Discovery and Characterization of Pyridoxal 5'-Phosphate-Dependent Cycloleucine Synthases

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Supporting Information Placeholder

ABSTRACT: Pyridoxal 5'-phosphate (PLP)-dependent enzymes are the most versatile biocatalysts for synthesizing non-proteinogenic amino acids. α,α-disubstituted quaternary amino acids, such as 1-amino-1-cyclopentanecarboxylic acid (cycloleucine), are useful building blocks for pharmaceuticals. In this study, starting with the biosynthesis of fusarilin A, we discovered a family of PLP-dependent enzymes that can facilitate tandem carbon-carbon forming steps to catalyze an overall [3+2]-annulation. In the first step, the cycloleucine synthases use SAM as the latent electrophile and an *in situ*-generated enamine as the nucleophile for γ-substitution. Whereas previously characterized γ-replacement enzymes protonate the resulting α-carbon and release the acyclic amino acid, cycloleucine synthases can catalyze an additional, intramolecular aldol or Mannich reaction with the nucleophilic α-carbon to form the substituted cyclopentane. Overall, the [3+2]-annulation reaction can lead to 2-hydroxy or 2-aminocycloleucine products. These studies further expand the biocatalytic scope of PLP-dependent enzymes.

INTRODUCTION

Nonproteinogenic amino acids, including those that are α,α-disubstituted, are important building blocks for pharmaceuticals and materials.[1–4] Numerous α,α-disubstituted quaternary amino acids are represented in drug molecules including eflornithine, fingolimod, and difelikefalin as an essential pharmacophore (Figure 1A).[5] Incorporation of α,α-disubstituted amino acids into peptides is also known to induce α-helix formation, which accounts for the membrane destabilization exerted by peptaibols, a class of peptidic broad-spectrum antibiotics.[6–8] Due to such prominent functions of α,α-disubstituted amino acids in biomolecules, new asymmetric strategies to synthesize these quaternary amino acids with stereoselectivity have gained attention.[9] While progress has been made in the past two decades[10–13], construction of amino acids bearing multiple stereocenters with full control of the absolute and relative configuration remains a challenging goal. This is one area where biocatalysis using enzymes can contribute new methodologies and reagents. [14]

Naturally occurring α,α-disubstituted amino acids can be found as building blocks for natural products or as precursors for signaling molecules (Figure 1B).[1,15] Nature employs a number of biosynthetic enzymes to construct these amino acids, often involving remarkable chemistry. For example, a recent study revealed that 2-aminoisobutyric acid (2-AIB, Figure 1B) found prevalently in peptaibol antibiotics, is biosynthesized from L-Val by a three-enzyme (TqaL/F/M) cascade via an unexpected aziridine intermediate (Figure S4).[16,17] The biosynthesis of L-isovaline was recently demonstrated to follow a similar oxidative rearrangement mechanism staring from L-Ile.[18] The biosynthesis of α-Me-L-serine (Figure 1B) uses the pyridoxal 5'-phosphate (PLP)-dependent enzyme FmoH that catalyzes hydroxymethylation at the α-position of D-alanine using methylenetetrahydrofolate (mTHF) as a cofactor.[19]

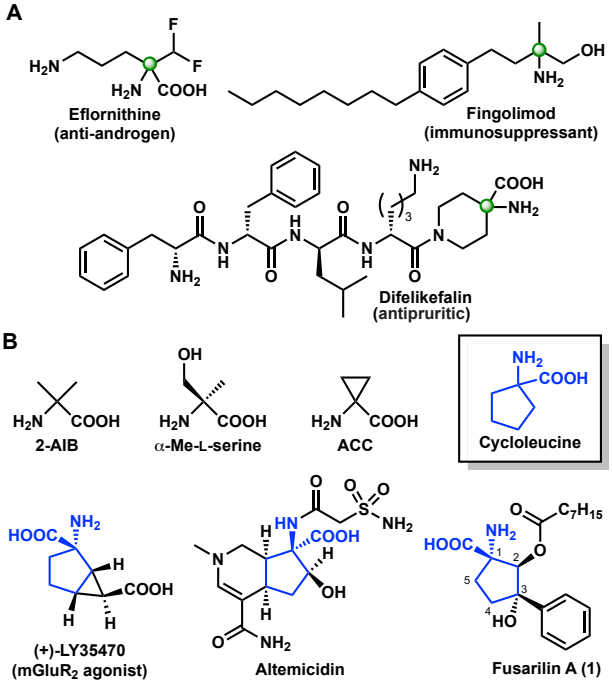

Figure 1. α,α-disubstituted amino acids. (A) Examples of FDA-approved drug containing α,α-disubstituted amino acid. (B)Structures of natural α,α-disubstituted amino acids, and cycloleucine-containing drug and natural products.

1-aminocyclopropane carboxylic acid (ACC, Figure 1B), an intermediate to plant ripening hormone ethylene[20], is biosynthesized from the intramolecular cyclization between the C_α and C_γ carbons of *S*-adenosyl-L-methionine (SAM) by the PLP-dependent ACC synthase (ACS).[21] Formation of the cyclopropane is accompanied by the cleavage of the C_γ-S bond and expulsion of thiomethyladenosine (MTA) (Figure S5).

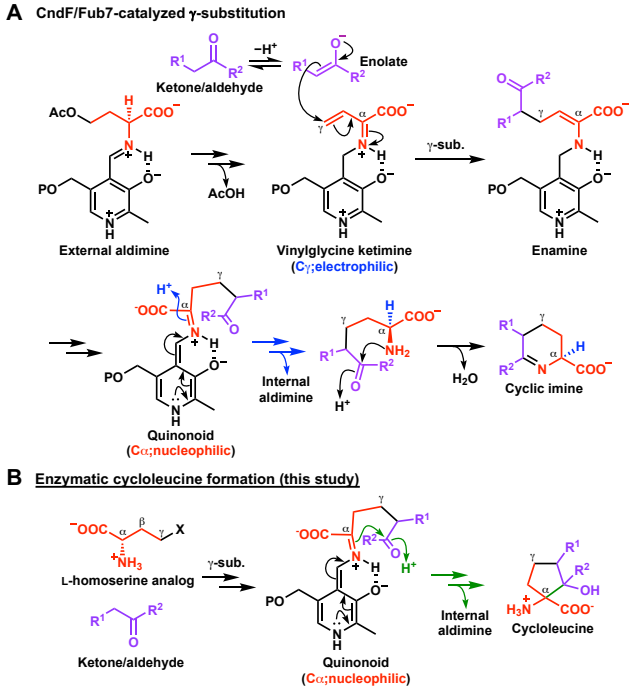

Figure 2. PLP-dependent enzymes as a cycloleucine synthase. (A) The proposed mechanism of CndF- and Fub7-catalyzed γ-substitution. (B) Proposed mechanism of a PLP-dependent cycloleucine synthase. After γ-substitution, it is mechanistically conceivable that the nucleophilic C_α-carbon in quinonoid form attacks the aldehyde intramolecularly to form cycloleucine.

1-aminocyclopentane carboxylic acid, also known as cycloleucine, is another prominent α,α-disubstituted quaternary amino acid that is a key pharmacophore for specific and reversible inhibition of nucleic acid methylation.[22–26] Cycloleucine is also found in pharmaceuticals such as metabotropic glutamate (mGlu) receptors 2 and 3 agonist (+)-LY35470 (Figure 1B).[27] While the biosynthesis of free-standing cycloleucine has not been reported to date, it is mechanistically conceivable that a PLP-dependent enzyme which catalyzes γ-substitution can form cycloleucine-containing product via tandem C_γ and C_α C-C bond-forming steps (Figure 2). In these PLP-dependent γ-substitution enzymes characterized so far, such as cystathionine-γ-synthase[28], CndF[29] and Fub7[30,31], a latent electrophile such as *O*-acetyl-L-homoserine is first bound to the PLP cofactor as an external aldimine (Figure 2A). Following ejection of acetate and formation of the PLP-bound vinylglycine electrophile, external nucleophiles attack the C_γ with the PLP as an electron sink to complete the γ-substitution reaction. In enzymes such as CndF and Fub7, the γ-substitution nucleophile is the enol form of a ketone or an aldehyde, respectively. Protonation of C_α of the resulting quinonoid species leads to release of the amino acid, which can undergo intramolecular Schiff base formation and subsequent reduction to give substituted pipecolate (Figure 2A). However, one can envisage that in a properly oriented enzyme active site, a second C-C bond forming step from the quinonoid intermediate can take place, in which the nucleophilic C_α attacks the electrophilic carbonyl stereoselectively to afford a substituted cyclopentane (Figure 2B). A PLP enzyme catalyzing tandem bond formations was recently identified, providing support for the proposal shown in Figure 2B: the bacterial enzyme SbzP catalyzes the condensation between SAM and β-NAD to form the 6-azatetrahydroindane (cycloleucine fused with tetrahydropyridine) ring system in altemicidin (Figures 1B and S6), albeit with different mechanism than proposed here.[32]

Here, we discovered a family of PLP-dependent cycloleucine synthases conserved in both bacteria and fungi. These enzymes perform tandem C-C bond forming steps using SAM and a transiently generated imine/enamine to form substituted cycloleucines.

