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Engineering the Substrate Specificity of a P450 Dimerase Enables the Collective Biosynthesis of Heterodimeric Tryptophan-Containing Diketopiperazines

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Abstract: Heterodimeric tryptophan-containing diketopiperazines (HTDKPs) are an important class of bioactive secondary metabolites. Biosynthesis offers a practical opportunity to access their bioactive structural diversity, however, it is restricted by the limited substrate scopes of the HTDKPs-forming P450 dimerases. Herein, by genome mining and investigation of the sequence-product relationships, we unveiled three important residues (F387, F388 and E73) in these P450s that are pivotal for selecting different diketopiperazine (DKP) substrates in the upper binding pocket. Engineering these residues in Nas_{F5053} significantly expanded its substrate specificity and enabled the collective biosynthesis, including 12 self-dimerized and at least 81 cross-dimerized HTDKPs. Structural and molecular dynamics analysis of F387G and E73S revealed that they control the substrate specificity via reducing steric hindrance and regulating substrate tunnels, respectively.

Tryptophan-containing diketopiperazines (DKPs) with a nonsymmetrical dimerization framework (HTDKPs) are a growing class of important natural products (Figure 1).[1] Their structural diversity and dimerization modes afford highly diverse bioactivities, such as anti-influenza, [1d] anti-HIV[1e] and neuroprotective activities [2] etc. While chemically accessing their complex structures is very challenging due to the polycyclic and densely functionalized backbones,[1c,1f,1g,3] HTDKPs can be succinctly biosynthesized via P450-mediated dimerization of DKPs.[2] In the past few years, a set of homologues of P450 dimerases with different regio- and stereo-specificity have been identified and characterized from the biosynthesis of various HTDKPs.[2a,4] By radical cascade reaction to form intra- and intermolecular carbon−carbon bonds with both regio- and stereospecificity,[2a] these enzymes are very efficient at constructing the HTDKP frameworks and were successfully used to synthesize >40 analogs employing different DKP substrates based on *Escherichia coli* and *Mycobacterium smegmatis* whole-cell biocatalysis.[2]

Very recently, we[4c] and another group[4d] further elucidated the conserved regulatory mechanism of the P450s in controlling the different regio- and stereospecificity of dimerization,

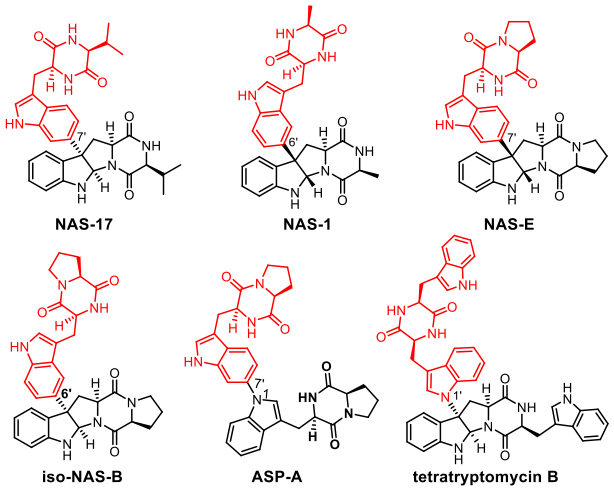

Figure 1. The representative HTDKPs produced by self-dimerized reactions. The DKP substrates accepted by the upper and lower binding pockets are shown in red and black, respectively.

