitted *trans*-AT PKSs to explore a vast realm of possible molecular configurations, yielding natural products with functionalities not accessible to PKSs restricted to the traditional processing reactions employed in fatty acid biosynthesis.

One of the additional processing domains available to *trans*-AT PKSs is the enoyl-isomerase domain (EI), which has been previously annotated as an inactive DH or DH* due to sequence alignments that predicted a DH double-hotdog fold.[9−11] This domain is located in what has been termed "shift-modules" that yield intermediates with β,γ double bonds, in contrast to the classic α,β-unsaturated products of DH catalysis (Figure 1). Olefins within polyketides impact the sterically available conformations of the final product more than any other functional group, and therefore several studies have recently been conducted to determine if this domain is

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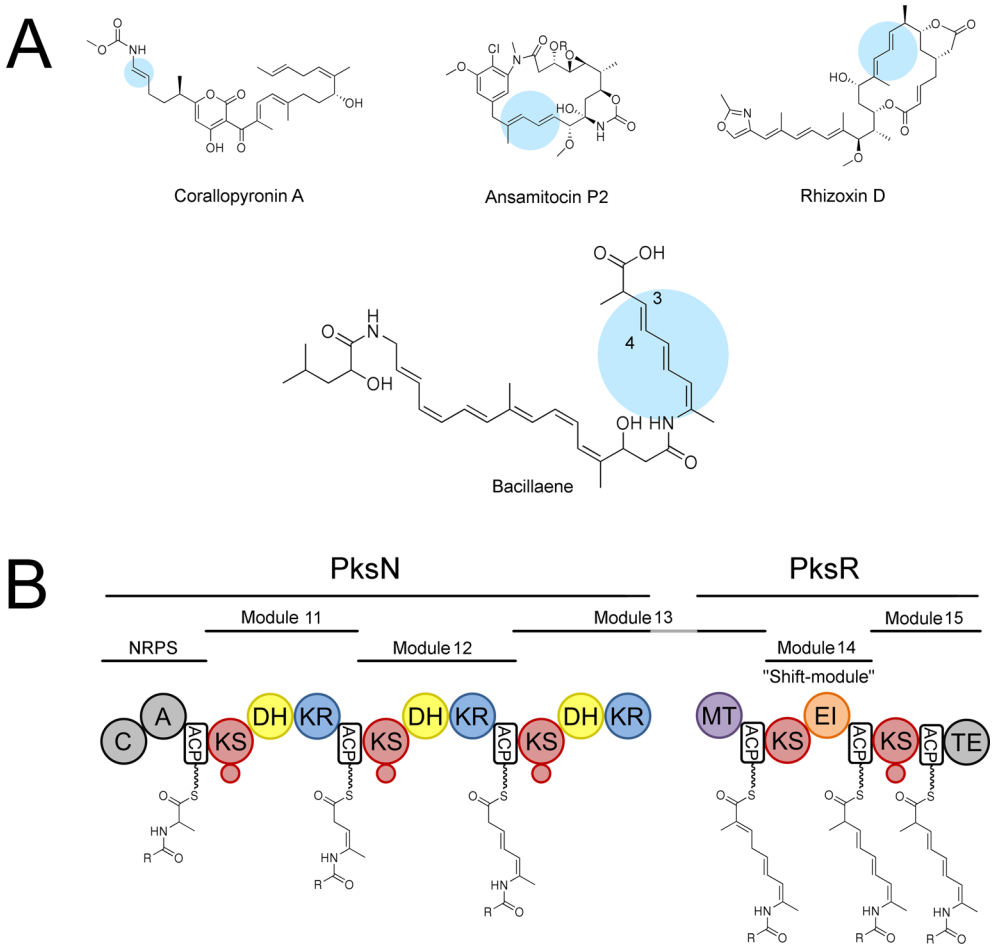