RESULTS

Fusarilins are substituted cycloleucine natural products. To mine a putative cycloleucine synthase from microbial biosynthetic pathways, we searched for natural products containing a cycloleucine substructure in databases (Reaxys and Dictionary of Natural Products) and in literature. In addition to altemicidin, four such natural products were found, including fusarilin A (**1**)[33] (Figure 1B), 1-amino-2-nitrocyclopentanecarboxylic acid,[34] stephadiamine,[35] and tagetitoxin[36] (Figure S1). Fusarilin A (**1**) was isolated from the endophytic fungus *Fusarium* sp., and contains a cycloleucine core with 2-acyloxy, 3-phenyl and 3-hydroxyl substituents.[33] Categorically, **1** can be classified as an unknown (core enzyme)-known (compound)-natural product, as it does not appear to be derived from well-studied families of biosynthetic core enzymes.[37] Based on our proposal in Figure 2B, the 3-phenyl-2-hydroxyl-cycloleucine core (**2**) could be derived from the proposed PLP-dependent enzyme catalyzed [3+2]-annulation between 2-phenylacetaldehyde and a L-homoserine-derived electrophile (Figure 2B). From **2**, 2-*O*-acylation with octanoyl-CoA and 3-hydroxylation with a Fe-dependent monooxygenase would complete the biosynthesis of **1**. Therefore, the biosynthetic gene cluster (BGC) of **1** should contain at least a PLP-dependent γ-synthase, a monooxygenase, an acyltransferase, and an enzyme that can generate 2-phenylacetaldehyde from L-Phe.

Identification of fusarilin BGCs. Although the producing strain[33] that enabled isolation of **1** was not available, we performed genome mining to identify potential BGCs from available *Fusarium* genomes deposited in the National Center for Biotechnology Information (NCBI) database. While a number of PLP-dependent γ-synthases, including CndF,[29] Fub7[30,31] and LolC[38], have been characterized, we decided to use FlvA[39] from flavunoidine biosynthetic pathway as a query. FlvA is a didomain enzyme consists of an *N*-terminal PLP-dependent γ-synthase and a *C*-terminal α-ketoglutarate-dependent, nonheme iron oxygenase (α-KG). FlvA has not been biochemically characterized, but has been confirmed to be involved in the biosynthesis of 5,5-dimethyl-L-dehydropipecolate.[39] In steps parallel to that shown in Figure 2A, the *N*-terminal domain is proposed to catalyze γ-replacement of *O*-acetyl L-homoserine (OAH) with isobutylaldehyde converted from L-Val, the latter which was proposed to be derived from the function of *C*-terminal α-KG domain.[30] Based on the proposal that 2-phenylacetaldehyde is involved in formation of **2**, we reasoned that a combination of an α-KG and a PLP-dependent γ-synthase homologous to FlvA domains could anchor the BGC of **1**.

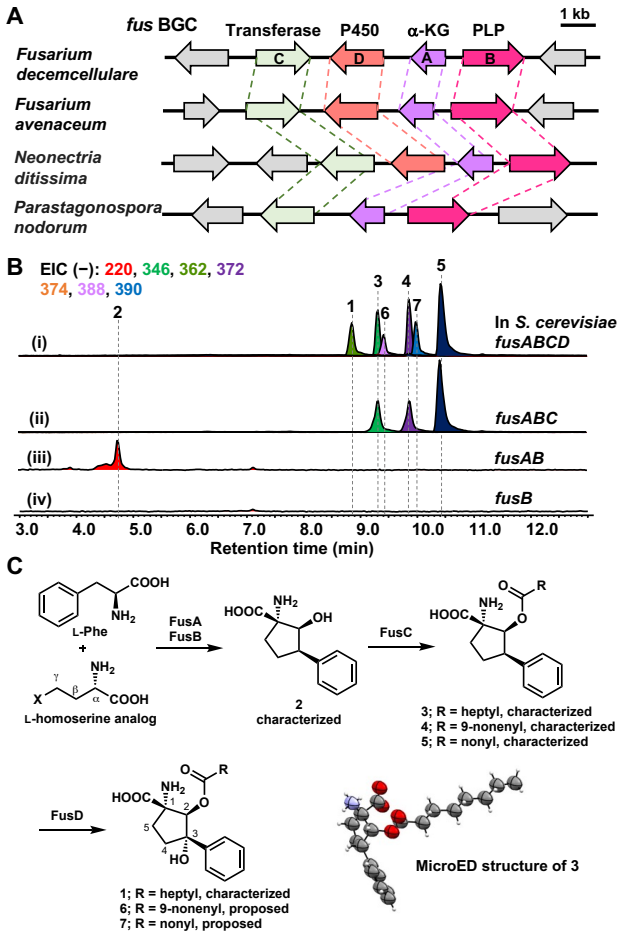

Figure 3. Discovery and heterologous reconstitution of the biosynthetic pathway of **1** and the analogs. (A) The BGCs identified. (B) Heterologous reconstitution of **1** and the analogs in *S. cerevisiae*. (C) The proposed biosynthetic pathway of **1** and the analogs. Absolute stereochemistry of **2**, **3**-**5** was deduced from the MicroED structure of **3** and the reported crystal structure of **1**.

We found more than ten *Fusarium* species encoding homologs (~50% identity) of α-KG and PLP domains of FlvA as separate proteins in a conserved BGC (FusA and FusB in *F. decemcellulare*, respectively) (Figure 3A). In these BGCs, other conserved genes are predicted to encode an acyltransferase (FusC) and a P450 monooxygenase (FusD). The four-gene cassette is also conserved in more distantly related fungal species including *Neonectria* spp., while *Parastagonospora* spp conserve the FusA-C three-gene cassette. The predicted functions of FusA-D are well-aligned with our retrobiosynthesis of **1**.

Reconstitution of fusarilin biosynthesis. The four genes FusA-D were synthesized, and heterologously expressed in the engineered *Saccharomyces cerevisiae* strain RC01.[40] Six new compounds absent in the control yeast strain were detected by LC/MS analysis, including one with the same molecular weight as **1** (Figure 3B, i). Large-scale cultivation of the yeast transformant in Yeast-Peptone-Dextrose (YPD) media was performed, followed by isolation and NMR characterization of the target compound. Both ^{1}H and ^{13}C NMR signals of **1**, as well as the optical rotation matched with published data in the same solvent,[33] confirming **1** produced by yeast is indeed fusarilin A (Table S4 and Figures S30-S34). Therefore, the connection between the *fus* genes, fusarilin A and cycloleucine biosynthesis was established.