permitting the precise tailoring of the frameworks. By combining the variations in the DKP substrates and the regio- and stereo-specificity of the reaction, enormous structural diversity and associated biological activities of HTDKPs are expected to be accessible. However, such efforts are impeded by the limited substrate scope of the dimerases. HTDKP dimerases have two binding pockets to individually accommodate the upper and lower DKP moieties. [4c,4d] While, the lower binding pocket is relatively flexible and can accept a range of cyclo-L-Trp-L-X (cW_LX_L, X denotes other proteinogenic amino acid residue) substrates, including cW_LA_L, cW_LP_L, cW_LV_L, cW_LI_L, cW_LL_L, cW_LM_L, cW_LF_L, cW_LY_L and cW_LW_L,[2] the upper binding pocket is rather restricted. Only three DKPs (cW_LP_L, cW_LA_L, cW_LV_L) were accepted by the identified dimerases Nas_{S1868}[2b] (AspB[4a]), NasbB[2b] (NasB[4a]), NascB[2a] and Nas_{F5053}[4c] (NzeB[4d]) and one cW_LW_L was found in the recently identified natural HTDKP tetratryptomycin B[4b] (Figure 1). Moreover, HTDKP dimerases show a poor tolerance to modifications of the substituent in the tryptophan group of DKP,

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only 7F-cW$_L$P$_L$ and 7Cl-cW$_L$P$_L$ can be accepted by the NascB[2a] which again restricts the introduction of structural variations into HTDKPs by employing Trp-modified DKPs.

Thus, to unleash the full potential of these fascinating reactions in the synthesis of HTDKPs, the substrate specificities of P450 dimerases, especially for the upper DKP moiety and tryptophan group, need to be elucidated and expanded. To this end, we herein employed genome mining and protein engineering to

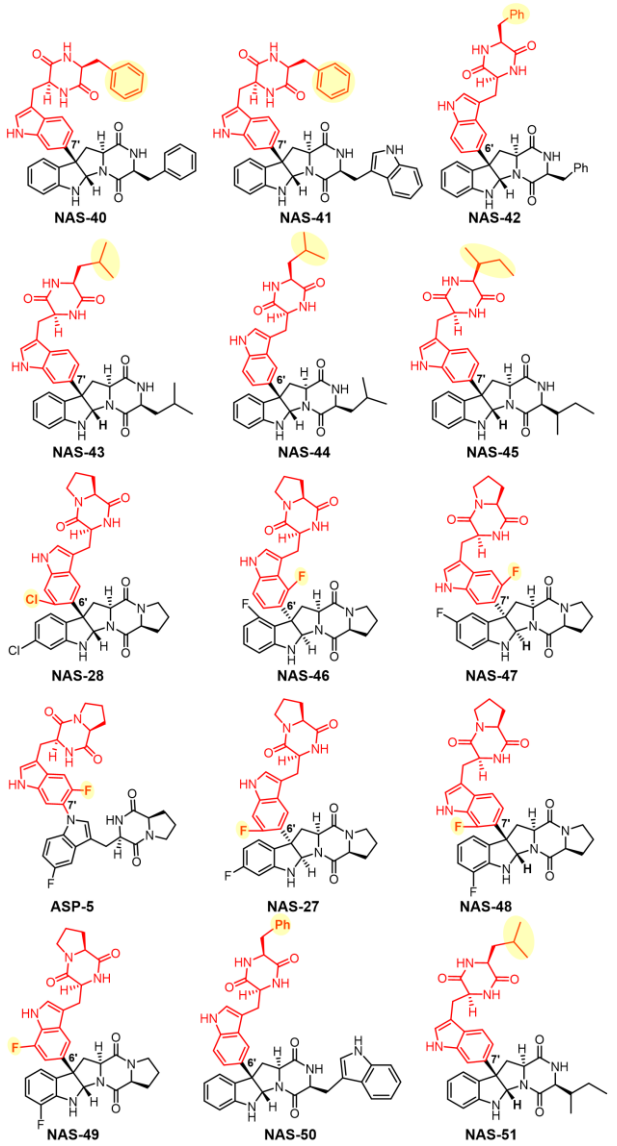

Figure 2. Products generated by the Nas$_{AO}$ and the mutants of Nas$_{F5053}$. The substrates accepted by the upper binding pocket are highlighted in red. The differences between these upper pocket substrates are highlighted in yellow.

broaden the substrate specificity of P450 dimerase reactions. By employing the engineered P450 Nas$_{F5053}$, we successfully achieved a collective biosynthesis of HTDKPs, which greatly expanded their structural diversity and provides an excellent platform for their efficient assessment.