Figure 1. Polyketides with shifted double bonds. (A) Several polyketides with isomerized double bonds are highlighted with blue circles. The C3-C4 double bond of bacillaene, isomerized by PksEI14, is annotated. (B) The last two PKS subunits responsible for the final catalytic steps of bacillaene biosynthesis are PksN and PksR. The proposed route involves the installation of β,γ double bonds by the DH domains of modules 11 and 12 (PksDH11 and PksDH12), while the EI domain of module 14 is a dedicated isomerase for the α,β double bond installed by the DH from module 13 (PksDH13). The KS domain of the terminal module (PksKS15) is not condensationally competent and likely acts as a gatekeeper to ensure that the isomerization has taken place before the final product is released from the synthase. The small red circles appended to the KS domains indicate the presence of a flanking subdomain.

responsible for the isomerization of double bonds to positions "out-of-register" with classical PKS biosynthetic logic. Some DH domains contain intrinsic isomerase activity, which has been shown for DHs from the bacillaene PKS (Figure 1B).[10] Both dehydration and $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization for C5−C6 and C7−C8 in bacillaene were demonstrated to occur within the first two DHs of PksN, without the participation of a dedicated EI domain. Whether the isomerization of the β,γ double bond at C3−C4 of bacillaene was also catalyzed by the inherent isomerase activity of the third DH remained undetermined, and sequence alignments revealed that the following module could potentially be a shift-module.

The EI domain of the rhizoxin PKS (at the N-terminus of RhiE) has been shown to be responsible for $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization, and a strain with an EI knockout prevented polyketide transfer downstream of the disruption.[9] Whether this is due to KS selectivity for the properly isomerized product or interference with docking between RhiD and RhiE is unclear. A mechanism was proposed for EI-catalyzed isomerization that involved a histidine conserved between DH and EI domains, as well as an unknown residue that could act as a general base. More recently, the EI domain from the corallopyronin A synthase was isolated and studied.[11] *In vitro* reconstitution of isomerase activity coupled with site-directed mutagenesis revealed that the proposed active site histidine is indeed required for catalysis; however, the unknown residue that acts in cooperation with this histidine could not be determined through mutagenic knockouts of proposed active site residues.

Here we report the 1.73 Å resolution crystal structure and reconstituted *in vitro* activity of the EI domain from the 14th module of the bacillaene PksX synthase (PksEI14). The structure reveals how a key active site substitution of a proline residue conserved among PKS DH domains permits the catalytic histidine to act as both a general base and general acid in the isomerization of a double bond. Experiments conducted in deuterated solvent reveal that the mechanism proceeds through the relocation of a substrate γ-proton to the α-position without solvent interference, similar to the mechanism of double bond isomerization catalyzed by ketosteroid isomerase.[12] The structure also shows that the EI domains may not contribute significantly to the dimerization of *trans*-AT PKS polypeptides, in contrast to what has been observed for *cis*-AT PKSs.[13−15] The structural and biochemical evidence presented here reveals an unprecedented mechanism for double bond isomerization and suggests that PksEI14 is responsible for the

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Table 1. Crystallographic Data and Refinement Statistics[a]

	native	CH_3-Hg derivative
	Data Collection	
wavelength (Å)	1.0332	0.9763
space group	$P2_12_12_1$	$P2_12_12_1$
cell dimensions, *a*, *b*, *c* (Å)	48.8, 68.3, 77.8	48.7, 68.2, 78.1
resolution (Å)	35.37−1.73	51.3−1.88
R_{merge}	0.113 (0.702)	0.066 (0.520)
$I/\sigma(I)$	48.3 (2.5)	26.1 (5.1)
no. of reflections	25889 (1429)	21801 (3088)
completeness (%)	97.6 (74.4)	99.5 (98.9)
redundancy	3.5 (2.4)	14 (13.7)
Wilson *B* value (Å^2)	41.3	24.7
no. of heavy atom sites		2
figure of merit		0.37
	Refinement	
resolution (Å)	35.37−1.73	
no. of reflections	25889 (1429)	
R_{work}/R_{free}	0.214/0.239	
no. of atoms		
protein	1932	
water	131	
av *B* factors (Å^2)		
protein	41.1	
water	50.2	
RMS deviations		
bond lengths (Å)	0.010	
bond angles (deg)	1.424	
Ramachandran statistics (%)		
preferred regions	98.72	
allowed regions	1.28	
outliers	0.0	

[a]Values in parentheses refer to the highest resolution shell (1.76−1.73 Å).

final step in the formation of the C3−C8 triene system characteristic of bacillaene.