To understand the steps in the biosynthesis of **1**, individual genes were removed followed by metabolite isolation and analysis. Removal of the P450 FusD abolished the production of **1**, **6**, and **7**, while compounds **3**, **4** and **5** remained in the extract (Figure 3B, ii). Characterization of the three compounds showed these are 3-deoxy derivatives of **1,** each acylated with a different medium-chain length fatty acid at the C2-OH group (octanoic acid for **3**, 9-decenoic acid for **4**, and decanoic acid for **5**) (Tables S6-S8 and Figures S42-S56), The octanoyl group is the same for **3** and **1**. 9-decenoic acid is a known metabolite of yeast[41], and is used as a substrate for acylation to form **4**. The differences in mass of 16 mu between **4** and **6**, and between **5** and **7**, suggests **6** and **7** are the 3-hydroxylated products of **4** and **5**, respectively (Figure 3C). The stereochemistry of **3** was assigned using microcrystalline electron diffraction (MicroED), which is consistent with the reported absolute stereochemistry[33] of **1** (Figure 3C). Based on the stereochemistry at C_3 as seen in the MicroED structure of **3,** the FusD-catalyzed hydroxylation of **3**-**5** is proceeds with retention of stereochemistry.

Formation of **3**-**5** by FusA-C also indicates the putative acyltransferase FusC catalyzes lipidation of **2**. Indeed, coexpression of only FusA and FusB abolished the production of **3**-**5**, but resulted in the formation of a much more polar compound that matched the molecular weight (MWT =221) of **2** (Figure 3B, iii). Isolation and characterization of **2** (0.2 mg/L) confirmed the compound to be 2-hydroxy-3-phenyl cycloleucine (Table S5 and Figures S35-S39). Individual expression of either FusA and FusB did not produce any detectable new metabolites (Figure 3B, iv and Figure S10). To confirm the hypothesis that L-Phe is a precursor to the phenyl ring in the biosynthesis of **1**, L-Phe-d_5 was supplemented to the transformant expressing FusA, FusB, FusC, and FusD. The perdeuterated phenyl ring was incorporated into **1** as the mass of **1** increased by 5 mu (Figure S11). These data are in line with our hypothesis that FusA catalyzes formation of 2-phenylacetaldehyde (**8**) from L-Phe (Figure 4), while FusB forms the cycloleucine core.

FusA is a non-heme iron-dependent decarboxylative desaturase. FusA was expressed and purified from *E. coli* C41 (DE3) (Figure S7), and subjected to in vitro biochemical analysis. As anticipated, L-Phe was readily consumed in the presence of FusA and typical cofactors of α-KG enzymes (Figure S12). To capture the product 2-phenylacetaldehyde (**8**), 3-nitrophenylhydrazine (3-NPH) was added as a derivatization reagent after quenching. The corresponding hydrazone 3-NPH-**8** ($[M–H]^-$ = 254) was detected from the derivatized reaction mixture and matched an authentic standard (Figure 4A). Excluding FusA from the reaction abolished the formation of 3-NPH-**8**. While D-Phe can also be converted to **8**, FusA prefers L-Phe as the substrate (Figure 4A, iii-v).

While 3-NPH-**8** was detected in the reaction mixture, a direct decarboxylation product from L-Phe could be phenylethylamine (**9**) (and the enamine tautomer styrylamine), which can then be hydrolyzed to **8**. To test whether **9** is formed during the FusA-catalyzed reaction, $NaBH_3CN$ was added to reduce the imine/enamine to 2-phenylethylamine **10** after quenching the reaction, followed by amine derivatization using dansyl chloride (DNS-Cl). The dansylated phenylethylamine (DNS-**10**) was clearly detected in the presence of FusA, while it cannot be detected in the absence of FusA or $NaBH_3CN$ (Figure 4B, i-iv). 2-phenylethylimine can be detected as DNS-**10** even after overnight enzymatic reaction (Figure S13), suggesting that the imine/enamine product is sufficiently stable to exist in the reaction mixture prior to hydrolysis into **8**.

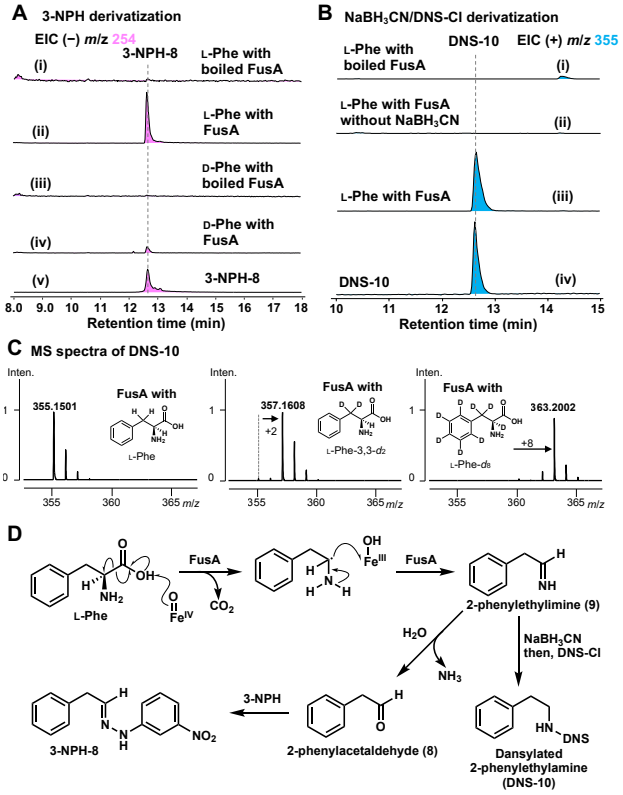

Figure 4. Biochemical characterization of FusA. (A) In vitro assay of FusA with L- and D-Phe. The product was derivatized by 3-nitrophenylhydrazine (3-NPH) (B) Detection of 2-phenylethylimine (**9**) from FusA reactions by $NaBH_3CN$ reduction followed by dansyl chloride (DNS-Cl) derivatization. (C)MS analysis of FusA-catalyzed reaction using deuterium labeled L-Phe. +2 Da and 8 Da mass shift of DNS-**10** were observed when L-Phe-3,3-d_2 and L-Phe-d_8 were used. (D) Proposed mechanism of FusA-catalyzed decarboxylative desaturation.

Several decarboxylative desaturases have been reported including IsnB,[42] P450 OleT,[43] radical-SAM enzyme MftC,[44] and diiron enzyme UndA.[45] The proposed mechanisms of these enzymes are initiated by β-hydrogen abstraction. For example, IsnB catalyzes the decarboxylation of L-Tyr isonitrile to form vinyl isonitrile via hydrogen abstraction at benzylic position by iron (IV)-oxo species. Sequence analysis of FusA showed it is most similar to the α-KG domain of FlvA[39] (~52% identity, used as query for BGC) and aziridine synthase TqaL[16,17] (~50% identity). TqaL is similarly proposed to perform β-hydrogen abstraction on L-Val, followed by intramolecular aziridine formation. Therefore, a plausible mechanism of FusA is to initiate decarboxylation of L-Phe through abstraction of the C_β hydrogen, followed by oxidative decarboxylation.

To test whether FusA follows a similar decarboxylation mechanism, in vitro reaction of FusA with L-Phe-3,3-d_2 was performed, followed by $NaBH_3CN$ reduction and DNS-Cl derivatization. Surprisingly, LC/MS analysis showed that both benzylic deuteriums of L-Phe-3,3-d_2 are retained in DNS-**10** (Figure 4C), excluding benzylic hydrogen abstraction as the first step. The same retention of all deuterium atoms was seen when L-Phe-d_8 was used as the substrate (Figure 4C), showing C-H activation of L-Phe is not required for formation of **9**. We therefore propose that the iron (IV)-oxo species of FusA abstracts the hydrogen from the carboxylic acid to generate the carboxylic radical (Figure 4D), which can undergo decarboxylation to form the radical intermediate. Further one-electron oxidation by the Fe(III)-OH species generates the C_α carbocation, which can be quenched by the neighboring amine to form 2-phenylethylimine (**9**). Such oxidative decarboxylation of amino acids has been noted in synthetic chemistry, during which a C_α radical contiguous to a nitrogen substituent can be oxidized to a cation.[46] The chemical logic of the unexpected radical decarboxylation may be to avoid formation of aziridine ring as observed in TqaL-catalyzed aziridine formation from L-Val.[16,17]