We initially intended to compare the upper pocket sequences of P450 dimerases to identify critical residues for different substrate specificity. However, all the P450s (NascB, NasbB, Nas$_{F5053}$ and Nas$_{S1868}$) [2,4a,4c,4d] discovered in previous works share high homology and show identically narrow substrate selectivity. Therefore a sequence homology search was performed to identify putative P450 dimerases with potentially different substrate specificities. Because bacterial HTDKP P450s mostly are clustered with cyclodipeptide synthases (CDPSs) that generate the DKP substrates, both P450 and CDPS were used as searching criteria. The obtained sequences further underwent substrate prediction [5] (for CDPS) and phylogenetic analysis to filter out possible new tryptophan-containing DKP P450 dimerases. This effort led to the identification of a P450 from *Actinomadura oligospora* named Nas$_{AO}$ (accession: WP_026414936.1) and it shared only 50% identity to NascB, which is much lower than any other previously identified homologue (Figure S1).

Nas$_{AO}$ could only successfully be expressed and purified in/from a *Mycobacterium* heterologous expression system.[2] By screening its activity towards different cW$_L$X$_L$ substrates *in vitro* in the presence of spinach flavodoxin (Fdx) and flavodoxin reductase (FdxR) as well as an NADPH recycling system (NADP$^+$, glucose and glucose dehydrogenase), we were delighted to find that Nas$_{AO}$ could catalyze the self-dimerization of two molecules of cW$_L$F$_L$s to produce **NAS-40** (Figure 2, Figure S2, Figure S10 and Tables S1). Moreover, Nas$_{AO}$ could also cross-dimerize it with cW$_L$W$_L$ to produce **NAS-41**, in which cW$_L$F$_L$ was accepted by the upper pocket (Figure 2, Figure S2 Figure S11 and Table S2). Recently, Li et al. reported a P450 TtpB2 which can catalyze the C$_3$-N$_{1'}$ dimerization of two cW$_L$W$_L$ substrates to synthesize tetratryptomycin (Figure 1).[4b] Although the catalytic mechanism of TtpB2 is apparently different from other HTDKP-forming P450s because the intermolecular radical addition[2a] counterintuitively takes place on the electron negative indolic nitrogen, TtpB2 still shows a 40% identity to NascB suggesting that it may have a similar substrate specificity for the upper pocket.

The unique catalytic property of Nas$_{AO}$ and TtpB2 to be able to accommodate large DKPs in the upper cavity suggested that their pockets may have some key residues for the different substrate specificity. To identify this difference, we compared Nas$_{AO}$ and TtpB2 with three other P450s (NascB, [2a] NasbB[4c] and Nas$_{F5053}$[4c])

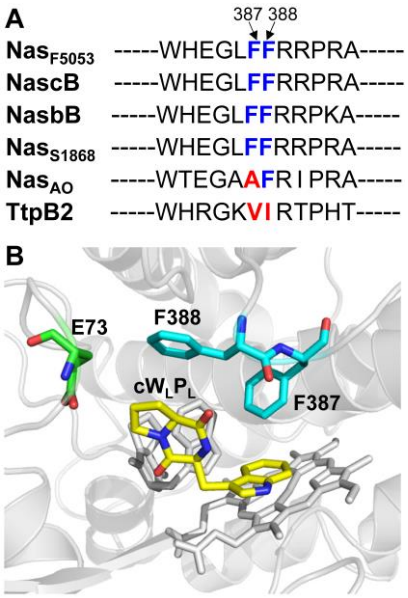

Figure 3. Identification of the residues pivotal for substrate specificity. A) Sequence alignment of Nas$_{F5053}$, NascB, NasbB, Nas$_{S1868}$, Nas$_{AO}$ and TtpB2; B) Key residues around the upper substrate binding pocket of Nas$_{F5053}$ (PDB: **6VXV**). The substrates cW$_L$P$_L$ in the upper and lower pockets are indicated by yellow and grey, respectively.

