A Validamycin Shunt Pathway for Valienamine Synthesis in Engineered *Streptomyces hygroscopicus* 5008

Li Cui,* Xiaodong Wei, Xinran Wang, Linquan Bai, Shuangjun Lin, and Yan Feng*

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**ABSTRACT:** Valienamine is the key functional component of many natural glycosidase inhibitors, including the crop protectant validamycin A and the clinical antidiabetic agent acarbose. Due to its important biomedical activity, it is also the prominent lead compound for the exploration of therapeutic agents, such as the stronger α-glucosidase inhibitor voglibose. Currently, the main route for obtaining valienamine is a multistep biosynthetic process involving the synthesis and degradation of validamycin A. Here, we established an alternative, vastly simplified shunt pathway for the direct synthesis of valienamine based on an envisioned non-natural transamination in the validamycin A producer *Streptomyces hygroscopicus* 5008. We first identified candidate aminotransferases for the non-natural ketone substrate valienone and conducted molecular evolution in vitro. The WecE enzyme from *Escherichia coli* was verified to complete the envisioned step with >99.9% enantiomeric excess and was further engineered to produce a 32.6-fold more active mutant, VarB, through protein evolution. Subsequently, two copies of VarB were introduced into the host, and the new shunt pathway produced 0.52 mg/L valienamine after a 96-h fermentation. Our study thus illustrates a dramatically simplified alternative shunt pathway for valienamine production and introduces a promising foundational platform for increasing the production of valienamine and its valuable N-modified derivatives for use in pharmaceutical applications.

**KEYWORDS:** valienamine, valienone, aminotransferase, protein evolution, shunt pathway

Valienamine is a functional C7N aminocyclitol moiety with significant α-glucosidase inhibitor activity due to its structural similarity to D-glucose. It serves as the core component of many aminocyclitol natural products, including the crop protectant validamycin A, the antidiabetic agent acarbose, and various bioactive microbial secondary metabolites, many of which are useful for the treatment of viral and bacterial infections and inflammation. As a prominent lead compound for glycosidase inhibitors, chemical analogues and N-modified derivatives of valienamine have been developed due to their specific and potent biomedical activities. β-Valienamine and voglibose have been explored as therapeutic agents for lysosomal storage diseases and type II diabetes, respectively.

Although valienamine has attracted increasing attention for decades due to its basic science applications and biomedical uses, its chemical synthesis is hampered by insufficient stereospecificity. Extra steps, including functional group protection and deprotection and chiral resolution, are unavoidable to manage its multiple stereocenters in the valienocyclitol framework. Currently, the synthesis of valienamine is mainly achieved through a complex multistep biosynthetic route that requires two fermentation processes. First, validamycin A is produced by *Streptomyces hygroscopicus* 5008 (S5008); it is then degraded by *Pseudomonas denitrificans*, *Flavobacterium saccharophilum*, or *Stenotrophomonas maltophilia*. This complicated process involves navigating the intricate pathways of two different bacterial species and includes at least 13 enzymatic reactions.

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The enzymes and synthetic intermediates involved in validamycin A biosynthesis pathway have been systematically characterized using genetic and biochemical analyses in the SS008 strain by performing feeding experiments,17 heterologous expression,18 gene inactivation,19 and enzymatic assays.20,21 Structurally, the intermediate valienone is the most similar to the desired valienamine product because these two compounds have identical cyclohexene carbon backbones and 3 of the same hydroxylated stereocenters. The only difference between valienone and valienamine is at the position of the prochiral carbonyl carbon in valienone, at which the identical position in valienamine is an S-amino group.

The present study focused on the catalysis of the formation of valienamine from valienone in a single transamination step, which would potentially be catalyzed by a single aminotransferase enzyme. Here, we report the identification and engineering of a suitable candidate aminotransferase based on in vitro enzymatic assays and evolution. The aminotransferase WecE from E. coli converted the prochiral carbonyl group of valienone into the desired S-conformation with high stereospecificity, and it possessed the required activity and stereospecificity to facilitate an alternative shunt pathway to produce valienamine from valienone. We further engineered WecE using a structure-guided computational analysis and saturation mutagenesis to achieve the improved variant VarB, which exhibited a 32.6-fold improvement in catalytic activity compared to wild-type WecE. We then successfully introduced this dramatically simplified alternative shunt pathway into the SS008 strain by replacing the gene responsible for valienone phosphorylation, valC, with the varB gene in situ, and introduced an extra copy of varB driven by the kasOp8 promoter through the integrative plasmid pPM927.