FusB is a SAM and PLP-dependent cycloleucine synthase. Reconstitution data suggested that the formation of **2** requires both FusA and FusB. As proposed in Figure 2B, FusB may catalyze tandem C-C bond formations between a PLP-bound vinylglycine and **8** to give **2**. To test this, purified FusB from *E. coli* C41(DE3) was mixed with **8** and possible latent γ-replacement electrophiles, including *O*-acetyl-L-homoserine, *O*-phospho-L-homoserine, *O*-succinyl-L-homoserine, and SAM. However, **2** cannot be detected (as DNS-**2**) from any of these reactions using **8** (Figure 5A and Figure S14). Instead, a product with MW that is same as dansylated homoserine lactone (SAM degradation product), DNS-HSL (Figure 5C), appeared in the reaction containing FusB and SAM. Also detected in this reaction is another DNS-Cl derivatized product with MW matching that of modified ACC or vinylglycine. Synthetic standards of both were prepared and showed the product to be dansylated vinylglycine (DNS-VG) (Figure 5A, iii and Figure S14). When derivative with Marfey's reagent 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA) and compared to synthetic standards of FDAA-derivatized (*S*)- and (*R*)-vinylglycine, the stereochemistry of the enzymatically formed vinylglycine was determined to be *S* (Figure S15). Vinylglycine has not been reported as a product of any PLP-dependent γ-synthases. Therefore, FusB is selective for SAM as the first substrate to generate PLP-bound (*S*)-vinylglycine and MTA, which is released from the enzyme in the absence of a competent γ-replacement nucleophile, which is not the aldehyde **8.**

When the in vitro reaction was repeated using both FusA and FusB, in the presence of L-Phe, SAM and necessary cofactors, accumulation of DNS-**2** was clearly observed (Figure 5A, ii and Figure S18). This hinted that the γ-replacement nucleophile for FusB could be 2-phenylethylimine (**9**) instead of **8**. Indeed, when an excess amount of NH_4Cl was added to the reaction containing FusB, SAM and **8**, DNS-**2** was detected from the assay (Figure 5A, iv), supporting the cosubstrate of FusB to be **9**. A +1 mu mass shift of DNS-**2** was observed when the in vitro reaction with FusA and FusB was performed using L-Phe-3,3-d_2 and SAM (Figure S23). This suggests loss of one deuterium during the reaction, which can result from the tautomerization of 2-phenylethylimine (**9**) to the enamine styrylamine, which can subsequently attack the PLP-bound vinylglycine.

Mechanistic analysis of FusB-catalyzed cycloleucine formation. When the combined assay of FusA and FusB was performed in >95% D_2O, a +1 mass shift of DNS-**2** (Figure 5B) was detected. This is in line with one deprotonation/protonation step during catalysis. If FusB uses the elimination–addition mechanism through the formation of the vinylglycine ketimine proposed for known PLP γ- synthases[28–31], proton abstraction events are expected to occur at the C_β and C_α positions of SAM. The labeled [3,3,4,4-d_4]-SAM and [2,3,3,4,4-d_5]-SAM were prepared in situ using human MAT isoform 2A (hMAT2A) and L-[3,3,4,4-d_4]-Met and L-[2,3,3,4,4-d_5; methyl-d_3]-Met, respectively. In the presence of [3,3,4,4-d_4]- SAM, a +3 mass shift of DNS-**2** was observed (Figure 5B), suggesting one deuterium from SAM is exchanged. When we repeated the same as-

say in D_2O, we observed +4 mass shift of DNS-**2**, confirming deprotonation and protonation occur at the same carbon of SAM, which is likely C_β of SAM. Similarly, a +3 mass shift of DNS-**2** was observed when we used [2,3,3,4,4-d_5]-SAM, while repeating the assay in D_2O gave +4 mass shift of DNS-**2** (Figure S23). This is agreement with the C_α proton abstraction to form the new C_α-C bond during catalysis. Overall, these observations are in agreement with the canonical elimination–addition mechanism via formation of the vinylglycine ketimine for γ-replacement.[28–31] It is also consistent with the exclusive formation of (*S*)-vinylglycine in the absence of **9**.

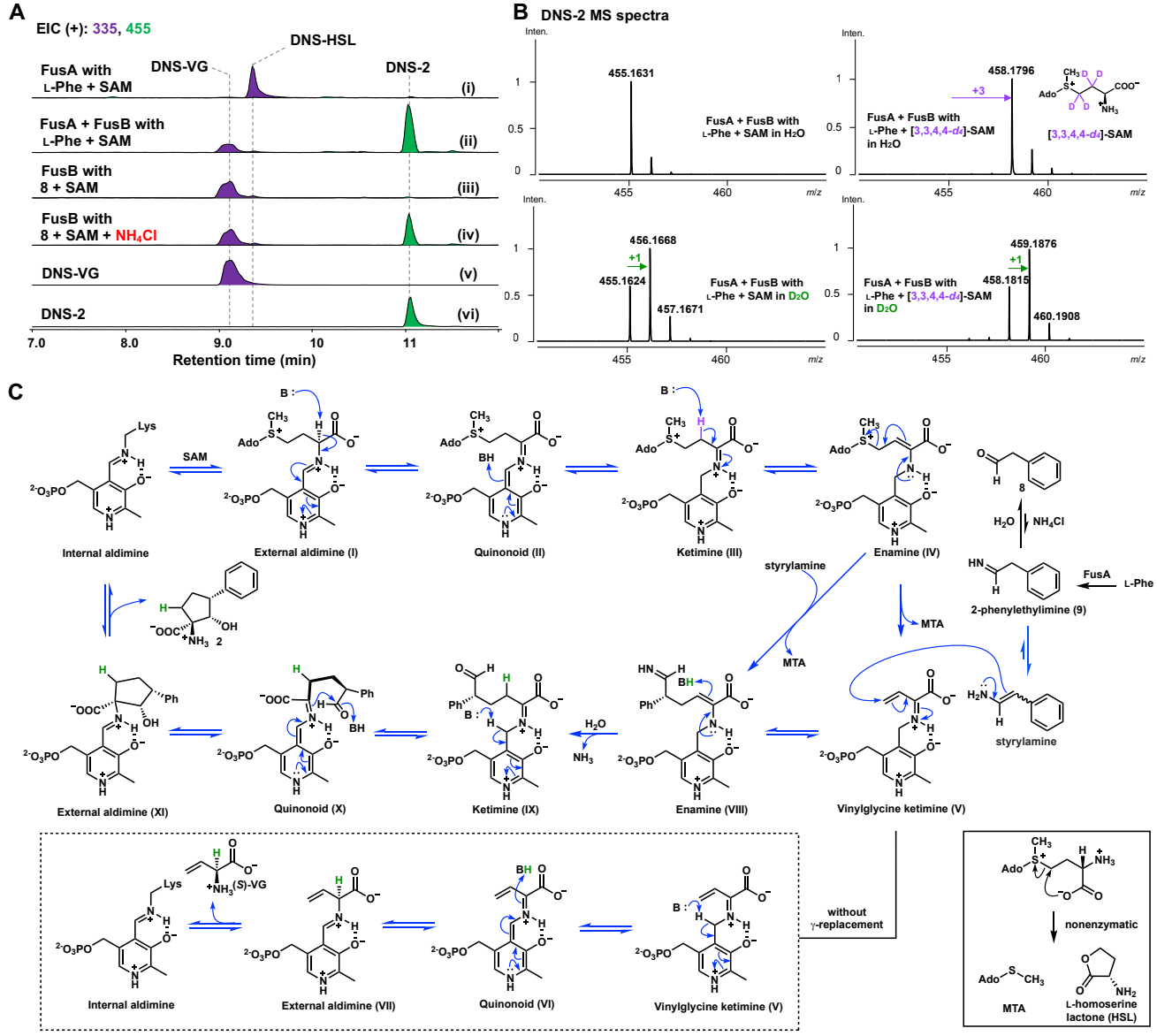

Figure 5. Biochemical characterization of FusB. (A) In vitro assay of FusB. The product was derivatized by dansyl chloride. DNS-VG: dansylated vinylglycine; DNS-HSL: dansylated homoserine lactone. (B) MS analysis of FusB-catalyzed reaction using D_2O and deuterium labeled SAM. +1 mass shift of DNS-**2** was observed when the combo in vitro reaction was performed in D_2O. +3 Da and +4 Da mass shift of DNS-**2** were observed when [3,3,4,4-d_4]-SAM were used for the assay in H_2O and D_2O, respectively. (C) Proposed mechanism of FusB-catalyzed [3+2]-annulation.