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that can only accept cW_LP_L, cW_LA_L and cW_LV_L. Sequence alignment revealed that the two residues 387 and 388 in the upper pocket were obviously different in size between the two groups of P450s (Figure 3A). The three P450s accepting small substrates all have bulky phenylalanines at this position (F387-F388), while corresponding residues in Nas_{AO} and TtpB2 consist of smaller amino acids with A387-F388 and V387-I388, respectively. In addition, these phenylalanine residues in the crystal structure of Nas_{F5053} (PDB: **6VXV**) [4c] are close to the DKP substrates by forming the belly of the upper pocket, which may potentially cause steric hindrance to the larger substrates (Figure 3B). Therefore, cW_LW_L (Figure S2), which could be due to their different microenvironment in the binding pockets.

Next, we proceeded to evaluate the substrate specificity of Nas_{F5053}-F387A mutant by using the previously chemically synthesized 28 cyclodipeptide substrates (**S1**-**S28**, Figure S3), [2a] however, this mutant only accepted cW_LF_L for self-dimerization (data not shown). To explore the beneficial effect of other mutants, residues F387 and F388 in Nas_{F5053} were individually targeted by saturated mutagenesis. As the *Mycobacterium*-based whole-cell biocatalysis system is efficient, clean and robust, [2b] the mutated plasmids were directly transferred into *M. smegmatis* MC^2 155 for

Table 1. Combinatorial Biosynthesis of Cross- and Self-Dimerized HTDKPs by the Nas_{F5053} Variants

	F387A	**F387G**		**F388N**	**E73S**			
Substrates[a]	cW_LF_L	cW_LI_L	cW_LL_L	7Cl-cW_LP_L	5F-cW_LP_L	6F-cW_LP_L	7F-cW_LP_L	8F-cW_LP_L
cW_LP_L	Y[b]			Y	Y	Y	Y	Y
cW_LV_L	Y	Y	Y		Y	Y	Y	Y
cW_LA_L	Y	Y	Y		Y	Y	Y	Y
cW_LL_L	Y	Y	**NAS-43** **NAS-44**	Y	Y	Y	Y	Y
cW_LI_L	Y	**NAS-45**	Y **(NAS-51)**		Y	Y	Y	Y
cW_LF_L	**NAS-40** **NAS-42**			Y	Y		Y	
cW_LW_L	Y **(NAS-41** **NAS-50)**							
cW_LP_D	Y	Y	Y		Y	Y	Y	Y
cW_DP_D	Y	Y	Y	Y		Y	Y	
cW_DP_L			Y			Y		Y
6Cl-cW_LP_L				Y		Y		Y
7Cl-cW_LP_L				**NAS-28**	Y			Y
8Cl-cW_LP_L					Y	Y		
5F-cW_LP_L	Y		Y	Y	**NAS-46**	Y	Y	Y
6F-cW_LP_L	Y	Y	Y		Y	**NAS-47** **ASP-5**	Y	Y
7F-cW_LP_L	Y		Y		Y	Y	**NAS-27**	Y
8F-cW_LP_L	Y		Y		Y	Y	Y	**NAS-48** **NAS-49**

[a] Substrates in the row and column are for acceptance by the upper and lower pockets of Nas_{F5053}, respectively.
[b] Y indicates the reaction can produce cross-dimerized HTDKPs. Except for **NAS-41**, **NAS-50** and **NAS-51** characterized by NMR, other cross-dimerized products are only confirmed based on HR-MS analysis.

combining the results of sequence alignment and crystal structural analysis, we assumed that residues 387 and 388 play key roles in substrate selectivity.

