Complete stereoinversion of L-tryptophan by a fungal single-module nonribosomal peptide synthetase

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Supporting Information Placeholder

ABSTRACT: Single-module nonribosomal peptide synthetases (NRPSs) and NRPS-like enzymes activate and transform carboxylic acids in both primary and secondary metabolism; and are of great interests due to their biocatalytic potentials. The single-module NRPS IvoA is essential for fungal pigment biosynthesis. Here, we show that IvoA catalyzes ATP-dependent unidirectional stereoinversion of L-tryptophan to D-tryptophan with complete conversion. While the stereoinversion is catalyzed by the epimerization (E) domain, the terminal condensation (C) domain stereoselectively hydrolyzes D-tryptophanyl-S-phosphopantetheine thioester and thus represents a noncanonical C domain function. Using IvoA, we demonstrate a biocatalytic stereoinversion/deracemization route to access a variety of substituted D-tryptophan analogs in high enantiomeric excess.

Nonribosomal peptide synthetases (NRPSs) are modular enzymes employing an assembly-line logic to synthesize a myriad of peptide-based secondary metabolites with diverse structures and biological activities.[1] Single-module NRPS and NRPS-like enzymes adopt similar thiotemplated enzymology with a single set of adenylation (A) and thiolation (T) domain. These enzymes have important functions in transforming carboxylic acid substrates in primary and secondary metabolism;[2] and have increased interests as biocatalysts due to their functional diversity (**Figure 1**).[3] Following selection and thermodynamic activation of the carboxylic acid by the A domain, the substrate is preserved as phosphopantetheine (Ppant) thioester on the T domain. Depending on the type of downstream releasing domains, the thioester intermediates are subjected to a broad range of modifications (**Figure 1a**), including but not limited to: esterification/amidation by a condensation (C) domain in A-T-C;[4] Dieckman/aldol condensation or cyclization by a thioesterase (TE) domain in A-T-TE;[5,6] 2- or 4-electron reduction by reductase (R) domain in either A-T-R or A-T-R-R;[2b,7] 2-electron reduction followed by PLP-dependent aldol condensation in A-T-R-P.[8] In natural product biosynthetic pathways, these enzymes generate natural product scaffolds with structural diversity that complements the chemical space of canonical nonribosomal peptides, such as dihydroxybenzoquinone, furanone, butyrolactone, and dihydropyrazine (**Figure 1A**).

Recently, a single-module NRPS, encoded by the gene *ivoA* from *Aspergillus nidulans* with an unusual domain architecture annotated as A-T-C-C* was proposed to acetylate L-tryptophan.[9] The enzymatic product *N*-acetyl-L-tryptophan was suggested to be further oxidized by a P450 enzyme IvoC and a phenol oxidase IvoB en route to the conidiophore pigment (**Figure 1B**). Although genetic studies provided compelling evidence implicating *ivoA* in the biosynthesis of *N*-acetyl-hydroxytryptophan,[10,11] the proposed acetyltransferase activity of IvoA is an unlikely fit for a single-module NRPS. Furthermore, the mechanistic proposal for IvoA is at odds with accepted logic of NRPS enzymology for the following reasons:1) It is metabolically wasteful to activate the carboxy group of a substrate at the expense of one equivalent of ATP in order to accomplish *N*-acetylation by acetyl-CoA; 2) It is against the NRPS directionality rule for a downstream C domain to carry out a condensation reaction (acetylation here) with an upstream T domain as the acceptor.[1,12]