The proposed mechanism of the FusB-catalyzed [3+2]-annulation is shown in Figure 5C. The external aldimine (**I**) between PLP and SAM can be deprotonated at C_α of SAM to give quinonoid (**II**). Next, protonation of **II** gives the ketimine (**III**), which can be tautomerized to enamine (**IV**). In the absence of 2-phenylethylimine (**9**)/styrylamine (dash box), **IV** can undergo expulsion of methylthioadenosine (MTA) to form the vinylglycine ketimine (**V**). **V** can be deprotonated to the quinonoid (**VI**), which can be reprotonated at C_α to give the vinylglycine aldimine (**VII**). Release of (*S*)-vinylglycine as product regenerates the internal enzyme-PLP aldimine. In D_2O experiments where FusB was mixed with (*S*)- or (*R*)-vinylglycine as the substrate, +1 mass shift of (*S*)-vinylglycine was observed, while no mass shift was observed when (*R*)-vinylglycine was used (Figure S16). This suggests that the conversion between vinylglycine ketimine (**V**) to the external aldimine (**VII**) could be a reversible process. However, DNS-**2** cannot be detected when FusA and FusB were mixed with L-Phe and (*S*)-vinylglycine (Figure S17).

In the presence of 2-phenylethylimine (**9**), the enamine tautomer styrylamine can attack the C_γ of SAM in vinylglycine ketimine (**V**) to form the first C-C bond. Given the electrophilic character of the permanently charged trigonal sulfonium cation in SAM, it is also

possible that styrylamine directly attacks the C_γ of SAM in the enamine (**IV**) to form the C-C bond, which results in the direct formation of the enamine (**VIII**). In either mechanism, MTA is formed as a byproduct of the γ-substitution reaction (Figure S19). The resultant enamine (**VIII**) can undergo imine hydrolysis to the aldehyde and tautomerization to the ketimine (**IX**), which is further tautomerized to the quinonoid (**X**). To form 2-hydroxycycloleucine found in **2**, the imine derived from **9** must be hydrolyzed to aldehyde. This proposed step was validated when a +2 mass shift of DNS-**2** was observed when the assay was repeated in H_2O^{18} (Figure S23). From **X**, two electrons from the quinonoid nitrogen can be pushed to C_α carbon to attack the aldehyde in a 5-*exo-trig* fashion from the *si*-face, which results in the formation of the cyclopentane with stereoinversion at C_α. Release of **2** as the product from the new external aldimine (**XI**) regenerates the internal enzyme-PLP aldimine.

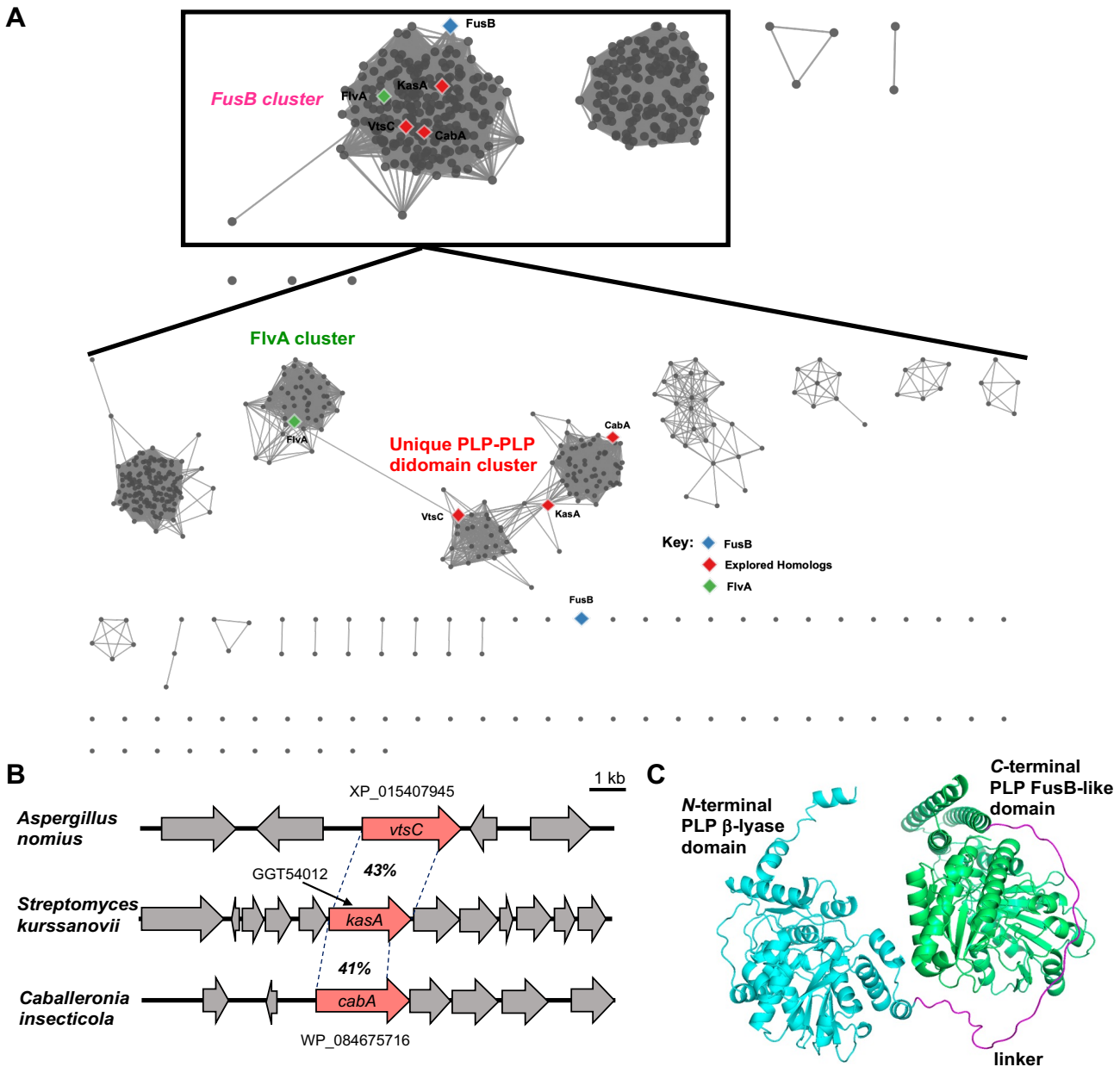

Figure 6. Sequence Similarity Network (SSN) of FusB and homologs. (A) SSN of FusB and FlvA with increased alignment score threshold from 20 to 150 along with node consolidation of 90% identity. (B) Homologs of FusB and their surrounding genes in *Aspergillus nomius*, *Caballeronia insecticola* and *Streptomyces kurssanovii*. (C) AlphaFold predicted structure of VtsC (AF-A0A0L1J5K8-F1).

SSN of FusB identified a new family of PLP-dependent cycloleucine synthases. To explore if other PLP-dependent cycloleucine synthases could be discovered, we constructed a sequence similarity network (SSN) using the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST)[47] with well-characterized γ–synthases along with FusB as the query (Figure S3). By increasing the minimum alignment score threshold, distinct clusters are formed including a cluster with FlvA and FusB. This becomes the new query for building a new SSN to explore this cluster (Figure 6A). By increasing the alignment score threshold to find subclusters in this new network, a unique cluster distinct from the dehydropipecolate synthase FlvA

cluster emerged, containing enzymes from both bacteria and fungi. Surprisingly, enzymes from this cluster, such as VtsC from *Aspergillus nomius*, KasA from *Streptomyces kurssanovii*, and CabA from *Caballeronia insecticola* are all didomain enzymes in which both domains are predicted to be PLP-binding (Figure 6B-C). The *C*-terminal domains show moderate sequence identity to FusB (~40%). The *N*-terminal domains of VtsC and KasA are predicted to have β-lyase function, and have high sequence similarity to L-serine dehydratase that catalyzes β-dehydration of L-serine to dehydroalanine.[48] The product dehydroalanine from a standalone L-serine dehydratase can tautomerize to 2-iminopyruvate that is readily hydrolyzed to pyruvate.[49,50]