To validate this assumption, three protein variants were generated with mutants at positions 387 and 388 in both P450s Nas_{F5053} and Nas_{AO}, and the activity of resulting mutant proteins was tested. For Nas_{F5053}, we exchanged these two residues with the corresponding amino acids of Nas_{AO} and TtpB2, resulting in Nas_{F5053}-F387A and Nas_{F5053}-F387V-F388I, respectively. Similarly, the phenylalanine at the position of 387 in Nas_{F5053} was also introduced into Nas_{AO} to create Nas_{AO}-A387F. To our gratification, Nas_{F5053}-F388A showed activity towards cW_LF_L and produced two self-dimerized products, **NAS-40** and **NAS-42** (Figure 2, Figure S2 and Table S3). In addition, Nas_{AO}-A388F lost its original catalytic activity and became unable to accept cW_LF_L as its substrate (Figure S2). These results clearly confirm that F387 is a pivotal factor for the restriction of binding steric hindered substrates in the upper binding pocket. In contrast to Nas_{F5053}-F387A, the mutant generated by copying the residues of TtpB2 into Nas_{F5053} (Nas_{F5053}-F387V-F388I) failed to accept cW_LF_L and assaying their activities. To our delight, one mutant Nas_{F5053}-F387G tolerated two new DKPs cW_LL_L and cW_LI_L to produce two cW_LL_L-derived products **NAS-43** and **NAS-44** and one cW_LI_L-derived product **NAS-45** (Figure 2; Figure S2 and Table S4-S6). More so, Nas_{F5053}-F388N was found to accept 7Cl-cW_LP_L for self-dimerization and produce **NAS-28** (Figure 2 and Figure S2).

The discovery of Nas_{F5053}-F388N is inspiring as the introduction of halogen atoms into natural products is an important way to create structural diversity by various coupling reactions.[6] Considering fluorine is important to improve the metabolic stability and bioactivity of molecules,[7] we were interested in exploring the possibility of the resulting mutants to accept fluorinated cyclodipeptide substrates. By using the fluorine-substituted L-tryptophan, we synthesized four 5/6/7/8F-cW_LP_L substrates (**S29**-**S32**, Figure S3). However, screening them against the mutant libraries of Nas_{F5053}-F388X as well as Nas_{F5053}-F387X failed to identify active mutants, indicating that further unknown residues remain to be discovered. When carefully analyzing the residues around the upper substrate binding pocket of Nas_{F5053}, residue E73 attracted our attention. E73 located on the αB'-αC loop was in close proximity to the substrate and may affect the ways that

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the halogenated substrate enters the active pockets. Saturation mutagenesis on this position and screening the activity of mutants allowed the identification of the mutant E73S, which could accept all the four fluorine-substituted 5/6/7/8F-cW$_L$P$_L$ substrates to indicated that at least 81 HTDKPs could be identified, which will greatly increase the structural diversity of HTDKPs. Inspired by the broad substrate scopes exhibited by these three mutants, we further constructed two combined variants (F387A-F388N-E73S