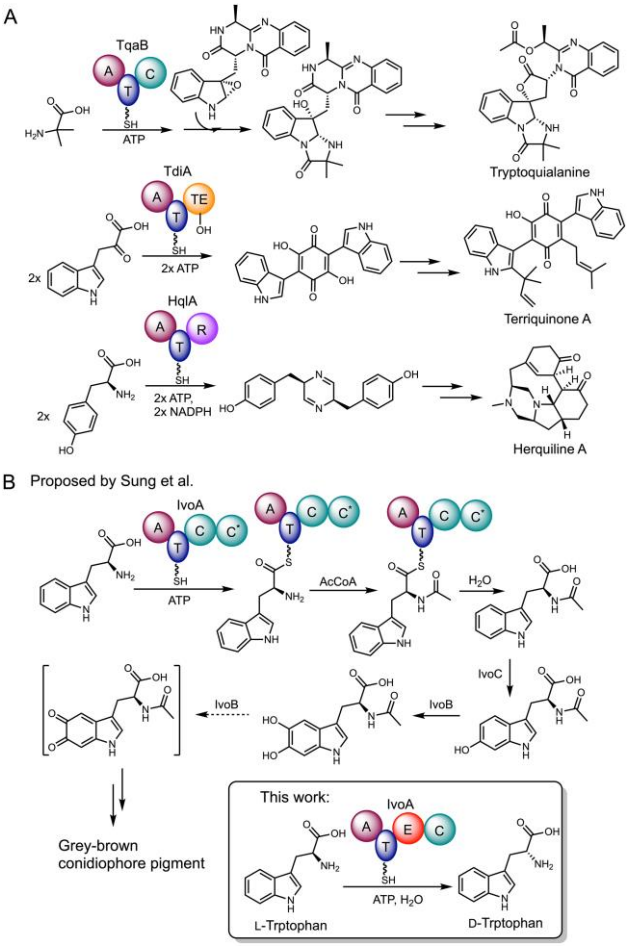


Figure 1. Diverse functions of single-module NRPS and NRPS-like enzymes. **A**) Characterized examples. **B**) IvoA studied in this work.

To elucidate the enzymatic function of IvoA, we first re-analyzed its domain architecture. Since epimerization (E) domains show sequence and structure homology to C domains, and are often inserted between T and C domains in the NRPS assembly lines,[12] we hypothesized that the true domain organization of IvoA is A-T-E-C. Embedding a functional E domain could rationalize the necessity of involving NRPS machinery: activation of the α-carboxy group can lower the pKa of the C_α proton, thereby facilitating stereoinversion. To test this hypothesis, we overexpressed IvoA by using *S. cerevisiae* JHY686 strain as a heterologous host.[13] Consistent with the previous report, we were able to detect *N*-acetyltryptophan formation. However, the purified product from yeast cell culture was found to be exclusively D-enantiomeric (ee > 99%) as confirmed by chiral-HPLC analysis (**Figure 2A**). The inverted stereochemistry of tryptophan supports our hypothesis that an E domain is present within IvoA.

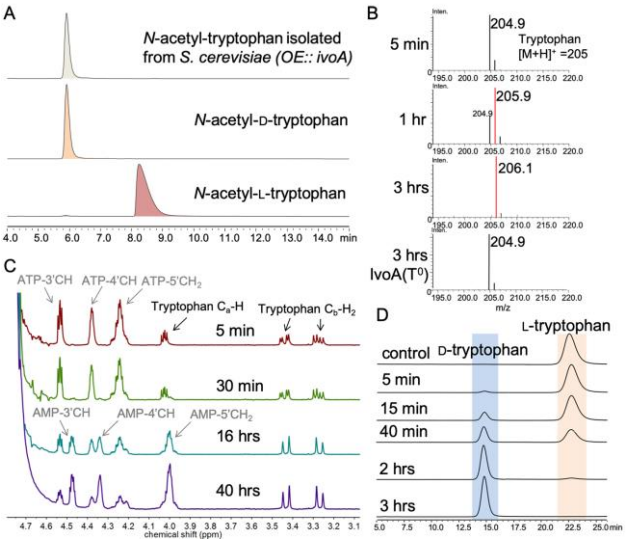


Figure 2. Characterization of IvoA activity. **A**) Stereochemistry determination for isolated *N*-acetyl-D-tryptophan. **B**) Mass spectrometry shows the mass shift of tryptophan when the assay was performed in D_2O. **C**) ^{1}H-NMR spectra indicate incorporation of deuterium at the α position: 1) the change of splitting pattern of the diastereotopic β proton signal due to smaller coupling constant ($^3J_{H\text{-}D}$)); 2) the disappearance of α proton signal. **D**) Chiral HPLC resolution of tryptophan enantiomers from IvoA reaction demonstrated complete stereoinversion of L-tryptophan to D-tryptophan.

To interrogate the function of IvoA, particularly the cryptic acetyltransferase activity, we purified IvoA from *S. cerevisiae* and assayed its activity in vitro (**Figure S1**). We first examined the substrate specificity of IvoA adenylation domain (**Figure S2**). As expected, L-tryptophan is the preferred substrate, while D-tryptophan is activated with 64% lower efficiency (**Figure S2**). We were unable to detect formation of acetylated tryptophan starting from either L/D-tryptophan in the presence of ATP, provided with either acetyl-CoA or acetyl-phosphate as the acetyl-donor. Increasing enzyme concentration to > 50 μM, plus prolonged overnight incubation only led to trace amount of *N*-acetyl-D-tryptophan (k_{obs}^{app} < 0.1 h^{-1}, **Figure S3**).