Thus, we simplified the multistep validamycin A biodegradation process requiring two different industrial strains to generate an alternative shunt biosynthetic pathway for valienamine in a single engineered host, which will be notably easier to target in future engineering applications. Our study also helps define important design and experimental considerations for similar engineering strategies and provides a technical platform for the further development of pathways used to synthesize higher value derivative drugs based on subsequent enzymatic nitrogen modifications of valienamine.

■ RESULTS

Designing a Simplified Alternative Shunt Biosynthetic Pathway for Valienamine. On the basis of the results of isotopically labeled feeding experiments17 and intensive genetic studies,18 valienone was identified as an explicit intermediate for the biosynthetic pathway of validamycin A in the SS008 strain. The configurations of all the hydroxylated stereocenters in the structure of valienone are absolutely identical to those in valienamine, except for the single prochiral carbonyl group corresponding to the amino moiety of valienamine. We speculated that the prochiral carbonyl group would be transformed into an amino group with the prospective chirality in a single stereospecific reaction conducted by an engineered aminotransferase (Figure 1).

Figure 1. Current pathway for the multistep synthesis of valienamine that combines the fermentation of Streptomyces hygroscopicus 5008 and Flavobacterium saccharophilum (gray), and the envisioned shunt pathway through valienone in engineered SS008 for valienamine biosynthesis in this study (red).

The shunt pathway for valienamine via an envisioned transamination reaction

Degradation of validamycin A by Flavobacterium saccharophilum

Biosynthesis of validamycin A in Streptomyces hygroscopicus 5008

Figure 1. Current pathway for the multistep synthesis of valienamine that combines the fermentation of Streptomyces hygroscopicus 5008 and Flavobacterium saccharophilum (gray), and the envisioned shunt pathway through valienone in engineered SS008 for valienamine biosynthesis in this study (red).

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pathway from valienone to valienamine that would simplify the multistep synthetic process to 4 explicit steps, eliminating the need for the validamycin A isolation and degradation steps.

**Selection of the Aminotransferase for the Valienone Precursor.** The fundamental challenge in developing an alternative biosynthetic pathway for valienamine was to identify a suitable aminotransferase with the envisioned activity and stereospecificity to shunt the validamycin pathway from the non-natural substrate valienone to valienamine. In our previous study, we used a bioinformatics integrative platform (RxnP)\(^2^2\) to collect enzyme information based on the similarity of the substrate and built a synthetic pathway for \(\beta\)-valienamine in the engineered SS008 strain.\(^2^3\) The in vitro enzymatic screen revealed that the sugar aminotransferase (SAT) ArnB_Eco catalyzed the transamination of the non-natural substrate valienone to produce a mixture of enantiomers with an enantiometric excess (e.e.) of \(-61.3\%\) for valienamine. It was the only candidate protein we tested that generated valienamine as a product. To assemble additional candidates for the desired introduction of the amine group, we used ArnB_Eco as the starting sequence to explore the potential candidates based on sequence alignment and genome neighborhood analysis in the Protein Data Bank (PDB) and the protein families (Pfam) databases.

Forty-nine sequences were assembled from the PDB and Pfam databases. The candidates showed 14.1–81.0% identity and 21.8–88.6% similarity with the starting ArnB_Eco sequence, for which the information is listed in Table S1. Further subsequent analysis of the phylogenetic evolutionary relationships revealed that 42 sequences of the assembled candidates belonged to the DegT_Dnmj_EryC1 sugar aminotransferase subfamily and were divided into three subgroups, Vl₄₀, Vl₂₀, and Vl₁₀, according to the phylogenetic evolutionary relationship.\(^2^4\) Fourteen of the SATs have been experimentally confirmed to perform the transamination of keto-sugars, and published crystal structures are available to help guide efforts in possible structure-guided engineered (Figure 2A). We prioritized the 14 candidates with certain functions and crystal structures and successfully overexpressed them in *E. coli* BL21 (DE3) for the in vitro enzymatic assays after purification using Ni-NAT affinity chromatography.