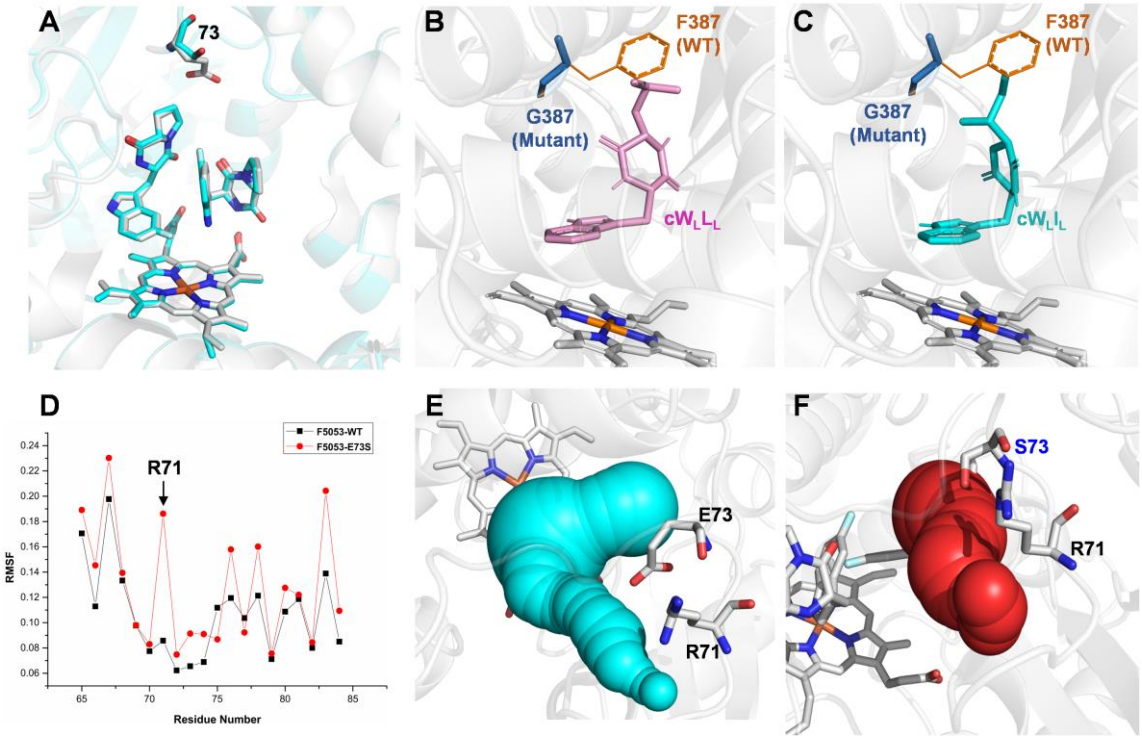

Figure 4. Analysis of the substrate specificity of the upper binding pocket in Nas$_{F5053}$-WT and mutant enzymes. A) Superposition of the active sites of cW$_L$P$_L$ bonded Nas$_{F5053}$-WT (grey, PDB:**6VXV**) and 6F-cW$_L$P$_L$ bonded Nas$_{F5053}$-E73S (cyan, PDB:**8HNZ**); B) Docking result of cW$_L$L$_L$ into apo-F387G mutant (PDB:**8HO1**); C) Docking result of cW$_L$I$_L$ into apo-F387G mutant (PDB:**8HO1**); D) RMSF values of the loop region (residues from 65 to 84) in MD simulations; E) The curved tunnel found in Nas$_{F5053}$ WT (PDB:**6VXV**); F) The new tunnel found in Nas$_{F5053}$-E73S mutant (PDB:**8HNZ**).

result in six new self-dimerization products, including one 5F-cW$_L$P$_L$ dimerization product (**NAS-46**), two 6F-cW$_L$P$_L$ dimerization products (**NAS-47** and **ASP-5**), one 7F-cW$_L$P$_L$ dimerization product (**NAS-27**) and two 8F-cW$_L$P$_L$ dimerization products (**NAS-48** and **NAS-49**) (Figure 2; Figure S2 and Table S7-S11). The successful identification of the pivotal residue E73 was again clearly very important to provide an effective way to expand the structural diversification of HTDKPs using substituted DKP.