When we performed the assay ([E] = 2 μM) in D_2O and analyzed the reaction mixture by LC-MS, we readily observed a gradual +1 Da mass shift of tryptophan (**Figure 2B**). This deuterium "wash-in" result is phosphopantetheinylation-dependent as inactivating the T domain (IvoA-T^0, S785A mutation) completely abolished the incorporation. Monitoring the reaction by ^{1}H-NMR confirmed that the hydrogen-deuterium exchange took place at the C_α–H (**Figure 2C**), indicating that epimerization of tryptophanyl-S-Ppant occurred. The complete hydrogen-deuterium exchange is also consistent with the "two-base" mechanism proposed for NRPS E domain.[14]

Table 1. Apparent steady-state kinetic constants of L-tryptophan stereoinversion by IvoA and mutants.

Enzyme	k_{cat}^{app} (min^{-1})	K_m^{app} (μM)	$(k_{cat}/K_m)^{app}$ (M^{-1} s^{-1})
WT[a]	38 ± 3	50 ± 10	1.3 x 10^4
T^0(S785A)	inactive	inactive	inactive
E^0(H963A)	0.09 ± 0.03	50 ± 10	30

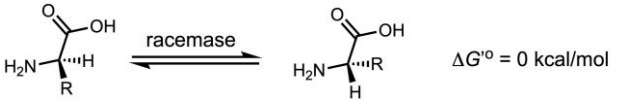
C^0(H1428A)	0.008 0.002	±	40 ± 10	3.5

[a]Substrate inhibition was observed with K_i^{app} of 4 ± 1 (mM).

We next followed the reaction by using chiral-HPLC and complete conversion of L-tryptophan (1 mM) to D-tryptophan was observed in 3 hours (**Figure 2D**). Chiral-resolution allowed us to determine the apparent steady-state kinetics by quantifying D-tryptophan formation (**Table 1, Figure S4**). The apparent k_{cat} (38 min^{-1}) is similar to that of other characterized single-module NRPSs and NRPS-like enzymes,[4,7] while the K_m (~50 μM) is close to the intracellular level of L-tryptophan (12 μM).[15] Both E and C domains are catalytically important for IvoA, as inactivating either domain by mutating the catalytic histidine residues (H963A and H1428A) substantially compromised the apparent turnover number k_{cat} (420-fold by E^0 while 4700-fold by C^0). In contrast, the apparent Michaelis constants were not changed, suggesting that substrate binding (at the A domain) was not affected.

Taken together, these data indicate that IvoA lacks acetyltransferase activity in vitro, but instead is a *bona fide* ATP-dependent enzyme catalyzing enantioselective stereoinversion of L-tryptophan to D-tryptophan. The observed acetylation of D-tryptophan in vivo must be carried out by an endogenous acetyltransferase. Because yeast histone acetyltransferase Hpa3 is known to act as a D-amino acid *N*-acetyltransferase for detoxification of D-amino acids,[16] we overexpressed IvoA in the *hpa3*-deleted yeast strain constructed by replacing *hpa3 with leu2* (SI Methods, Figure S5). Consequently, the culture medium was devoid of *N*-acetyltryptophan, whereas free D-tryptophan (ee = 98%) were still accumulated inside the cells (**Figure S6-S7**). Therefore, we conclude that IvoA does not acetylate tryptophan and the origin of the negligible acetyltransferase activity of IvoA observed in vitro may derive from trace amount of contaminated yeast Hpa3.

Scheme 1. Stereoinversion of amino acid enantiomer into its mirror-image counterpart by common racemases and IvoA.



L-tryptophan + ATP + H_2O $\xrightarrow{IvoA}$ D-tryptophan + AMP + PPi

$\Delta G'^{o}$ = -12 kcal/mol

Distinct from common PLP-dependent or PLP-independent amino acid racemases (**Scheme 1**), which often catalyze bidirectional stereoinversion and also inevitably lead to racemization (equilibrium constant approaches unity),[17] IvoA catalyzes unidirectional stereoinversion, completely converting L-tryptophan to its enantiomer D-tryptophan. The complete conversion is driven by coupled ATP hydrolysis, which is thermodynamically favored (**Scheme 1**),[18] and is enabled by the thiotemplate enzymology of IvoA (**Figure 3**). We reason that the activated tryptophan is delivered to the E domain as tryptophanyl-S-Ppant thioester, which undergoes epimerization to give a mixture of D/L-tryptophanyl-S-Ppant diastereoisomers in equilibrium. We propose that dynamic kinetic resolution may be accomplished by the C domain in a releasing step, which stereoselectively hydrolyzes the D-tryptophanyl-S-Ppant thioester to achieve irreversible conversion.