RESULTS AND DISCUSSION

The boundaries chosen for PksEI14 were based on the boundaries of domains upstream and downstream within PksR (PksKS14 and PksACP14, respectively). Crystals provided diffraction data enabling a model of PksEI14 to be refined to a resolution of 1.73 Å, with phasing obtained by single-wavelength anomalous dispersion from crystals soaked in methyl mercury acetate (Table 1). The structure revealed a double-hotdog fold with an overall architecture very similar to that of *cis*-AT PKS DH domains (C_α rmsd: 2.4 Å over 250 C_α when aligned with the DH from the fourth module of the erythromycin synthase, EryDH4; PDB code 3EL6) (Figure 2A).[13] In contrast to all known *cis*-AT DH structures, PksEI14 crystallized as a monomer, without the ∼20-residue N-terminal region observed in those DHs that mediates their dimeric interfaces.

A structural alignment of PksEI14 with EryDH4 reveals that the α-carbon of the catalytic histidine (His18 of PksEI14) conserved in both PKS DH and EI domains superposes with the α-carbon of the catalytic histidine of EryDH4; however, the imidazole rings are offset by ∼1.7 Å (as measured between N_ε atoms) (Figure 2B). In PKS DHs, the catalytic histidine lies in a highly conserved HX_8P motif, in which its imidazole stacks against the proline ring.[13] This motif is HX_8V in PksEI14, with the $C_{\gamma1}$ methyl group of the valine sterically repositioning the imidazole ring. In other PKS EI domains, leucine is observed to substitute for the proline (Supplementary Figure S1). The aspartic acid that completes the catalytic dyad of PKS DHs is often substituted for an asparagine in EI domains, and the structure reveals that this asparagine (Asn182) is also repositioned relative to the aspartic acid. A highly conserved glutamine or histidine in PKS DHs located four residues upstream of the catalytic aspartic acid is presumably responsible for increasing the pK_a of this residue, priming it as a proton donor during the dehydration reaction.[15] The conservation of this glutamine/histidine residue is not maintained among EIs and is substituted by either a valine or leucine (Leu186 in PksEI14, Supplementary Figure S1).

The previously suggested mechanism of $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization by the EI domain involves an unknown residue that acts as a general base and abstracts a proton from the γ-carbon, similar to the isomerization mechanism for FabA.[9,11,16] However, the structure reveals an active site pocket dominated by hydrophobic residues that cannot participate in acid−base catalysis. The only ionizable residue within the PksEI14 active site in position to play this role is Tyr185, although it is not conserved among available EI sequences. To determine whether Tyr185 is involved in catalyzing double bond isomerization, an assay was developed in which *in vitro* activity from the isolated PksEI14 domain was reconstituted. As obtaining an analogue of the natural polyketide substrate to observe the reaction in the forward direction was challenging natural polyketide substrate (a long, highly functionalized

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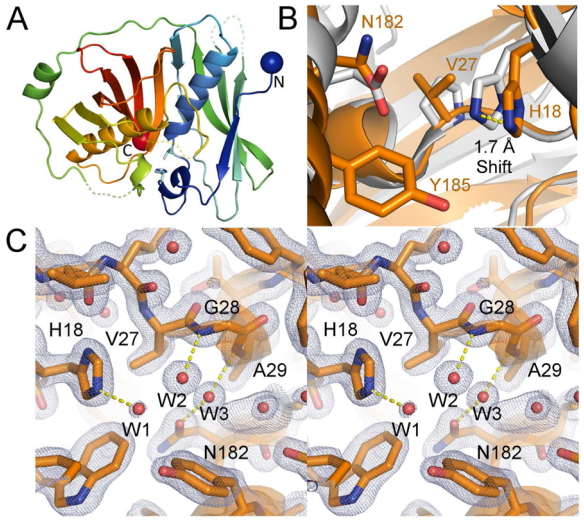