By engineering three residues (Phe387, Phe388 and Glu73), we successfully expand the substrate specificity of the upper substrate binding pocket of Nas$_{F5053}$ and generated 12 self-dimerized products. Considering that each of the HTDKPs is formed by two units of cWXs, we were interested in combining the newly discovered eight cWXs (cW$_L$F$_L$, cW$_L$I$_L$, cW$_L$L$_L$, 7Cl-cW$_L$P$_L$ and 5/6/7/8F-cW$_L$P$_L$) with other cWXs to further increase the structural diversity of HTDKPs. Each of these eight substrates was individually co-fed with one of the remaining 32 cWXs (in total 256 reactions) into the MC^{2}155 strains containing the corresponding P450 mutants. LC-HRMS analysis revealed that cross-dimerized products could be detected in 81 combinations (Table 1 and Figures S4). Among these combinations, two representative reactions (F387A/cW$_L$F$_L$+cW$_L$W$_L$ and F387G/cW$_L$I$_L$+cW$_L$L$_L$) were further investigated. Three cross-dimerized products **NAS-41**, **NAS-50** and **NAS-51** were successfully isolated and characterized by 1D and 2D NMR (Figure 2, Figure S2 and Table S12-S13). The simultaneous production of **NAS-41** and **NAS-50** in one co-feeding experiment and F387G-F388N-E73S) to potentially improve the catalytic efficiencies and accept more other DKPs. However, both of them could not express in *E.coli* and *Mycobacterium* systems although different expression conditions were tried.

Finally, in order to have a better understanding of the molecular basis of the new substrate selectivity demonstrated by different mutants, we tried to solve the crystal structures of the related mutants. Despite being unable to obtain structures of the F387A and F387N mutants, we were fortunate to get four high-resolution structures of the other two Nas$_{F5053}$ variants, including the substrate-free F387G (PDB: **8HO1,** 2.0 Å), E73S with one 5F-cW$_L$P$_L$ in the upper pocket (PDB: **8HNY,** 2.1 Å), E73S with two 6F-cW$_L$P$_L$s (PDB: **8HNZ,** 1.5 Å), and E73S with one 8F-cW$_L$P$_L$ in the upper pocket (PDB: **8HO0,** 1.7 Å) (Figure S5, Table S15). Comparing the apo-F387G structure with our previously reported apo-wild type structure (PDB: **6W0S**), and the E73S/ 6F-cW$_L$P$_L$ bonded structure to wild type Nas$_{F5053}$/ cW$_L$P$_L$ bonded structure (PDB: **6VXV**), we found that the overall protein structures, the heme and the substrates overlap very well, resulting very low RMSD vaules, only 0.20 Å and 0.16 Å respectively (Figure 4A, Figure S6). These findings indicate that all these structures share a common starting conformation.

To get more detailed information, two cW$_L$L$_L$ and two cW$_L$I$_L$ substrates were further individually docked into the active pockets of the apo structure of F387G. Consistent with the wide type Nas$_{F5053}$/ cW$_L$P$_L$ complex structure, all the substrates in the lower pocket of F387G showed U-shaped conformations. Besides, their

N_1-H are close to the heme, and the distances between their N_{10} and C_2 are in the reachable range around 3.2 Å (Figure S7) which is necessary for the radical generation at N_1 and migration, and intramolecular Mannich reaction between N_{10} and C_2 to form the pyrroloindoline C_3 radical.[2a] Analyzing the conformations of the upper substrates, we found that the L-leucine and L-isoleucine moieties of the corresponding DKPs are all oriented towards the F387G. Mutating F387 into the small residue glycine significantly reduced the steric hindrance around the pocket, thus enabling the mutant to recognize the bulky substrates cW_LI_L and cW_LL_L (Figure 4B-4C).

For mutant E73S, its unusual substrate specificities were not able to be deduced via comparing the crystal structures. Thus, we employed molecular dynamics (MD) simulations based on the two 6F-cW_LP_Ls bonded complex structure (PDB: **8HNZ**). As E73 is located on the αB'-αC loop, we mainly focused on the impact of mutation on this loop region (residues from 65 to 84). Most of the residues showed higher RMSF values in E73S, suggesting that the loop region is more flexible than that of the wild type enzyme. Especially, R71 which is very close in space to E73, showed the biggest difference in RMSF values among all loop residues (Figure 4D). For a deeper understanding of these changes, tunnel analysis was performed and we found that the substrate tunnel to upper pocket in the wild type was very curved due to the presence of R71 and E73 (Figure 4E). However, a new tunnel which located near S73 and R71 was detected in the E73S mutant (Figure 4F). We speculated that this new tunnel is likely to facilitate the passage of fluorinated substrates, and its generation should be attributed to the reduced steric hindrance and the increased flexibility of the R71 site after convert glutamate to serine.

