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Directed Evolution of a Tryptophan 2,3-Dioxygenase for Diastereoselective Monooxygenation of Tryptophans

Yanxin Wei,[†] Chen Lu,[†] Shengsheng Jiang, Yanyan Zhang, Qiuchun Li, Wen-Ju Bai, and Xiqing Wang**

Abstract: We report herein the first engineered enzyme that can monooxygenate unprotected tryptophans into their corresponding HPICs in a single, scalable step with excellent turnover number and diastereoselectivity. Taking advantage of directed evolution, we analyzed the stepwise oxygen insertion mechanism of tryptophan 2,3-dioxygenases, and transformed tryptophan 2,3-dioxygenase from *Xanthomonas campestris* into a monooxygenase for oxidative cyclization of tryptophans. It was revealed that residue F51 is vital in determining the product ratio of HPIC to *N*'-formylkynurenine. Our reactions and purification procedures use no organic solvents, representing an eco-friendly method to prepare HPICs for further applications.

The tricyclic 3*a*-hydroxyhexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (HPIC) scaffold is a common motif in alkaloids and peptides with marvelous structural diversity and biological activity (Figure 1, left).[1, 2] For example, kapakahine C is cytotoxic to P388 murine leukemia cells,[3] and most family members of the NW-G natural products,[4] such as NW-G01, exhibit potent and selective antibacterial activity against Gram-positive bacteria. Besides their antibacterial properties, himastatin[5] and chloptosin[6] also display anticancer activity. Therefore, HPIC-containing natural products are popular targets for synthetic and pharmacological investigations. Moreover, HPICs may be useful intermediates for synthesis of pyrrolobenzoxazine alkaloids, such as paeciloxazine, CJ-12662, and CJ-12663,[7] via oxidation.[8] Additionally, monocyclic peptides bearing an HPIC skeleton can be transformed into bicyclic peptides with Savige-Fontana tryptathionylation for potency improvement.[9] Thus, in addition to being present in a variety of bioactive molecules, HPICs are also important synthetic intermediates.

Construction of HPIC motifs from tryptophan (Trp) derivatives has been enthusiastically explored by synthetic chemists (Figure 1, right). Pioneering work by Danishefsky enabled diastereoselective preparation of HPICs from Trp in 3-4 steps, which represents a powerful strategy for synthesizing complex natural pruducts.[10] These cation-triggered cyclizations employ electrophiles like *N*-phenylselenophthalimide (*N*-PSP) or *N*-bromosuccinimide (NBS) to activate the indole ring; the activating heteroatom is then replaced with oxygen using oxidative deselenation or solvolysis (Figure 1, 1a).[11] Unlike the aforementioned stepwise methods, DMDO oxidation can convert Trp derivatives to HPICs directly, but the diastereoselectivity depends on the carboxylic acid and amine protecting groups (Figure 1, 1b).[12] Without bulky blocking groups like triphenylmethyl (Tr), 1:1 mixtures of HPIC diastereomers are obtained. Recently, radical-triggered cyclizations have also been used for stepwise preparation of HPICs from Trp derivatives.[13] Despite these notable advances, the step-economy[14] of existing HPIC preparations is negatively impacted by the need for multiple protecting group manipulations. Efforts to address this challenge have been tried via photosensitized oxygenation of unprotected Trp in a two-step/one-pot sequence (Figure 1, 2),[15] involving tricyclic peroxide formation and hydroperoxide cleavage. While straightforward, this method suffers from poor diastereoselectivity. In addition, the labile peroxide intermediate may rapidly decompose to *N*'-formylkynurenine (NFK).