Didomain enzymes with two PLP domains have not been discovered to date. Fusion of catalytic domains in a single enzyme is a strategy to minimize exposure of labile intermediates to water.[51,52] We therefore hypothesized that a transiently generated nucleophile by the β-lyase, such as 2-iminopyruvate/dehydroalanine, may be captured by the fused C-terminal domain for catalysis. To test this, the two domains of VtsC, the β-lyase domain (VtsC-β) and the FusB homolog domain (VtsC-γ), were dissected, expressed, and purified from *E. coli* C41(DE3). The β-lyase domain indeed uses L-Ser as a substrate and generated pyruvate as determined by the appearance of 3-NPH-pyruvate after 3-nitrophenylhydrazine (3-NPH) derivatization (Figure 7A, i-iv). Other known substrates[53] of PLP-dependent β-lyases such as L-Thr, L-Cys, and L-Trp are not accepted by VtsC-β (Figures S25 and S27). Assays with VtsC-γ showed that only SAM can be converted to (*S*)-vinylglycine following DNS-Cl and Marfey's derivatizations (Figure 7B and Figures S26-S28). When the dissected VtsC-β and VtsC-γ were mixed together with L-Ser and SAM, however, no new products besides pyruvate and (*S*)-vinylglycine were observed.

The full-length VtsC was then expressed and purified from *E. coli* C41(DE3) following optimization. When mixed with L-Ser and SAM, followed by derivatization with DNS-Cl, two new products with MW of 377 (major) and 610 (minor) were detected (Figure S24). The MW difference and shift in retention time suggest the presence of two free amine groups in the underivatized product. To increase the efficiency of derivatization of both amine groups, benzoyl chloride (BzCl) or 4-bromobenzoyl chloride (4-Br-BzCl) were used as reagents following quenching of the enzymatic reaction. New compounds with MW corresponding to the doubly acylated products, Bz-**11** with *m/z* 377 and 4-Br-Bz-**11** with *m/z* 510 were observed (Figure 7C, i and iii). This led us to establish the MW of the underivatized product **11** to be 144. L-Ser as a building block for **11** was evident by the incorporation of L-Ser-N^{15} into Bz-**11** (Figure 8, iv and Figure S29). Interestingly, when the bacterial didomain enzyme KasA was expressed and assayed under the same conditions, the same two products were formed based on MW and retention time (Figure 7C, ii and iv). This revealed that VtsC and KasA, despite being mined from two different kingdoms of life and with only moderate sequence identity (43%), synthesize the same compound.

To determine the structure of 4-Br-Bz-**11** (and by interference **11**), the VtsC reaction was scaled up to provide sufficient product for NMR analysis (Table S9 and Figures S57-S62). After derivatization, 4-Br-Bz-**11** was purified and NMR analysis showed the 4-Br-Bz groups are acylated at two vicinal diamines on a cyclopentane structure, one of which is α to a carboxylic acid. The structure of **11** was deduced to be 2-aminocycloleucine, a new α,α-disubstituted amino acid. NOESY correlations show two amide hydrogens in 4-Br-Bz-**11** have cross-peaks with the same hydrogen on C_3 (Table S9 and Figure S62), showing that **11** is the *syn* isomer. This is confirmed by MicroED structural analysis of 4-Br-Bz-**11** (Figure 7D).

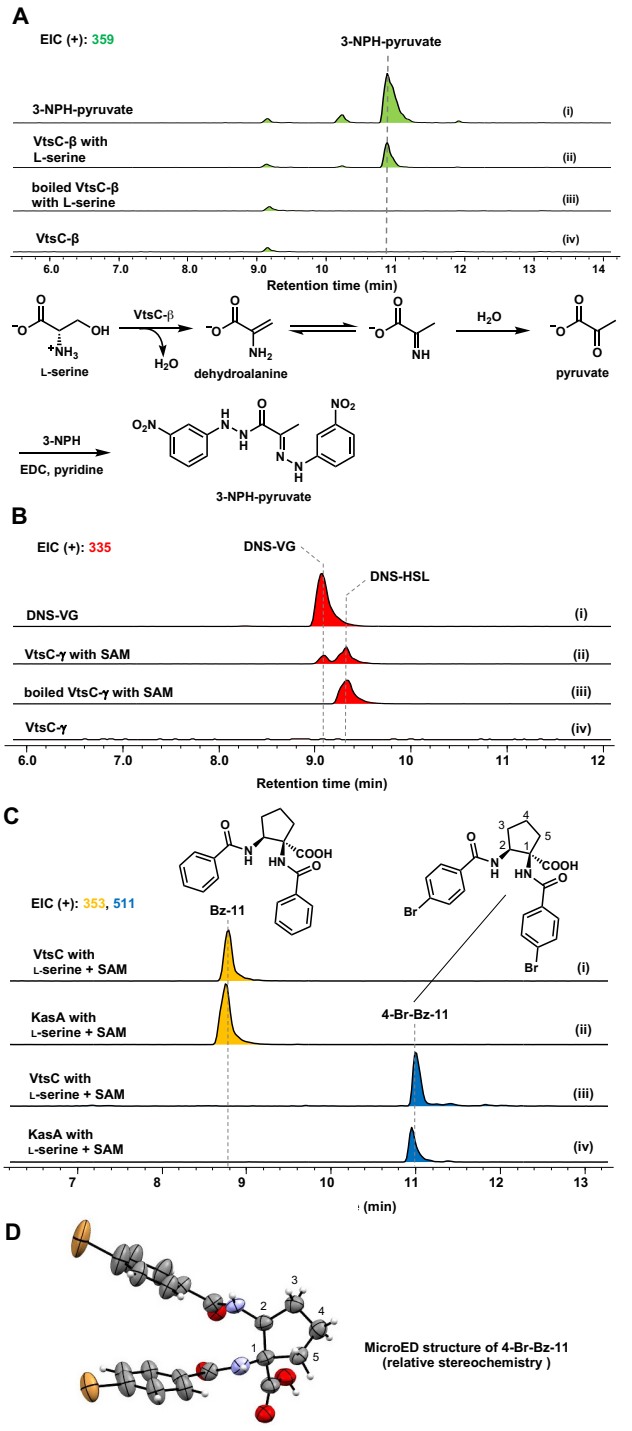

Figure 7. Biochemical characterization of VtsC and KasA. (A) In vitro assays of the β-lyase domain (VtsC-β) and L-Ser. Product was derivatized with 3-NPH based on dehydroalanine tautomerization and hydrolysis to pyruvate in buffer. (B) In vitro assays of FusB homolog domain (VtsC-γ) and SAM. Product was derivatized with DNS-Cl. (C) In vitro assays of VtsC and KasA in the presence of SAM and L-Ser. Products were derivatized with BzCl or 4-Br-BzCl. (D) MicroED structure of 4-Br-Bz-**11**.