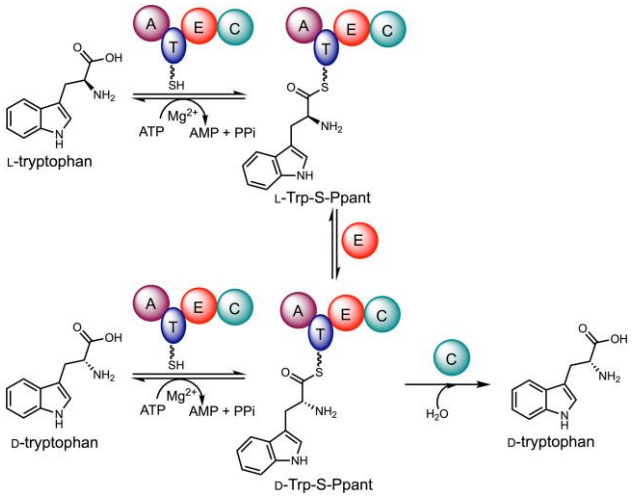


Figure 3. Working model of IvoA.

As mentioned earlier, even though IvoA A domain prefers L-tryptophan, D-tryptophan can still be adenylated and thioesterified (**Figure S2**). In addition, the loaded D-tryptophanyl-S-Ppant underwent epimerization by IvoA E domain as evidenced by similar, yet slower deuterium "wash-in" behavior under multiple-turnover condition (**Figure S8**). The slower turnover measured by deuterium incorporation reflects the lower adenylation efficiency of D-tryptophan. Nonetheless, the occurrence of hydrogen-deuterium exchange at D-tryptophanyl-S-Ppant C_α position not only suggests that epimerization is faster than the C-domain catalyzed D-specific tryptophanyl thioester hydrolysis, but also indicates that the D/L-tryptophanyl-S-Ppant equilibrium can be approached from either direction (Figure 3). However, IvoA cannot convert D-tryptophan to L-tryptophan, which suggests that the L-tryptophanyl-S-Ppant is not hydrolyzed by the C domain. A D-specific hydrolytic releasing C domain is therefore the key for unidirectional complete stereoinversion.

To directly demonstrate the stereoselectivity of IvoA C domain, we purified the standalone IvoA-C and assayed its activity in vitro. Addition of IvoA-C in equimolar to either IvoA(C^0) mutant or IvoA-ΔC truncation mutant successfully rescued the impaired stereoinversion activity, which proved that the standalone IvoA-C is active (**Figure S9**). We then synthesized both D- and L-tryptophanyl-S-*N*-acetylcysteamine as surrogate substrates mimicking the IvoA T domain bound tryptophanyl-S-Ppant intermediates. However, the enzyme did not catalyze hydrolysis significantly above the background nonenzymatic rate (**Figure S10**). Using D-tryptophanyl-S-pantetheine (D-Trp-pant) also did not improve enzymatic hydrolysis. We reason that the protein:protein interaction between T and C domain is important for substrate recognition, which has been shown in other studies of C domains.[19] Hence, we chose to enzymatically load D/L-tryptophan to IvoA-ΔC(E^0) by taking advantage of the promiscuous A domain. It is imperative to inactivate the E domain in this truncation mutant in order to minimize the epimerization. The formation of corresponding D/L-tryptophanyl-S-Ppant of IvoA-ΔC was confirmed by intact protein mass spectrometry (**Figure S11**). Free excess D/L-tryptophan substrates were quickly removed from IvoA-ΔC(E^0) by using desalting spin columns and the loaded D/L-tryptophanyl-S-IvoA-ΔC(E^0) were immediately subjected to IvoA-C catalyzed hydrolysis. The liberated free tryptophan was then quantified by LC-MS. As shown in **Figure 4**, IvoA-C stereoselectively hydrolyzed D-

tryptophanyl-S-IvoA-ΔC(E^0) over L-tryptophanyl-S-IvoA-ΔC(E^0). NRPS C domains that have thioesterase activity are rare, and to date only one example from crocacin PKS-NRPS hybrid assembly-line was known, but did not show stereoselectivity.[20] Therefore, the IvoA-C characterized here represents a novel C domain and we classify it as a $^{D}C_{H2O}$ subtype according to the universally acknowledged nomenclature.[12]