It is very interesting that Nas_{F5053} employs different mechanisms in controlling the selection of DKP substrates in the upper site. Especially, the discovery of the regulatory mechanisms of E73S in substrate tunnel significantly broadens our way in tailoring these fascinating enzymes. Previously, only three upper DKPs could be recognized by Nas_{F5053} and most other related HTDKP-forming P450s, resulting in only 46 HTDKPs identified during the past decades.[2,4a-d] In this study, we made a big step forward in the numbers of both tolerated upper DKPs (another eight cWXs became acceptable) and formed HTDKPs (at least 93 HTDKPs were identified here, twice as many as in the past). These new residues together with our previously elucidated the regio- and stereospecificity-conferring residues,[4c] play the critical role in shaping the highly complicated reaction specificity of the Nas_{F5053}-like P450 dimerases, and can be engineered coordinately to further create the structural diversity of HTDKPs.

In recent years, a number of pyrroloindoline alkaloids bearing with nucleobases at C_3 position were also revealed .[8] Interestingly, the three residues (E73, F387 and F388) revealed here were replaced by Tyr, Ile and Lys in all the P450s that could accept nucleobases. Besides, the residues on the αK-β3 loop (ranging from 284 to 295: NLSIGDALPRIA; according to the Nas_{F5053} numbering) are all conserved in the nucleobase-tolerating P450s but different from the HTDKP-forming P450s. These clues indicate that the three residues as well as the loop may play critical roles in nucleobases selectivity. C_3-monoindole substituted pyrroloindolines, such as leptosins,[9] T988s,[10] gliocladines,[11] and bionecins,[12] are another kind of interesting natural product. Although their biosynthetic pathways are still unknown, engineering the substrate selectivity of Nas_{F5053}-type P450s for indole and its analogs have the chance to get access to these products.

In summary, we have revealed three key resides (F387, F388 and E73) that are critical for substrate specificity in the upper pocket in P450 dimerases. Structural and molecular dynamics analysis revealed that F387 and E73 determine the substrate specificity via controlling substrate binding and entering into the upper pocket, respectively. Engineering of these residues in the Nas_{F5053} enable tolerance of eight further substrates cW_LF_L, cW_LI_L, cW_LL_L, 7Cl-cW_LP_L and 5/6/7/8F-cW_LP_L to generate 12 self-dimerized HTDKPs and at least 81 cross-dimerized HTDKPs between the eight DKPs and other remaining DKPs which significantly increase the numbers and structurally diversity of HTDKPs. The successful identification of the regulatory residues in the upper pocket provides a new way to further enormously expand the structural diversity of HTDKPs and potentially other kinds of pyrroloindoline alkaloids.

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Keywords: biosynthesis • biocatalysis • protein engineering • dimerization • P450

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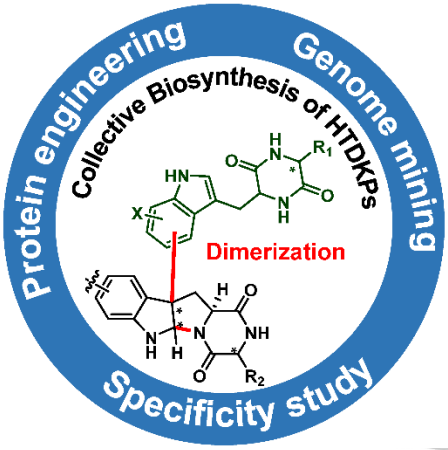

The biosynthesis of heterodimeric tryptophan-containing diketopiperazines (HTDKPs) is restricted by the limited substrate scopes of the HTDKP-forming P450 dimerases. Engineering of three important residues (F387, F388, and E73) in Nas_{F5053} significantly expanded its substrate specificity and enabled the collective biosynthesis of at least 93 HTDKPs.