Using OPA-precolumn derivative HPLC,\(^2^5\) we next determined that 6 of the detected candidates produced a variety of stereospecific amine products from the substrate valienone. Significantly, WecE from *E. coli* MG1655,\(^2^4\) which shows high homology and shares 31.1% identity and 45.3% similarity with ArnB_Eco,\(^2^6\) exhibited stringent stereospecificity, with >99.9% e.e. for the S-enantiomer valienamine. The amine product was verified by recording TQ-MS (Figure S1), \(^1\)H NMR, and \(^13\)C NMR spectra (Figure S2). Other candidates, however, converted valienone into racemic valienamine or \(\beta\)-valienamine with different e.e. values (Figure 2B).

**Semirational Engineering of WecE To Enhance Its Catalytic Activity toward Valienamine.** We chose the semirational design strategy\(^2^7\) to optimize the catalytic activity of the candidate aminotransferase WecE toward the non-natural substrate valienone (Figure S4A). The mechanism of WecE is thought to follow the well-characterized “ping pong” mechanism mediated by PLP (Figure S3).\(^2^8,2^9\) We docked the theoretical valienone-PMP external aldime intermediate into the reported active sites of WecE (PDB: 4ZAH)\(^3^0\) to explore the interaction between the enzyme and the substrate (Figure 3A). The active site of WecE is composed of the residues from both halves of the dimer: Loop7 (residues Arg213-Thr225) and Loop9 (residues Val318-Ile322) are located at the entrance of the substrate binding pocket. The evaluation of the mutation energy of the residues located within 8 Å of the docking valienone-PMP intermediate predicted that 16 residues might be involved in the binding of the enzyme to the intermediate (Figure S4B). We selected both the putative active site residues and the substrate binding-related regions Loop7 and Loop9 as the target residues for the catalytic activity evolution analysis to include all potentially important residues (Figure 3B and 3C).

Twenty-eight residues were screened using the high-throughput site-saturation mutagenesis method. Higher activity was observed for four mutants containing mutations at three critical residues (Val318, Phe319, and Tyr321) replicated by the OPA-precolumn derivative HPLC. Y321F showed the highest catalytic activity, with 4.4-fold increase in activity compared with the wild-type enzyme. The activities of V318Q and V318R were 3.6-fold and 3.9-fold higher than the activity of the wild-type enzyme, respectively. The activity of the F319V mutant was increased 2-fold. The best performing mutations were combined to obtain the optimal VarA (Y321F/V318R) and VarB (Y321F/V318R/F319V) mutants, which
exhibited 15.2-fold and 32.6-fold increased catalytic activities, respectively, compared to wild-type WecE.

Further comparisons of the kinetic performance of the wild-type and mutant enzymes were performed using a Michaelis–Menten kinetic analysis with L-GDH as a coupling enzyme (Table 1). Compared to the wild-type enzyme, the catalytic efficiency of the VarB mutant was increased 19.18-fold.

The positive residues of the optimal mutant VarB (Y321F/V318R/F319V) are concentrated in Loop9, which is located at the entrance of the substrate binding pocket of WecE (Figure S5A). In the Y321F mutant, the hydrogen atom of the phenylalanine residue replaced the hydroxyl group of the tyrosine residue on the aromatic ring, and the hydrophilic interaction was transformed to a hydrophobic interaction at this site. The hydrophobic interaction supplied by phenylalanine at 319 was changed to a van der Waals interaction when this residue was replaced with valine in the F319V mutant. The replacement of both residues 321 and 319 might weaken the interaction between Loop9 and the intermediate, according to the strength characters of the intramolecular interaction.31 The arginine in the V318R mutant has longer side chain and positively charged terminator group than the original valine. These changes might increase the reactivity and the steric hindrance at this site.