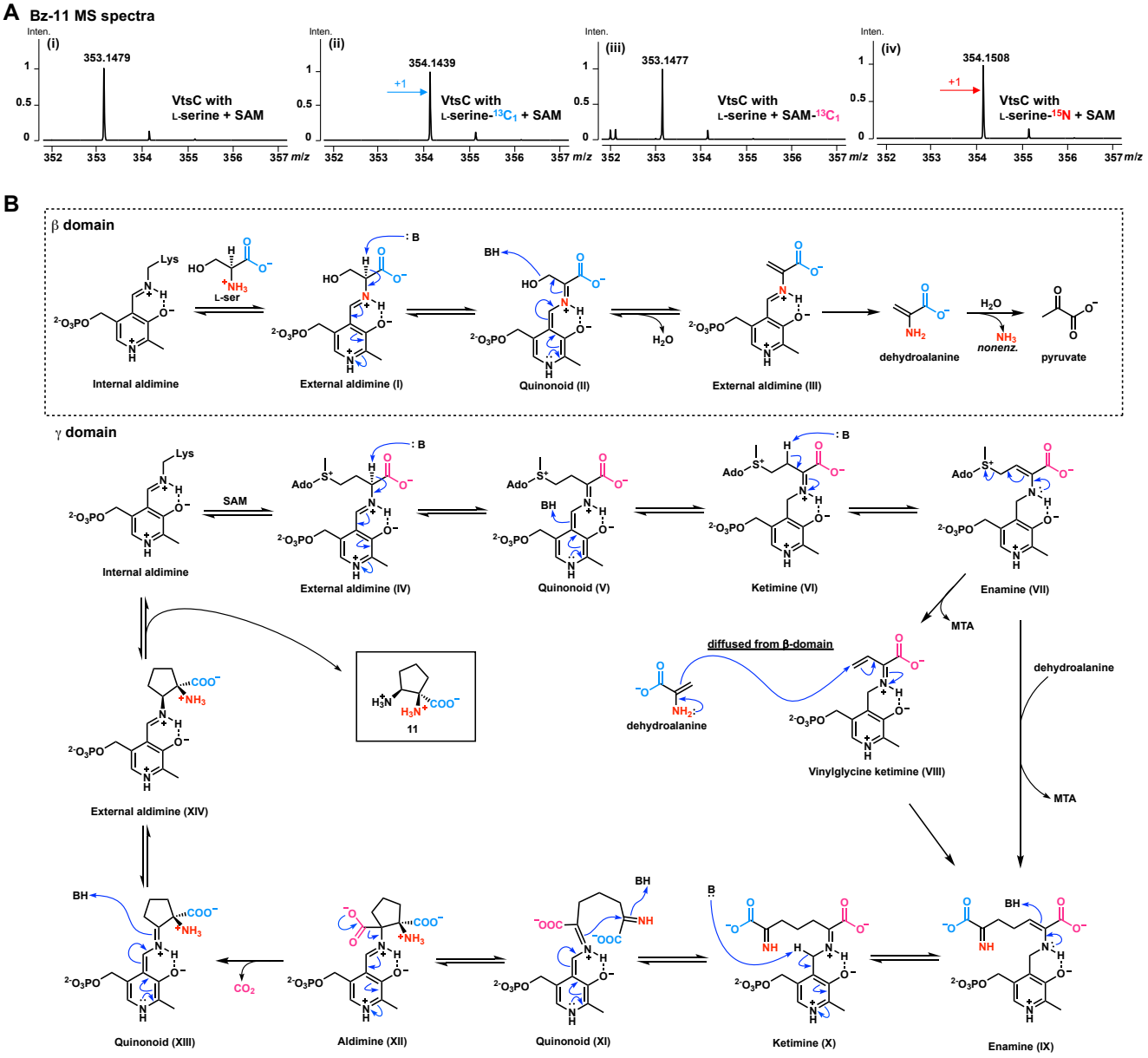

Figure 8. Mechanistic analysis of VtsC and KasA. (A) Labeling studies of VtsC reaction with (ii) $^{13}C_1$- L-Ser; (iii) $^{13}C_1$-SAM generated in situ from labeled L-Met; (iv) ^{15}N-L-Ser. (B) Proposed mechanism of VtsC and KasA.

Mechanistic analysis of VtsC/KasA. 11 contains one carboxylic acid, while both substrates of the γ-domain of VtsC/KasA, SAM and dehydroalanine, contain one carboxylic acid each. This suggests VtsC/KasA can catalyze one decarboxylation in addition to [3+2]-annulation to form **11**. Since the α-amine of SAM forms the external aldimine, the carboxylate of SAM would be the most logical to undergo decarboxylation. To test this, we prepared $^{13}C_1$-SAM using $^{13}C_1$-L-Met, ATP, and hMAT2A, and performed in vitro reactions of VtsC and KasA with L-Ser. No mass shift of Bz-**11** (Figure 8A, i, iii) was observed. When the reactions were repeated with $^{13}C_1$-L-serine and SAM, however, we observed +1 mu mass shift of Bz-**11** (Figure 8A, ii and Figure S29). These results confirm decarboxylation of the SAM-derived fragment occurs during catalysis.

The catalytic cycle of VtsC and KasA is shown in Figure 8B. The β-domain accepts L-Ser to form the external aldimine **I**. Expulsion of the β-hydroxyl group as water from the quinonoid **II** forms the dehydroalanine external aldimine (**III**). Release of dehydroalanine reforms the internal aldimine. The initial steps of the VtsC and KasA γ-domain mechanism proceed in a similar manner to FusB, with formation of the SAM-bound enamine (**VII**). In the presence of dehydroalanine, either a S_N2-type or elimination-addition mechanism as proposed for FusB can occur, resulting in loss of methylthioadenosine (MTA) and formation of enamine **IX**. **IX** can tautomerize to ketimine (**X**), which can be deprotonated to form quinonoid (**XI**). The C_α carbon in **XI** can attack the imine in 5-*exo-trig* fashion, mimicking an intramolecular Mannich cyclization, and form the cycloleucine product as the aldimine **XII**. **XII** can undergo decarboxylation to form the quinonoid **XIII**. Protonation of the α-carbon reforms the external aldimine (**XIV**) and releases the product **11** and regenerates the internal aldimine. Two reprotonation steps on the intermediates take place in the proposed mechanism (**XI** to **XII**, **XIII** to **XIV**), consistent with the observed mass shift of +2 Da for

Bz-**11** when the VtsC and KasA reactions were performed in ~80% D_2O (Figure S29).

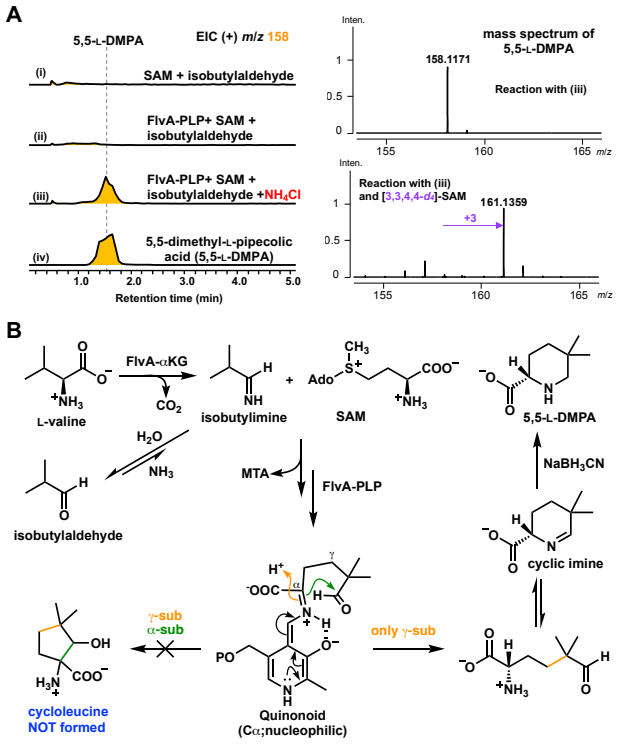

Figure 9. Biochemical characterization of FlvA-PLP. (A) In vitro assay of FlvA-PLP. The reaction was quenched by 2 eq. of acetonitrile, which was subject to $NaBH_3CN$ reduction to detect 5,5-L-DMPA. MS analysis of FlvA-PLP-catalyzed reaction with [3,3,4,4-d_4]-SAM is also shown. A +3 Da shift of 5,5-L-DMPA was observed. Additional results from using deuterium labeled SAM in H_2O or D_2O are shown in Figure S23. (B) FlvA-catalyzed γ-substitution using SAM and isobutylimine as a nucleophile.