An ideal but unrealized strategy is the direct monooxygenation of Trps. Besides reactivity and chemoselectivity, controlling the diastereoselectivity of such a process represents a key challenge. Since enzymatic oxidation of an indole moiety is believed to be fundamental in biosynthesis of HPIC-containing natural products, we believed that an appropriate enzyme would overcome these obstacles. Recent studies on the biosynthesis of some HPIC-containing natural products reveal that certain monooxygenases can promote oxidative cyclizations on the indole cores of complicated diketopiperazines and cyclodepsipeptides,[16] lending credence to our initial proposal. However, due to the substrate-specific nature of these enzymes, applying them to the monooxygenation of simple Trp is problematic and not surprisingly, oxygenases that are suitable for this transformation have not been reported. As a result, we examined several Trp oxygenases and were intrigued by two heme-containing Trp dioxygenase enzymes: tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). Mechanistic studies on these enzymes reveal that Trp is converted to NFK via stepwise oxygen insertions (Figure 2).[17] The first oxygen insertion from the heme iron-bound dioxygen generates a 2,3-epoxide intermediate, which is opened by the incipient iron-oxo species. This intermediate then undergoes oxidative cleavage of the C^2-C^3 bond to yield NFK (Path A). We envisioned that these two oxidations could be decoupled by impairing the coordination of the 2,3-epoxide to the metal center, possibly by using directed evolution.[18] Interrupting the second oxygen insertion would enable HPIC production by allowing for intramolecular, nitrogen-assisted epoxide opening followed by cyclization onto the resulting imine (Path B). Herein, we report our findings.

The use of wild-type TDOs and IDOs with Trp afforded NFK as the major product and delivered only trace amounts of HPIC.[17h] Among examined IDOs and TDOs,

[a] Y. Wei, C. Lu, S. Jiang, Prof. Dr. Q. Li, Prof. Dr. X. Wang
College of Bioscience and Biotechnology, Yangzhou University
Yangzhou, Jiangsu 225009, China
E-mail: xiqingwang@yzu.edu.cn
[b] Dr. Y. Zhang
Testing Center, Yangzhou University
Yangzhou, Jiangsu 225009, China
[c] Dr. W.-J. Bai
Department of Chemistry, Stanford University
Stanford, California 94305, USA
E-mail: bai.stanford@gmail.com
Present address: Amgen Inc., 1 Amgen Center Drive, Thousand Oaks, CA 91320.
[†] These authors contributed equally to this work.

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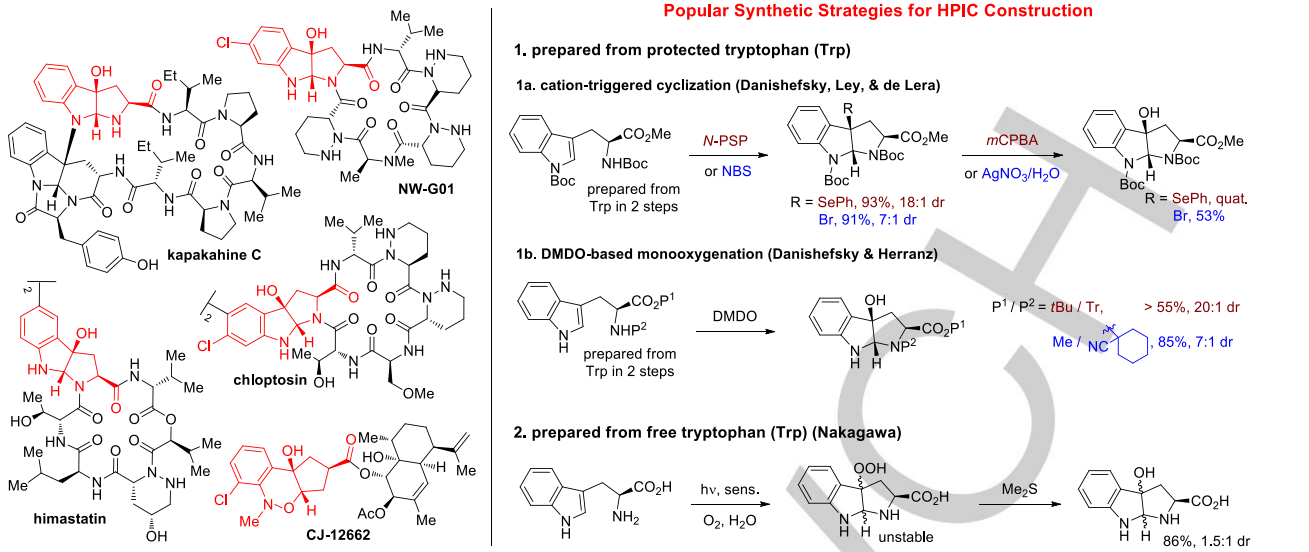

Figure 1. *Left:* Examples of HPIC-containing natural products & CJ-12662. *Right:* Synthetic approaches towards HPIC skeletons.