The results of homologous modeling and molecular dynamics simulations visualized an obvious shift in average stable orientation of the intermediate in the mutant VarB from Loop9 and closer to the theoretical transamination executive residue Lys181 than in wild-type WecE (Figure S5B and S5C). The change in the location of the intermediate is consistent with the deduced weaker interaction between Loop9 and the intermediate caused by the optimized residues, which might contribute to the closer catalytic distance and improved catalytic activity of the mutants toward valienone.

**Coupling of the Envisioned Pathway for Valienamine in S5008.** The carbocyclic framework of valienone in the S5008 strain was derived from sedoheptulose 7-phosphate in the pentose phosphate pathway mediated by successive reactions with the cyclase ValA,32 epimerase ValD,20 and dehydratase ValK. Valienone was then phosphorylated by a cyclitol kinase ValC21 and entered the downstream pathway for validamycin A (Figure 1). We engineered the valdamycin A biosynthetic pathway to accumulate the transamination precursor valienone and replaced the competitive kinase valC with the optimized aminotransferase mutant varB in situ using the Redirect methodology33 to establish the drastically simplified alternative shunt pathway for valienamine synthesis in the S5008 strain.

<table>
<thead>
<tr>
<th>enzyme mutations</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ min$^{-1}$)</th>
<th>fold change compared with the WT enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.133 ± 0.002</td>
<td>0.012 ± 0.003</td>
<td>0.090 ± 0.021</td>
<td>1</td>
</tr>
<tr>
<td>Y321F</td>
<td>0.092 ± 0.009</td>
<td>0.025 ± 0.002</td>
<td>0.272 ± 0.044</td>
<td>3.02</td>
</tr>
<tr>
<td>VarA Y321F/V318R</td>
<td>0.099 ± 0.005</td>
<td>0.065 ± 0.004</td>
<td>0.657 ± 0.022</td>
<td>7.29</td>
</tr>
</tbody>
</table>
| VarB Y321F/V318R/F319V | 0.062 ± 0.002 | 0.107 ± 0.003 | 1.726 ± 0.008 | 19.18

*WT: wild-type.*

Figure 3. (A) The docking orientation of the valienone-PMP external aldime intermediate in the active site of the aminotransferase WecE dimer. (B) The substrate binding-related regions Loop7 (blue), Loop9 (green), and the residues located within 8 Å of the intermediate (red) are shown. (C) The putative interaction between the docking valienone-PMP external aldime intermediate and the aminotransferase WecE.
The varB gene was inserted between 2.0 kb genomic fragments flanking both sides of valC in the pJTU1278 plasmid to produce the shuttle plasmid pJTU1801. Through a double crossover recombination mediated by pJTU1801, the 1,056 bp valC gene was replaced by the 1131 bp varB gene in situ (Figure 4A). The derivative, Val-01, was confirmed by PCR and gene sequencing. And the cells were cultured in 50 mL fermentation medium at 37 °C for 120 h in flasks. The levels of the varB transcript were monitored using quantitative real-time PCR (qPCR) every 24 h, and the production of valienamine was quantified using LC-TQMS and confirmed by comparing the retention time and the MRM fragmentation of the standard (Figure S6). Moreover, valienone and validamycin A were detected using a precolumn DNPH-derivative HPLC and HPLC analyses, respectively.

Previous analyses revealed that valC belonged to the ValABC operon and was transcribed in the same direction as valA and varB into one polycistronic mRNA under the control of the native valAp promoter. The heterologous aminotransferase gene varB completely replaced valC in situ and was expected to be under the control of the endogenous regulatory network of the valABC operon. On the basis of the results of the qPCR analysis of mRNA expression, the relative levels of the varB transcript in Val-01 were increased significantly from 10^2 to 10^3 compared to the wild-type S5008 during the fermentation process, with the highest levels of expression observed in the middle stage at 48 h. The upstream and downstream genes, valA and valD, respectively, were both expressed at levels similar to the wild-type S5008 (Figure 4B).