Figure 2. Structure of PksEI14. (A) The crystal structure of the PksEI14 monomer is shown in cartoon form, with N- and C-termini labeled with blue and red spheres, respectively. Flexible loops that could not be modeled into electron density are shown as dashed lines. The catalytic histidine (His18) and active site valine (Val27) are modeled as sticks. (B) A structural alignment of PksEI14 (orange) with the DH domain from the erythromycin PKS (gray, PDB code 3EL6) reveals that the highly conserved proline in the PKS DH HX_8P motif is replaced with a valine, inducing a steric repositioning of the catalytic His18 imidazole ring. Asn182 replaces the catalytic aspartic acid of the PKS DH domain. The only active site residue that could complete a catalytic dyad is Tyr185; however, mutation to phenylalanine did not abolish isomerase activity. (C) Stereodiagram of active site $2F_o - F_c$ electron density contoured at 1.5 Å rmsd. Yellow dashes indicate hydrogen bonds to ordered waters (each between 2.8 and 3.1 Å). See also Supplementary Figure S1.

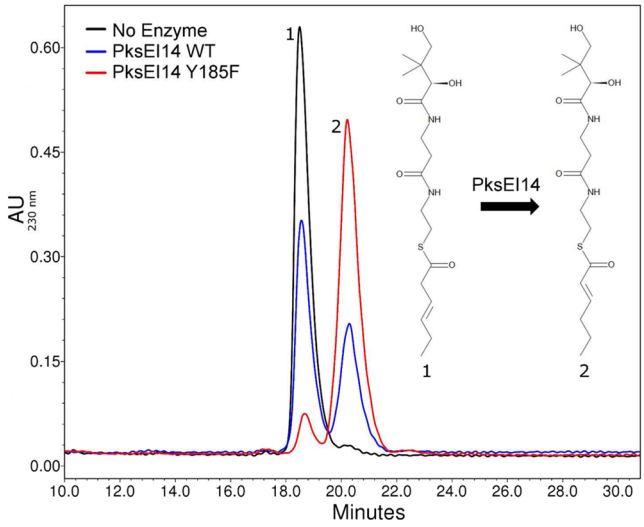

Figure 3. PksEI14 activity assay. Reversed-phase HPLC chromatograph of reconstituted *in vitro* isomerase activity observed after a 24 h incubation of the β,γ-unsaturated substrate analogue with isolated PksEI14. The black line represents a control reaction that did not include any enzyme, revealing a single peak for (*E*)-hex-3-enyl-*S*-pantetheine. The blue line represents the same reaction with the addition of wild-type PksEI14, revealing a new peak with properties consistent with a synthetic standard of (*E*)-hex-2-enyl-*S*-pantetheine, the $\beta,\gamma \rightarrow \alpha,\beta$ isomerized product. An identical reaction replacing wild-type PksEI14 with the Tyr185Phe mutant is shown in red, exhibiting increased isomerase activity over the same time course. See also Supplementary Figure S2.

polyene diamide), an analogue of the reaction product, (*E*)-hex-3-enyl-*S*-pantetheine, was prepared instead. PksEI14-mediated isomerization yielding (*E*)-hex-2-enyl-*S*-pantetheine would thus resemble the anticipated reverse direction for double bond migration, namely, $\beta,\gamma \rightarrow \alpha,\beta$ double bond isomerization. The reverse of the biological reaction is anticipated to be thermodynamically more favorable for this analogue since the double bond of the product is conjugated with the thioester carbonyl. Incubation of (*E*)-hex-3-enyl-*S*-pantetheine with PksEI14 followed by reversed-phase HPLC analysis revealed substrate conversion to a distinct species with a retention time equivalent to that of (*E*)-hex-2-enyl-*S*-pantetheine and UV absorbance maxima of 225 and 263 nm, characteristic of α,β-unsaturated thioesters (Figure 3 and Supplementary Figure S2).[17] LC–MS analysis revealed that the generated compound exhibited a molecular weight also consistent with the anticipated product. Surprisingly, the active site mutant in which the suspected tyrosine was replaced with a phenylalanine, PksEI14(Tyr185Phe), catalyzed the isomerization ~3.1-fold more rapidly, revealing that the tyrosine is not necessary for catalysis (Figure 3). The enhancement in rate may reflect a slightly better fit of the substrate analogue to the altered active site. The tyrosine-to-phenylalanine mutation is not expected to alter the mechanism for isomerization since a phenylalanine resides in this position within the Rhizoxin EI domain (Supplementary Figure S1). An *N*-acetyl-cysteamine (NAC) derivative of the β,γ-unsaturated substrate ((*E*)-hex-3-enyl-*S*-NAC) was also incubated with PksEI14 under similar conditions; however, isomerization to (*E*)-hex-2-enyl-*S*-NAC was negligible (Supplementary Figure S2b).