Xanthomonas campestris TDO (xcTDO) was chosen for further evaluation due to its highest activity (Table S1), no substrate inhibition,[19] and extensive mechanistic studies. Going forward, the amino acid residues surrounding the substrate and heme binding pocket of xcTDO were subjected to three rounds of site-saturation mutagenesis (Figure S1). Enzymatic reactions were carried out with cell-free lysates of *Escherichia coli* expressing xcTDO variants, then high-performance liquid chromatography (HPLC) was used to screen the libraries for HPIC yield. Authentic *cis* and *trans* HPIC diastereomers prepared via photooxygenation of Trp were used as the standards to assign and quantify the HPIC peak in the HPLC traces at 295 nm.[15]

The first-round of site-saturation mutagenesis targeted xcTDO residues located in the αB (F51 and H55) and αD (Y113, R117, and L120) helices and the amino-terminal residues from the other monomer (Y24, Y27, and L28). They are all situated on one side of the active site and within 7 Å from the substrate (Figure S1). During screening, most lysates in the F51X library exhibited significantly more HPIC production than that of wild-type xcTDO, whereas little or no HPIC was detected with lysates from the other libraries. Moreover, only *cis*-HPIC was observed, demonstrating superb diastereoselectivity of this enzymatic reaction. These results indicated that the replacement of F51 could change the reaction pathway to favor HPIC formation. Accordingly, we focused on the F51 site and purified a complete set of mutant xcTDO proteins with amino acid substitutions at this residue. At optimized pH 6.0 (Table S2), HPIC yields and total conversions of all xcTDO-F51X variants were evaluated (see Figures S3-S5) and summarized in Figure 3. Of the mutations that retained good activities (i.e. total conversion> 50%), xcTDO variants with smaller amino acid side chains (e.g. A, L, V, and I) at residue 51 exhibited higher HPIC productivity than those with sterically hindered side chains (e.g. F, W, and H). This suggests that the van der Waals forces imposed by bulky amino acid side chains at residue 51 preferentially orient the tryptophan-2,3-epoxide intermediate for the second oxygen insertion (Figure 4). The high conservation in all wild-type TDOs and IDOs and the same spatial conformation in the structures of xcTDO, human TDO, and human IDO (Figure S2) implies that F51 is important in selecting for NFK production by inhibiting the intramolecular cyclization pathway involved in HPIC formation. These findings further support the stepwise oxygen insertion mechanism proposed for tryptophan dioxygenases and validate our strategy of tuning a dioxygenase for HPIC production.

The F51X mutant plasmids that expressed xcTDO proteins with TONs > 250 were used as templates to further improve HPIC production. Residues 121-125, 127, and 253-255 in the αD-αE and αJ-αK loops, all of which reside on the other side of the active site and within 7 Å from the substrate (Figure S1), were targeted in the second-round of site-saturation mutagenesis. Again, only *cis*-HPIC was observed. xcTDO-F51M/Q127Y exhibited increased overall reactivity (>99% conversion), excellent TON for HPIC formation (3,312), and moderately enhanced HPIC/NFK ratio (2:1) (Table 1, entry 1; Figure S6).

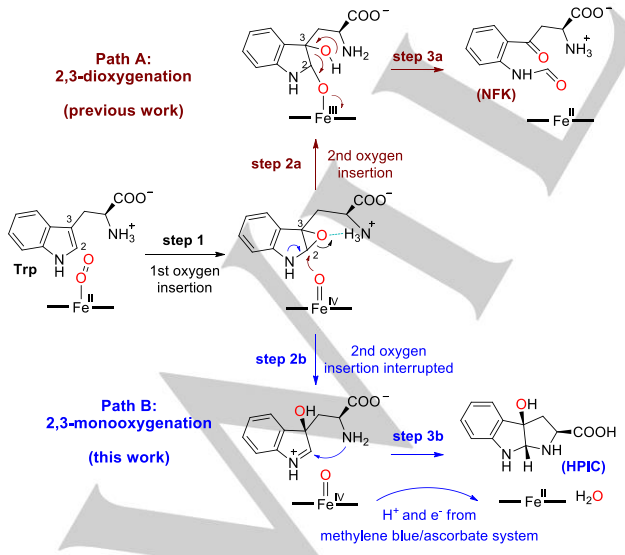

Figure 2. The stepwise oxygen insertion mechanism of xcTDO. Both radical and electrophilic addition mechanisms have been proposed for the first oxygen insertion (Step 1).