Compared with the wild-type S5008 strain, the production of validamycin A was abolished in the fermentation broth of the engineered derivative Val-01. The de novo product valienamine and its non-natural precursor valienone both accumulated in the broth, and their concentrations increased with increasing fermentation time, reaching their highest levels of 318 μg/L and 2.1 g/L, respectively, at 96 h (Figure 4C). Both the transcriptional and metabolic analyses indicated the presence of the envisioned pathway for valienamine synthesis under the control of the activated aminotransferase mutant in the metabolically engineered S5008 strain.

**Overexpression of the Aminotransferase Increases Valienamine Production.** We evaluated 4 constitutive promoters from different Streptomyces species in the engineered, valienone-accumulated mutant strain ZYR-1 (ΔvalC, Apr+) to overexpress the aminotransferase in the engineered Val-01 strain. Transamination cassettes were constructed in the pSAM2-derived integrative vector pPM927 containing the varB gene under the control of kasO* from Streptomyces coelicolor, ermEp* from Streptomyces erythraeus, SF14p from Streptomyces ghanaensis phage1937, and the native promoter valAp from the valABC operon. The constructed transamination cassettes were introduced into ZYR-1 through conjugation with E. coli ET12567 (PUZ8002) to generate the thiostrepton- and apramycin-resistant (ThioRApr+) exconjugants. The derivatives were confirmed by gene sequencing of the PCR products, and they were then cultivated in fermentation medium for 120 h under fermentation conditions. The real-time analysis indicated that the candidate promoters for the artificial pathway triggered the transcription of varB in the mutant strains; the strength of the promoters was as follows: kasOp* > SF14 > ermEp* > valAp. The highest expression of varB was observed at 48 h in the middle stage of fermentation (Figure 5A).

Valienamine accumulated with a prolonged incubation time, along with increased transcriptional activity in the varB-activated mutants. The strain under the control of the kasOp* promoter exhibited the highest concentration of 260 μg/L at 96 h (Figure 5B).

The optimal transamination cassette with an extra copy of varB under the control of the kasOp* promoter was then introduced into Val-01 by conjugation to obtain the mutant Val-02 strain. qPCR and LC-TQMS analyses confirmed that the transcription of varB in Val-02 reached a peak level that was 1.8-fold higher than Val-01 at 48 h (Figure 5C), with the accumulation of valienamine reaching 526 μg/L at 96 h (Figure 5D). Genetically, a copy of the heterologous aminotransferase gene varB utilized the regulatory network of the valABC operon controlled by the strong native valAp promoter, and an extra copy of varB overexpressed under the
control of the kasOp* promoter established the envisioned alternative shunt pathway for valienamine synthesis in the validamycin A producer.

**DISCUSSION**

Our study provides a vivid illustration of methods for the dramatic simplification of the biosynthesis of large and complex natural products by identifying and engineering suitable enzymes from other organisms. In addition to reducing the overall multistep biosynthetic process to 4 steps, our successful implementation of this shunt strategy also highlights how the selection of suitable nonendogenous enzymes results in an improvement in enzymatic properties. Previous studies of WecE have confirmed that it catalyzes the transamination of TDP-keto-sugars, but it does not display catalytic activity toward GDP-keto-sugars; the only structural difference between these substrates is the nucleotide moiety, and nucleotide moieties are widely understood as major determinants of substrate specificity among SAT subgroup enzymes.

From the opinion of the transamination mechanism, the NDP moiety, as a part of the substrate structure, is not involved in the Schiff-base circulated catalytic procedure mediated by PLP. The transamination reaction could occur directly on scyllo-inosose substrates without phosphorylation or NDP modification, such as the members in the VI subgroup of the SATs. This reveals that although the interactions between the aminotransferase and the different NDP moieties can potentially affect the substrate orientation with possible stereochemical consequences, there are still possibilities for transamination reaction without NDP modification, as long as the intermediate can stably bind to the active site in appropriate orientation.