Since the structure does not reveal any other active site residues that could catalyze acid–base chemistry, the general base in the biological reaction could simply be a solvent-supplied water molecule, similar to the isomerization mechanism proposed for the crotonase domain of multifunctional enzyme, type-1.[18] The reverse *in vitro* reaction described herein would require water to play the role of a general acid in donating a proton to the γ-carbon. The PksEI14-catalyzed reaction described above was conducted in D_2O (with the more catalytically proficient Tyr185Phe mutant) to observe whether a solvent-supplied deuteron would label the γ-carbon in the product, (*E*)-4-deutero-hex-3-enyl-*S*-pantetheine. The reaction afforded sufficient (~3 mg) isomerized product for analysis by ^{1}H NMR, revealing a characteristic β-carbon proton splitting pattern that corresponded to a completely protonated methylene γ-carbon (Figure 4). As the α,β-unsaturated reaction product was not purified from the β,γ-unsaturated substrate before ^{1}H NMR analysis, (*E*)-hex-3-enyl-*S*-pantetheine was still clearly visible in the spectrum (1:0.52 molar ratio by proton signal integration). Greater than 95% of the α-protons of (*E*)-hex-3-enyl-*S*-pantetheine had exchanged for deuterons during the course of the reaction, confirming that the degree of contamination by residual solvent protons was insignificant (Supplementary Figure S3C). The rate of solvent-catalyzed α-proton exchange is on approximately the same time scale as the concurrent background hydrolysis of the labile thioester in both substrate and product, complicating the analysis of reactions incubated longer than 24 h (Supplementary Figure S3D).

To determine if the remarkable decrease (>10-fold) in catalytic rate of the reaction in D_2O was the result of an

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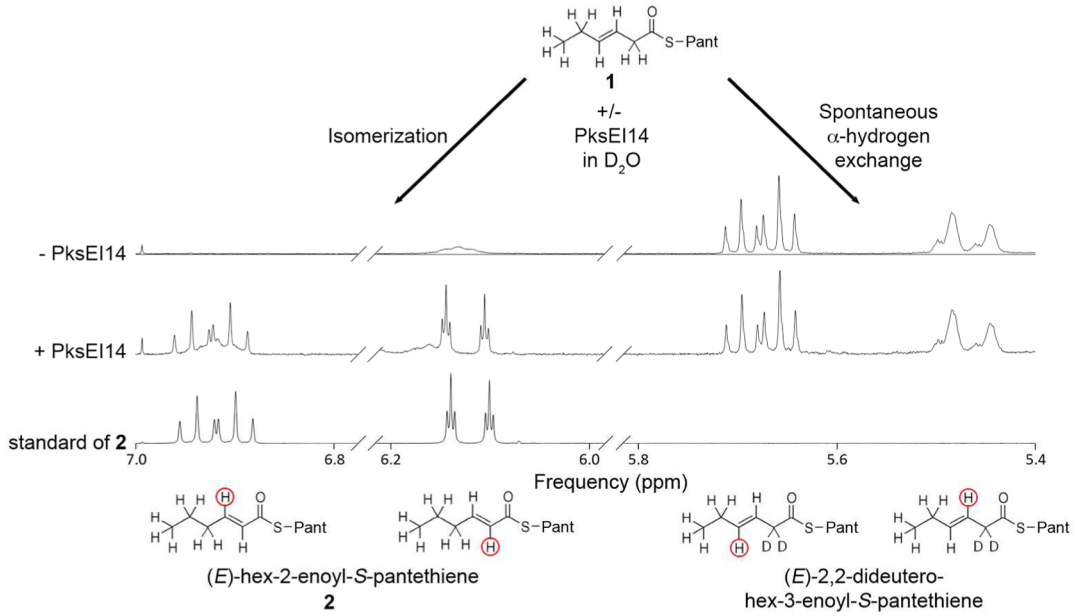