Revisiting FlvA: Biochemical characterization of FlvA-PLP that forms 5,5-dimethyl-L-dehydropipecolate. Having biochemically characterized a series of cycloleucine synthases, we revisited the α-KG-PLP didomain enzyme FlvA,[39] which was used as an initial query to identify FusA and FusB. The didomain FlvA (α-KG-PLP) is involved in the biosynthesis of 5,5-dimethyl-L-dehydropipecolate, which is reduced by an accompanying reductase to 5,5-dimethyl-L-pipecolate (5,5-L-DMPA, Figure 9B). Although FlvA was proposed to be a γ-replacement PLP-dependent enzyme, the substrates of the reaction are not known and only isobutylaldehyde was proposed as the nucleophile for γ-substitution.[30,39] With characterization of FusB and VtsC, we can now propose that the PLP domain of FlvA (FlvA-PLP) likely uses SAM as a latent electrophile, and isobutylimine (and the corresponding enamine) generated by the α-KG domain of FlvA (FlvA-α-KG) from L-Val as the nucleophile. While we were not able to purify the full-length FlvA and FlvA-α-KG in a soluble form from *E. coli*, FlvA-PLP was highly expressed and purified from *E.* coli C41 (DE3) (Figure S7). When SAM was used as the substrate with FlvA-PLP, (*S*)-vinylglycine was formed as with FusB, VtsC, and KasA (Figure S22). Gratifyingly, 5,5-L-DMPA formation can be reconstituted by FlvA-PLP in the presence of isobutylaldehyde and excess NH_4Cl, together with $NaBH_3CN$ for the final imine reduction step (Figure 9A, iii). In contrast, 5,5-L-DMPA was not detectable by omitting NH_4Cl from the reaction mixture (Figure 9A, ii). Therefore, FlvA-PLP uses the enamine tautomer of isobutylaldimine as the carbon nucleophile to catalyze the γ-substitution (Figure 9B), the mechanism of which is similar as that of FusB, as supported by the mass shift of 5,5-L-DMPA from the assays in D_2O (Figure S23) as well as with deuterated SAM (Figure 9A and Figure S23). In contrast to FusB, FlvA-PLP only performs γ-replacement via C-C bond formation, as no cycloleucine-like product was observed (Figure 9B). This clear functional difference between FlvA-PLP and FusB raises the mechanistic question of how each enzyme controls C_α protonation or substitution after the γ-substitution. To answer this question, our efforts on the crystallization of FlvA-PLP and FusB are currently underway.

DISCUSSIONS

In this study, we discovered and biochemically characterized a PLP-dependent cycloleucine synthase FusB from the biosynthesis of fusarilin A (**1**). Furthermore, SSN-guided genome mining of FusB homologs led to characterization of 2-aminocycloleucine synthases VtsC from fungi and KasA from bacteria, which are didomain enzymes containing PLP-dependent β-lyase fused with a PLP-dependent cycloleucine synthase. These enzymes, while all cycloleucine synthases, have important mechanistic distinctions. FusB catalyzes the [3+2]-annulation of SAM and 2-phenylethylimine/enamine through tandem γ-substitution and α-substitution by intramolecular aldol reaction. On the other hand, VtsC and KasA use SAM and dehydroalanine to catalyze the 2-aminocycloleucine **11** formation via tandem γ-substitution and α-substitution by intramolecular Mannich cyclization. While the PLP-dependent enzyme LolT from loline biosynthesis was shown to catalyze a 5-*endo-trig* Mannich cyclization[14], VtsC and KasA catalyze a 5-*exo-trig* Mannich cyclization in formation of **11**.

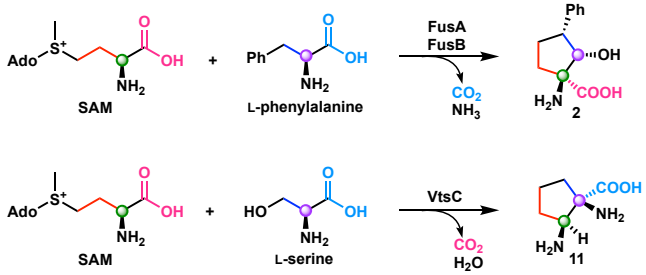

Figure 10. Difference fates of the α-carbons from the substrates during cycloleucine formation catalyzed by FusA/FusB and VtsC (KasA) system.

The differences in the mechanisms of FusB and VtsC/KasA, as well as functions of the partnering enzyme/domain that generate the enamine nucleophile, result in different origins of the quaternary carbons in the cycloleucine products (Figure 10). In the case of FusB, the quaternary carbon in **2** comes from the α-carbon of SAM, whereas in VtsC/KasA, the quaternary carbon in **11** is derived from the α-carbon of L-Ser. This is due to the timing of decarboxylation relative to the [3+2]-annulation, as well as the additional decarboxylation activity of VtsC and KasA compared to FusB. In the case of the *fus* system, FusA first catalyzes the decarboxylation of L-Phe to yield the carbon nucleophile phenylethylimine (**9**), which leaves the α-carbon of SAM as the quaternary carbon in **2**. In contrast, the PLP γ-domains of VtsC and KasA use dehydroalanine to form cycloleu-

cine with two quaternary carbons, followed by selective decarboxylation of the carboxylate group from SAM. This results in the quaternary carbon originating from dehydroalanine. The *C*-terminal domains of VtsC and KasA represent the first examples of PLP enzymes that can catalyze three reactions: γ-substitution, α-substitution with Mannich cyclization, and α-decarboxylation. Further biochemical and structural characterization of VtsC and KasA is required to fully understand the molecular basis of these unprecedented multifunctional PLP-dependent enzymes, as well as orientation of the active relative to that of the β-domain.

The use of SAM in the reactions characterized here further adds to the versatility of this cofactor in metabolism. The sulfonium cation in SAM activates fragmentation of the C_γ-S bond when bound to PLP as an external aldimine. A few PLP-dependent enzymes such as ACC synthases,[21,54,55], Mur24,[56] and SbzP[32] have been reported to use SAM as the substrate. While Mur24 and SbzP have been proposed to perform the γ-substitution through formation of the vinylglycine ketimine and the vinylglycine quinonoid, respectively, ACC synthases have been shown to go through an intramolecular S_N2-like substitution of C_α to C_γ to form the cyclopropane ring. Our current data cannot conclusively distinguish between the elimination-addition or S_N2-like substitution mechanism (Figures 5 and 8). Comparison of SbzP and the enzymes discovered here shows intriguing mechanistic differences. While SbzP is proposed to form [3+2]-annulation using C_γ as a nucleophilic carbon and C_α as an electrophilic carbon on PLP-bound SAM,[32] cycloleucine formation by FusB, VtsC, and KasA takes place with opposite parity of the PLP ketimine/enamine: the tandem C-C bond formations using electrophilic C_γ carbon and nucleophilic C_α carbon. Such stark mechanistic differences underscore the versatility of PLP-dependent enzymes.

SSN analysis revealed that potential cycloleucine synthases are widely distributed to not only fungi but also bacterial genomes. Genes encoding PLP-dependent cycloleucine synthases are surrounded by other putative biosynthetic enzymes, indicating the cycloleucine moiety is a building block for more elaborate structures in natural products. Using the *fus* pathway as an example, the PLP-dependent FusB can be clearly assigned as a core building enzyme in natural product biosynthesis, distinct from other cores enzymes such as polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs) and terpene synthases (TSs). The *vts* BGC contains 8 additional modification enzymes including oxygenases such as nonheme iron-dependent oxygenases and P450s, while the *kas* cluster encodes numerous enzymes most of which are predicted to be hypothetical proteins. We therefore expect that new cycloleucine bearing natural products will be discovered through genome mining and heterologous expression of these complete pathways. Collectively, discovery of the new cycloleucine synthases from this study highlights the potential of mining biosynthetic pathways anchored by PLP-dependent enzymes for exploration of the unknown-unknown biosynthetic dark matter.[37]

CONCLUSIONS

In summary, a new family of PLP-dependent enzymes that can form cycloleucine structures has been uncovered from both fungi and bacteria. These enzymes use *in situ* generated enamines as nucleophiles and SAM-derived vinylglycine as electrophile to form the first C-C bond, followed by either an aldol or Mannich reaction to complete cyclopentane formation. Mining of these enzymes in biosynthetic gene clusters will lead to new products with the cycloleucine core.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details, materials, and methods, including photographs of experimental setup. 1D, 2D NMR spectra for all compounds. Micro-ED structures of **2** and 4-Br-Bz-**11** were deposited to CCDC. This material is available free of charge via the Internet at http://pubs.acs.org.

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A. N. D. A., K. S., and M. O. conceived the project. M. O., H. M. N., and Y. T. directed the experimental plans. A. N. D. A., K. S., K. K. N., M. L., and M. O. performed the biochemical experiments and analyzed the data. D. A. D., and L. S. M. performed MicroED analysis and refined the structures. All authors contributed to writing and approval of the submitted manuscript.

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Notes

The authors declare no competing financial interest.

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