Through the enzymatic assays in vitro, WecE was selected for our envisioned pathway due to its excellent stereospecificity (e.e. > 99%) in the production of valienamine, and other 5 SATs, which catalyze the NDP-keto-sugars and scyllo-inosose as natural substrates in the DegT_DnrJ_EryC1 subgroup, recognized the non-natural valienone substrate to yield amine products with different configurations. This revealed that the SAT enzymes, which utilize NDP-keto-sugars as preferred substrates, could perform transamination reaction on independent keto-sugar moieties (i.e., without the nucleotide group), and the function of the NDP moiety may be to stabilize the configuration of the intermediate during the reaction. This observation provides a new perspective for studies aiming to determine how SATs can be employed to synthesize a potentially large number of variations of novel amino sugar moieties used in pharmaceutical and chemical industry applications. The performance of a transamination reaction using a particular keto-sugar structure may not necessarily be restricted to enzymes based on the assumed interactions between the enzyme and a particular nucleotide moiety.

Subtle alterations in the active sites of these enzymes control the distinct orientations of identical keto-sugar substrates, yielding amines with different stereochemical configurations. Thus, further in-depth comparisons of these structurally and

![Figure 5](https://dx.doi.org/10.1021/acssynbio.9b00319)
functionally related SAT enzymes represent an excellent starting point for both basic studies and applied projects aiming to understand and exploit an improved ability to stereochimically control the amine synthesis. Specifically, an understanding of the mechanisms by which key amino residues affect the stereospecificities of products enables the precise synthesis of various amino sugars for use in new functional compounds. Both mutagenesis and structural biology research will likely facilitate ongoing discoveries in this area.

An important concept that was illustrated in this study was the truly drastic simplification of the biosynthetic pathway in question and the simplified dimensionality for the engineering of future strains and protein engineering to produce the desired compound. More generally, our study emphasizes how retaining a chemical structure-oriented perspective while navigating genetic and other biological parameters represent practical strategies that enable researchers to harness the metabolism of various organisms and obtain the desired pharmaceutical/chemical outcomes.

**CONCLUSIONS**

In this study, we implemented a shunt strategy for valienamine biosynthesis by evolving and integrating a non-natural transamination reaction in the S5008 strain through protein and metabolic engineering, which simplified a process involving two bacteria into a pathway requiring 4 genes in a single host. This strategy not only produced a promising route for valienamine synthesis with artificially designed catalytic reactions, but also illustrated how chemical structure-guided searches for enzymes that conduct non-natural reactions can be harnessed to gainfully manipulate biological metabolism for focused pharmaceutical/chemical applications. These studies also allowed us to explore a biosynthetic starting platform for the production of more valuable N-derivatives through the rational consideration and molecular evolution of promiscuous enzymes.

**METHODS**

**Bacterial Strains and Plasmids.** The bacterial strains and plasmids used in this study are listed in Table S3. General gene cloning was performed using the plasmid pBluescript II SK(+) (Stratagene, USA) in *E. coli* DH5α cells (Invitrogen, USA), and the candidate aminotransferases were cloned into the plasmid pET28a (Novagen, USA) and expressed in *E. coli* BL21 (DE3) cells (Novagen, USA). The S5008 strain was used as the host strain to build the envisioned shunt pathway through conjugation with the *Streptomyces* shuttle vector pJTU1278 and *E. coli* ET 12567 (pUZ8002). The valienone supplier ZYR-121 was used as the basis to evaluate valienone with tools available at the Swiss-model Web site. Molecular Dynamics Simulation.

**Assembly and Phylogenetic Analysis of Candidate Aminotransferases.** Candidate aminotransferases were selected using the NCBI BLAST alignment search tool with the sugar aminotransferase ArnB_Eco26 as the starting sequence. Multiple alignments of the candidate sequences were performed using ClustalX software with the following parameters: protein weight matrix = Gonnet, gap open = 10, and gap extension = 0.2. The phylogenetic analysis of the evolutionary relationships among the sequences was performed using MEGA7 software with Maximum Likelihood algorithms.

**Candidate Expression and Purification.** The gene sequences of the candidates were synthesized with synonymous mutations to eliminate restriction endonuclease sites by GENEWIZ Inc. Company (SuZhou, China) according to their NCBI database entries. The fragments were ligated into pET28a using NdeI and EcoRI restriction sites and then transformed into *E. coli* BL21 (DE3) using standard procedures. The sequence-verified transformants were cultured in LB media (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 100 μg/mL kanamycin at 37 °C and shaking at 220 rpm. Gene expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside for 15 h at 16 °C when the bacterial OD600 reached approximately 0.3–0.5. The cells were harvested by centrifugation and disrupted by sonication in 20 mM sodium phosphate buffer (pH 7.5). The N-terminal His-tagged aminotransferases were purified using Ni-NAT columns and confirmed to be homogeneous using SDS-PAGE.