Figure 4. PksEI14-catalyzed isomerization in D_2O. To determine whether PksEI14 shuttles a proton within the substrate or employs water in the catalytic mechanism, (*E*)-hex-3-enyl-*S*-pantetheine (**1**) was incubated with PksEI14 in D_2O, and the reaction was analyzed by NMR. The negative control (top spectrum), in which PksEI14 was not added, shows only uncatalyzed α-hydrogen exchange. When PksEI14 is supplied (middle spectrum), isomerization to (*E*)-hex-2-enyl-*S*-pantetheine (**2**) is observed. If the mechanism of isomerization involved a solvent- or enzyme-donated proton, the γ-position would be monodeuterated, and different splitting patterns would be observed. The bottom spectrum is of synthetically prepared (*E*)-hex-2-enyl-*S*-pantetheine (**2**). Each of the four possible olefinic proton signals are aligned with the corresponding region of the spectrum and denoted with red circles. Pantetheine moieties are represented by "Pant". See also Supplementary Figure S3.

abnormally large kinetic isotope effect, (*E*)-2,2-dideutero-hex-3-enyl-*S*-pantetheine was incubated with PksEI14 in H_2O. Although isomerization to (*E*)-hex-2-enyl-*S*-pantetheine was observed, it could not be excluded that the product was generated primarily from a substrate species that had first undergone background exchange of α-deuterons for solvent protons, compromising any conclusions drawn from analysis of the product. The reduced catalytic rate observed for the reaction conducted in D_2O may have resulted from an alteration of the PksEI14 active site geometry caused by deuteration, as observed for haloalkane dehalogenase.[19]

To our knowledge, the only polyketide from *cis*-AT PKSs that contains a β,γ double bond is ansamitocin (Figure 1A). Intriguingly, the ansamitocin PKS does not contain an EI domain, yet studies have shown that the formation of the β,γ double bond occurs while the polyketide is tethered to the synthase, and the positioning of this double bond is critical for downstream transfer of the growing intermediate.[20] A sequence alignment of DH domains reveals that the highly conserved catalytic aspartic acid in the responsible DH (AsmDH3) is replaced by a glutamic acid, which may help enable an unusual β,ε-dehydration of the substrate. Although the migration of double bonds by polyunsaturated fatty acid synthases has also been proposed to be catalyzed by dedicated shift modules, no *cis*-AT PKS pathway has been discovered that employs a dedicated EI domain to catalyze $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization.[21]

As suggested by the results presented here, the forward biological reaction ($\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization) would be thermodynamically unfavorable if the substrate did not harbor a moiety at the δ-position available for conjugation with a β,γ double bond (e.g., double bond, amide, carbonyl). For PksEI14, this functional group is supplied by the δ,ε double bond installed by PksDH12. This δ,ε double bond is in conjugation with the double bond installed by PksDH11, which is in conjugation with the amide formed by the preceding NRPS module. While the amide likely sets a thermodynamically favorable foundation for the three subsequent double bond shifts, sequence alignments do not provide a clear explanation for how PksDH11 and PksDH12 are able to catalyze both dehydration and isomerization without a dedicated EI domain. These DHs could mediate isomerization after normal α,β-dehydration, similar to the mechanism proposed for FabA.[16] Also possible is that the DHs of these modules directly dehydrate the β,γ-positions through the elimination of a γ-proton and β-hydroxyl; this type of dehydration may be facilitated by the increased acidity of the γ-proton adjacent to the amide or double bond at the δ,ε-position. In addition to the bacillaene EI domain, the EIs of both the rhizoxin and corallopyronin PKSs isomerize double bonds into conjugation with preexisting functional groups at the δ,ε-position (a double bond and amide, respectively). Whether PksDH13 is capable of catalyzing isomerization is unknown; perhaps PksEI14 is more compatible with the substrate (e.g., due to its geometry or presence of an α-substituent).