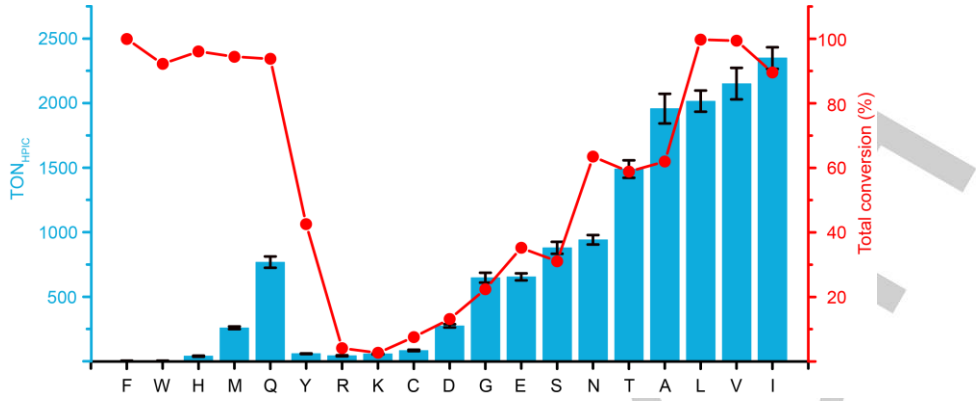

Figure 3. The effects of amino acid substitution at residue 51 on HPIC production and overall activity of xcTDO. [xcTDO]=1 μM; [Trp]=5 mM; total conversion=([HPIC]+[NFK])/(5 mM). Note that F51P mutant was not tested due to poor stability.

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Q127, which is also a highly conserved residue in the tryptophan dioxygenase family (Figure S2A), resides on the opposite side of F51 in all of the xcTDO, human TDO, and human IDO structures (Figures 4 and S2B). Replacing it with a Tyr residue might indirectly affect the active site via interactions with the amino-terminus from the other monomer and the αD-αE loop. Even though the F51M substitution was not prominent (Figure 3), the xcTDO-F51M/Q127Y mutant showed higher HPIC productivity than xcTDO-F51I/Q127Y (TON_{HPIC}=457) and xcTDO-F51L/Q127Y (TON_{HPIC}=633) (Table S3), suggesting that the F51M and Q127Y substitutions imposed a cooperative effect on the enzyme activity (Figure 4). Furthermore, two other mutants, xcTDO-F51S/T254S and xcTDO-F51Q/G255S, were found to enhance the HPIC/NFK ratio to 6.2:1 and 3.1:1, respectively, at the expense of catalytic activity (Table 1, entries 2 and 3). Molecular dynamic simulations suggest that T254 modulates the H-bond interaction between the NH_3^+ group and epoxide oxygen of the 2,3-epoxide intermediate.[20] Thus, a subtle change from Thr to a structurally similar Ser residue or a perturbation from the mutation in the neighboring G255 might further destabilize the transition state for the second oxygen insertion, thereby increasing the likelihood of the desired intramolecular cyclization (Figure 2). Further improvement efforts by combining the mutations of F51M, Q127Y, and T254S resulted in a decrease in the TON for HPIC production (Table 1, entry 4).

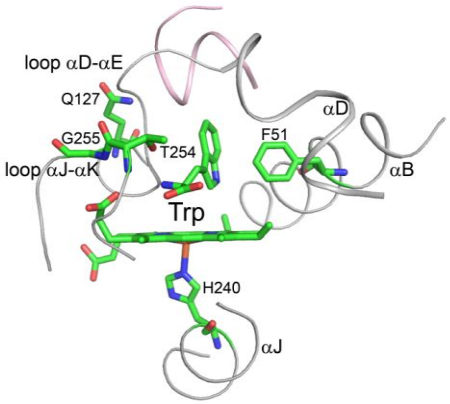


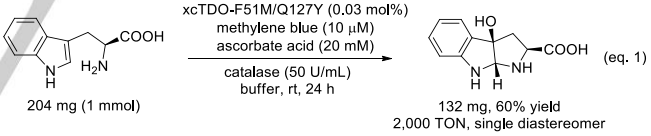
Figure 4. Schematic of the active site of xcTDO (PDB: 2NW8). The fragment colored in pink comes from the other monomer in the crystal structure of xcTDO.