**Enzymatic and Kinetic Assays.** Purified aminotransferase candidates (1 mg/mL) were incubated in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM valienone (WuXi AppTec Company, WuXi, China) as the amino acceptor, 10 mM L-glutamine as the amino donor, and 0.3 mM pyridoxal 5’-phosphate as the cofactor at 37 °C for 2 h. The reaction mixture was precipitated by the addition of an equal volume of methanol and centrifuged at 12,000 rpm for 10 min. The transamination products were derivatized with o-phthalaldehyde in borate buffer (pH 9.0) at room temperature for 30 s, separated on an Eclipse XDB-C18 (5 μm, 4.6 × 150 mm) column with 22% acetonitrile at 30 °C for 18 min, and quantified by detecting the fluorescence at an emission wavelength of 445 nm and excitation wavelength of 340 nm.

The kinetic parameters of the candidates were determined by coupling with 0.5 U of NADH-dependent L-glutamate dehydrogenase, 0.5 mM NADH, and 2.5 mM NH₄Cl in the enzymatic assay system as the concentration of valienone ranged from 0.05 to 2.5 mM. The activity of 1 unit of enzyme was defined as the amount of enzyme required to consume 1 μmol of NADH per min, which was monitored continuously at 340 nm with a multiscan spectrum microplate spectrophotometer.

**Semirational Design of Aminotransferase Evolution.** The docking of the theoretical valienone-PMP intermediate into the crystal structure of WeC and the evaluation of the residues were conducted using the CDOCKER and the Calculate Mutation Energy (Binding) model of the Discovery Studio 3.5 (Accelrys, USA). The site-saturation mutagenesis library of the selected hot spots was constructed in the pET28a-WeC plasmid through whole plasmid amplification with degenerate primers (NNK). For full coverage of the amino acid exchange mutants, at least 200 colonies were examined for each site using a high-throughput method in 96-well microplates based on the aminotransferase-glutamate dehydrogenase coupling system. The target transamination was screened by coupling with an L-glutamate dehydrogenase indicative reaction system that consisted of 0.5 U/mL of coupling enzyme and 0.4 mmol/L NADH. The oxidation of NADH to NAD⁺ was monitored by detecting the decrease in absorbance at 340 nm, which indicated the activity of the aminotransferase.

**Molecular Dynamics Simulation.** The structure of the optimal mutant VarB was predicted using homology modeling with tools available at the Swiss-model Web site. Molecular dynamics simulations were performed using the GROMACS 5.1.4 simulation package. The system was subjected to equilibration and production runs of 100 ns. The long-time behavior of the system was analyzed using the GROMACS analysis tool. The results obtained from the simulations were compared with the experimental data to validate the model.

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dynamics simulations were conducted using the WecE and VarB structures along with the theoretical valienone-PMP external aldimine intermediate in GROMACS (version 2019.3) for a 100 ns simulation under a CHARMM36m force field for proteins and the CHARMM general force field for ligands. Distance calculations, the root-mean-square deviation (RMSD) analysis, and the visualization of protein–ligand structures were performed using GROMACS packages and PyMol software.

**Chemical Analysis.** The triple quadrupole mass spectrometry (TQ-MS) analysis was performed using the Agilent QQQ MS 6470 system in positive ion mode. The structures of the purified products were identified by recording $^1$H NMR and $^{13}$C NMR spectra (Bruker AV-400 spectrometer) in D$_2$O.