Our results suggest that the mechanism for double bond isomerization by the EI domain proceeds via the shuttling of a substrate proton by a single active site histidine, similar to the mechanism of triose phosphate isomerase.[22] An alignment of the six crystal structures of *cis*-AT PKS DHs solved to date reveal that the imidazole groups of the catalytic histidines superpose nearly perfectly,[15] yet the imidazole group of PksEI14 is relatively shifted ~1.7 Å (as measured between N_ε atoms) (Figure 2B). Since the EI domain possesses the same double-hotdog fold as the DH domain, the ACP-bound substrate may be presented to the active site residues of these two domains in a similar manner. The shifting of the catalytic histidine from its position in DH domains is

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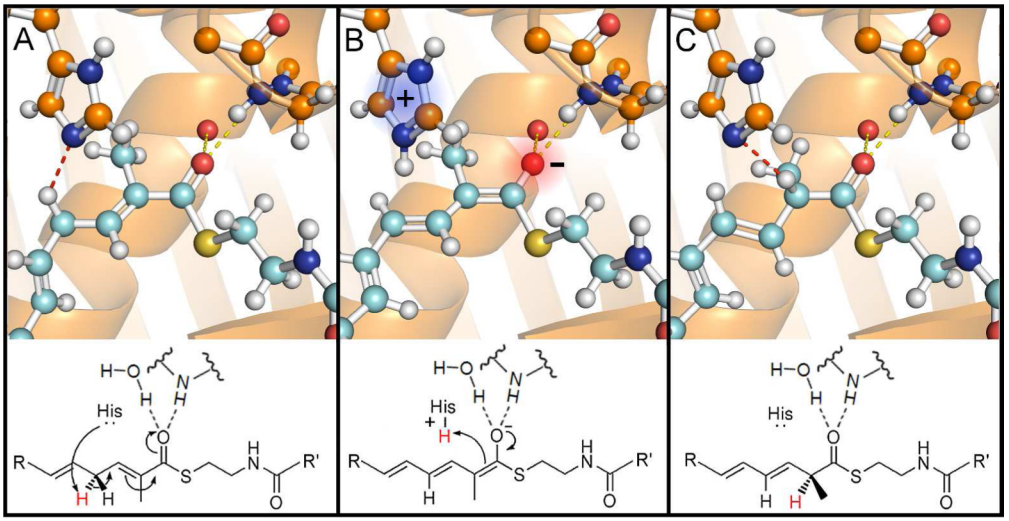

Figure 5. Proposed mechanism for double bond migration. Shown in three panels is the proposed mechanism for the biological reaction catalyzed by the PKS EI domain. Bacillaene carbon atoms are modeled in cyan, "R" represents bacillaene atoms not shown in the figure, and "R′" represents the ACP phosphopantetheinyl arm. Each yellow dashed line indicates a distance of 2.8−3.1 Å, and the red dashed lines indicate a distance of 2.2 Å. (A) The substrate modeled contains an α-methyl branch and is unsaturated at the α,β and δ,ε positions to resemble the biological intermediate. The thioester oxygen atom is modeled in the place of water W2 (see Figure 2C). Catalysis is initiated by the abstraction of a γ-proton by the catalytic histidine. (B) The enolate is stabilized by the polarization of the thioester carbonyl, which forms interactions with the positive dipole of a central α-helix. (C) In the final panel, the proton abstracted from the γ-position has been shuttled to the α-position.

approximately the distance of a carbon−carbon bond and could position the imidazole group between the sites of proton abstraction and donation. The shuttling of a substrate proton catalyzed by a single residue to isomerize a double bond has been proposed for several other isomerases, including ketosteroid isomerase, human mitochondrial enoyl-CoA isomerase, cyclohexenylcarbonyl-CoA isomerase, and $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase.[12,23−25]