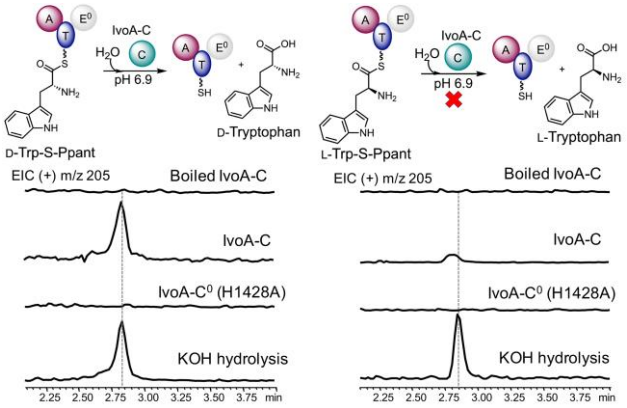
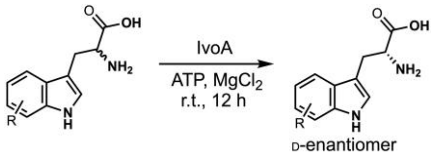


Figure 4. Characterization of IvoA-C activity in vitro by LC-MS.

The verified stereoinversion activity of IvoA prompted us to explore its biocatalytic potential. D-tryptophan and its substituted analogues are important building blocks for many peptide pharmaceuticals, such as FDA approved lanreotide, pasireotide, octreotide, macimorelin, triptorelin, etc. Recently, there is growing interest in developing biocatalytic processes for syntheses of substituted D-tryptophans by stereoinversion and deracemization from the L-enantiomers and *rac*-tryptophans, respectively.[21] However, to overcome the entropically unfavorable deracemization process (ΔG^{o} = 0.4 kcal/mol),[22] the current methods are based on multi-step cascade reactions to establish non-equilibrium conditions for enrichment of D-enantiomers.[21] In contrast, IvoA offers a concise one-step, direct nonredox stereoinversion/deracemization process, and allows us to access a library of D-tryptophan analogues in high enantiomeric excess (ee >99%) at millimolar level. Different substitution groups, either electron-withdrawing or electron-donating, at most positions (4, 5, 6 and 7) on the indole ring can be tolerated (**Table 2**). No conversion of 2-Me-DL-tryptophan is due to inefficient activation by A domain (**Figure S12**), which suggests that substitution at 2-position may interfere with substrate recognition. The poor substrates are generally those with larger substituents (e.g. 5-NO_2, 5-CN, 6-Br, 7-Br), which reflects the size limit by IvoA A domain. In light of recent success in A domain engineering,[23] it is conceivable that the substrate scope can be expanded in the future by enlarging the substrate binding pocket of A domain.

In summary, our biochemical study uncovered the unusual activity of IvoA, and our findings expand the function diversity of single-module NRPSs. The reassigned function of IvoA also provides insight to fungal pigment biosynthesis. By inverting the chirality of tryptophan, IvoA perhaps can modulate amino acid flux to pigment biosynthesis in vivo. Considering the proposed role of IvoB and IvoC, one can speculate that the D-configuration generated by IvoA may be retained in the final uncharacterized conidiophore pigment.

Table 2. Biocatalytic stereoinversion or deracemization of substituted tryptophans.[a]



Entry	Substrate		ee (%)
	Stereochemistry	R	
1	L	H	>99
2	L	5-OMe	>99
3	*rac*	5-CN	63
4	*rac*	5-NO_2	44
5	*rac*	4-F	>99
6	*rac*	5-F	>99
7	*rac*	6-F	>99
8	*rac*	5-Cl	>99
9	*rac*	6-Cl	98
10	*rac*	5-Br	>99
11	*rac*	6-Br	75
12	*rac*	7-Br	87
13	*rac*	2-Me	1.3
14	*rac*	4-Me	>99
15	*rac*	5-Me	>99
16	*rac*	6-Me	97
17	*rac*	7-Me	>99

[a]Expt. Cond.: 1.5 mM substrates, 5 μM IvoA, 5 mM ATP, 10 mM $MgCl_2$, 100 mM K_2HPO_4 buffer, pH 7.5.

ASSOCIATED CONTENT

Supporting Information.
This material is available free of charge via the Internet at http://pubs.acs.org. Experimental procedures, chromatograms, and spectroscopic data.

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Notes

No competing financial interests have been declared.

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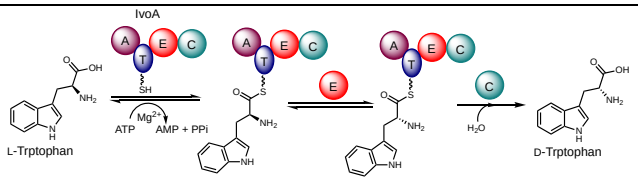

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