**Plasmid Construction and Conjugation.** The codon-optimized DNA sequence of the aminotransferase was synthesized by GENEWIZ (SuZhou, China) for insertion in the SS008 strain. The fragments of the upstream homologous arm of $valC$, the $varB$ gene, and the downstream homologous arm of $valC$ were amplified using the homologous primers listed in Table S2, and then the fragments were successively assembled in pTU1278 using Gibson Assembly Cloning Kit (NEB, USA) to generate the Streptomyces shuttle vector plasmid pTU1801. The pTU1801 plasmid was transformed into the SS008 strain for double crossover recombination through conjugation mediated by *E. coli* ET12567/pUZ8002 to replace $valC$ with the engineered aminotransferase gene in situ.

**Overexpression of the Candidate Aminotransferase in Vivo.** The native $valA$ promoter was amplified from the genomic DNA of the SS008 strain using the primer sets $valA$p-$F/valA$p-$R$ listed in Table S2. The heterologous promoters kasOp*, ermA$p*$, and SF14p were synthesized by GENEWIZ (SuZhou, China) with MfeI and NdeI restriction sites at both ends. The promoters were ligated with the optimal aminotransferase mutant into the pPM927 plasmid using the process outlined in Figure S7. The ligations were transformed into ET12567/pUZ8002 and conjugated into ZYR-1 to evaluate the transcriptional activity of the candidate promoters based on a qPCR analysis and valienamine concentration. The optimal integrative plasmid was conjugated into the Val-01 strain to obtain the Val-02 strain with an extra copy in the engineered valienamine producer.

**Fermentation of the Derivatives and qPCR Analysis.**

The SS008 strain and its derivatives were cultured in fermentation media (10% corn powder, 2.5% soybean flour, 0.5% yeast extract, 0.1% NaCl, and 0.15% KH$_2$PO$_4$) at 37 °C with shaking at 220 rpm for 120 h. The cells were collected every 24 h for total RNA extraction using Redzol reagent according to the manufacturers’ protocol (SBS Genetech Co., Ltd. Beijing, China). Genomic DNA contamination was eliminated by treatment with RNase-free DNase I (Thermo Scientific, USA). The RNA concentration was quantified by measuring the absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). All RNA samples were reverse transcribed into cDNAs using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). The transcript levels were determined using qPCR with TransStart Top Green qPCR SuperMix (SYBR Green) (Transgen Biotech, Beijing, China) and the ABI Step One Plus system (Applied Biosystems, USA). The mRNA levels were quantified in triplicate using a standard curve with the major sigma factor in the *Streptomyces hrdB* gene as the internal reference.

**Analysis of Metabolites.** Fermentation samples were collected every 24 h and centrifuged at 12 000 rpm for 10 min. The supernatant was combined with chloroform to remove the proteins and low levels of polar organic impurities prior to the metabolite analysis. The HPLC analysis of validamycin A was performed using an Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm) with 3% acetonitrile at 30 °C and monitored at 210 nm using a diode array detector. The precolumn derivative HPLC assay for valienone was performed using an Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm) at 30 °C with 50% acetonitrile, and the absorbance was monitored at 380 nm after derivatization with 2,4-dinitrophenylhydrazine in a phosphate solution at 37 °C for 45 min. TQMS for valienamine was conducted on Agilent QQQ MS 6470 using an Eclipse XDB-C18 (5 μm, 4.6 × 150 mm) column; the 22% acetonitrile eluent at 30 °C was directed to the MS with an ESI source gas temperature of 300 °C, a gas flow rate of 5 L/min, and a nebulizer pressure of 45 psi. The quantification of small molecular compounds was based on the integrated peak area of the MRM chromatograms. The MRM transitions used ranged from 176.08 to 39.1, which were determined using the MassHunter Optimizer software with appropriate standards. MRM peak areas were compared to a calibration curve of external standard peak areas to determine the concentrations.

**ASSOCIATED CONTENT**

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00319.

Information on the candidate aminotransferases; primers and plasmids used in this study; the identification and detection of the transamination product; the putative catalytic mechanism and the evolution of WecE (PDF)

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Author Contributions

Li Cui designed the shunt pathway and conducted most of the experiments. Xiaodong Wei performed the experiments under supervision of Li Cui and Prof. Yan Feng. Xinran Wang and Prof. Linquan Bai supported the genetic manipulation and the transcriptional detection of the Streptomyces host. Prof. Shuangjun Lin helped with the chemical analysis including MS and NMR. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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