The possible intermediate of the mechanism proposed here involves either the transient protonation of the thioester carbonyl to an enol or the formation of an enolate that is stabilized by an oxyanion hole. To better understand how the single active site histidine catalyzes isomerization unaided and what the intermediate may be, substrate and product analogues were modeled into the active site of PksEI14. The location of the substrate and product were subjected to several restraints. First, a conserved positively charged residue and phenylalanine have been suggested to form the ACP-docking site for *cis*-AT PKS DHs, and these residues are retained in PksEI14 (Lys257 and Phe204).[13] The modeled substrate and product were therefore oriented so that the phosphopantetheinyl arm would be in close proximity to these residues. Second, since the active site histidine must interact with both the α- and γ-carbons of the polyketide, a second restraint limited the distance of these carbons to within 3.0 Å of the ε-nitrogen of the catalytic histidine. Finally, the remaining atoms were manually adjusted to minimize steric clash. These restraints revealed that the thioester carbonyl would be positioned to replace Wat2, forming hydrogen bonds with both the backbone amide of Gly28 and Wat3 (Figure 5). Gly28 is located on the N-terminal end of the first central α-helix, which contributes a partial positive charge from the helix dipole. Wat3 is coordinated by the carboxamide side chain of Asn182 (which replaces the catalytic aspartic acid of PKS DHs) and the backbone amide of Ala29, the second residue of this central helix (Figure 2C). An oxyanion hole formed by these two interactions could stabilize the thioester in an enolate form. No ionizable side chain is available in this region to donate a proton to the thioester carbonyl to form an enol intermediate. The participation of water molecules as oxyanion hole hydrogen bond donors is quite common among enolizing enzymes.[26] If the model presented in Figure 5 is an accurate representation of how the substrate binds to the active site of PksEI14, the stereoselectivity of the *in vivo* reaction would generate an *S* stereochemistry at the methyl-bearing α-carbon (C2 of bacillaene).

In contrast to each of the structures of *cis*-AT PKS DHs that have been solved to date, PksEI14 crystallized as a monomer. This raises questions regarding the architecture of *trans*-AT PKS shift-modules, represented by module 14 of PksX (generally composed of a condensation-incompetent KS, an EI, and an ACP domain). *cis*-AT PKS DHs dimerize primarily through interactions between their 20 N-terminal residues.[13−15] However, an equivalent N-terminal stretch is absent in both PksEI14 and the homologous domain from the Bae synthase. The recently published structure of the KS-B didomain from the rhizoxin *trans*-AT PKS reveals that the B domain, structurally homologous to a DH domain, dimerizes quite differently from *cis*-AT PKS DHs with a less extensive interface.[5] This smaller interface may also be the only interaction between the EI domains of shift modules.

This study reports the first structure of the *trans*-AT PKS EI domain, revealing how divergent evolution of the double-hotdog fold has rendered an additional PKS domain capable of distinct chemistry.[27−29] The results presented here strongly suggest that a key active site modification between PKS DH and EI domains enables the EI histidine to shuttle a proton between the α- and γ-carbons of an enoyl intermediate. Characterization of the reconstituted *in vitro* activity catalyzed by PksEI14 in deuterated solvent supports a mechanism whereby a γ-proton (C4 of bacillaene) is abstracted by the active site histidine and is relocated to the α-position (C2) without the participation of solvent protons. To our knowledge, this enzymatic mechanism is the first example of a histidine

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independently shuttling a substrate proton to catalyze $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization.

METHODS

Cloning, Expression, and Purification. The gene corresponding to PksEI14 was amplified from *B. subtilis* ssp. 168, cloned into the LIC expression vector pGAY28b, and expressed heterologously in *Escherichia coli* BL21(DE3).[30] Hexahistidine-tagged PksEI14 was purified from cell lysate using immobilized metal affinity and size-exclusion chromatography. The mutation of the active site tyrosine to phenylalanine (Tyr185Phe) was accomplished using standard gene mutation techniques, and the resulting protein was purified in a similar manner to the wild-type.

Crystallization and Structure Determination. Crystals of PksEI14 grew over 1−4 days by sitting drop vapor diffusion at 22 °C in a condition composed of ammonium sulfate and Tris-HCl. The structure was initially solved by single-wavelength anomalous dispersion using crystals soaked in a solution containing methyl mercuric acetate. The experimental phases provided an initial map, which was used to construct a model that was further refined with a 1.73 Å resolution native data set. The coordinates for PksEI14 have been deposited in the Protein Data Bank, accession code 4U3V.

ASSOCIATED CONTENT

Supporting Information

Complete details on protein purification, crystallization, syntheses of thioester substrates and activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

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Author Contributions

D.G. solved the native and derived crystal structures, synthesized thioester substrates, and conducted *in vitro* assays. P.S. cloned the PksEI14 gene, purified the protein, and optimized crystals. D.G. and A.T.K. designed the experiments and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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