The third-round screening employed xcTDO-F51M/Q127Y and xcTDO-F51S/T254S as templates and targeted residues involved with the heme-iron binding (W102, L105, F126, Y131, R132, W236, H240, V244, I248, G256, F262, and L263) (Figure S1). No improvement was observed, suggesting that residues coordinated with the heme-iron are trivial for HPIC/NFK product distribution.

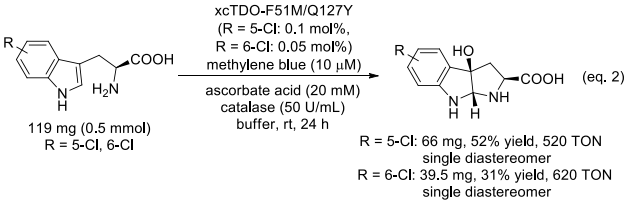
Table 1. Improved product distribution during the 2nd round screening.

Entry	xcTDO variant	HPIC (TON)[a]	NFK (TON)[a]	HPIC/NFK ratio
1	F51M/Q127Y	3312	1655	2.0:1
2	F51S/T254S	468	76	6.2:1
3	F51Q/G255S	809	264	3.1:1
4	F51M/Q127Y/T254S	340	133	2.6:1

[a]Determined by HPLC.



To demonstrate the scalability and practicality of this enzymatic reaction, the xcTDO-F51M/Q127Y mutant was selected for scale-up because of its impressive TON. At a catalyst loading of 0.03 mol% and optimal pH 6.0 (Table S4), 1 mmol of Trp was smoothly converted to *cis*-HPIC[15] in 60% yield and perfect diastereoselectivity, making this transformation synthetically useful (eq. 1).



Given that some HPIC-containing natural products bear chlorine substituents on the aromatic ring (e.g. NW-G01 and chloptosin in Figure 1), monooxygenation of unprotected 5-Cl- and 6-Cl-tryptophans was also investigated. Without further engineering, direct application of xcTDO-F51M/Q127Y delivered

5-Cl- and 6-Cl-HPICs as single diastereomers in 52% and 31% yield, respectively (eq. 2). It is worth noting that our green enzymatic reaction and subsequent purification with ion exchange chromatography use no organic solvent.

In conclusion, by evaluating the stepwise oxygen insertion mechanism of tryptophan dioxygenases and taking advantage of directed evolution, we interrupted the dual oxygen insertion process and transformed the xcTDO tryptophan dioxygenase into a monooxygenase. Our engineered xcTDO exhibits excellent TON and high diastereoselectivity, and enables the scalable synthesis of HPICs from Trps in a single step. To the best of our knowledge, this is the first enzyme that can catalyze the oxidative cyclization of unprotected Trps in a highly diastereoselective fashion, a process that has not been synthetically realized before. It was found that residue F51 was identified as being critical in determining the HPIC/NFK product distribution and enzyme activity. In addition, both our reaction and purification processes require no organic solvent, making this eco-friendly method for synthesizing HPICs for further applications.

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Keywords: biocatalysis • monooxygenation • protein engineering • pyrroloindole • tryptophan 2,3-dioxygenase

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Layout 1:

COMMUNICATION

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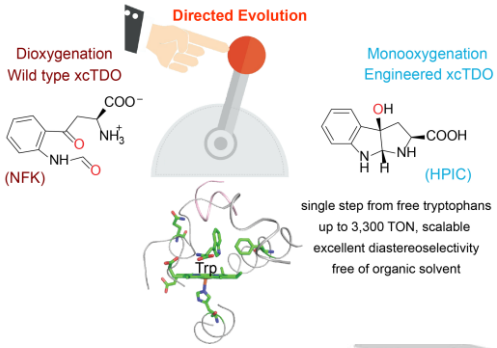

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