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Engineering a Highly *meta*-Selective Biphenyl Monooxygenase for the Biosynthesis of High-Value Polymer Precursors

Christopher J. Vavricka,^{*,#} Takeshi Matsui,^{*,#} Satoshi Yuzawa, Hiroto Ida, Ryota Hidese, Akihiko Kondo, and Tomohisa Hasunuma^{*}



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ABSTRACT: The *meta*-selective C–H functionalization of aromatic substrates lacking *meta*-directing electron-withdrawing groups remains challenging. To engineer the regioselective enzymatic conversion of biphenyl (BP) to 3,3'-dihydroxybiphenyl (33DHBP), an industrial precursor to high-performance polymers, substrate positioning within the active site must be precisely controlled. Here, we report the regioselective catalytic promiscuity engineering of toluene/*o*-xylene monooxygenase (ToMO) to achieve sequential, *meta*-selective hydroxylation of the non-natural biaryl BP, where distinct ToMO variants were engineered to catalyze *meta*-selective hydroxylation of BP, 3-hydroxybiphenyl (3HBP), or both. Variants to catalyze the *meta*-selective hydroxylation of BP were designed using an automated docking workflow, with predicted binding poses consistent with the observed regioselectivity; this resulted in the development of the I100V-E103V-F205G variant that hydroxylated BP with 100% apparent *meta*-selectivity. Additional substitutions, especially L268A and L402A, were introduced to widen the long hydrophobic active-site access channel, further improving *meta*-selective BP hydroxylation in *Pseudomonas putida*. Variants to catalyze the *meta*-selective hydroxylation of 3HBP were selected through a structure-based residue scan of 437 active-site substitutions, resulting in the identification of the I100V-E103V-F176H variant that hydroxylated 3HBP with over 90% apparent *meta*-selectivity and no activity toward BP. In addition, the combined I100V-E103V-I162Y-F205G variant hydroxylated 3HBP with over 90% apparent *meta*-selectivity and improved hydroxylation of BP. This study demonstrates structural and computational monooxygenase engineering for the regioselective hydroxylation of non-natural biaryls, enabling the production of 33DHBP as a valuable precursor to specialized polymers.

KEYWORDS: monooxygenase engineering, *meta*-selective C–H hydroxylation, computational enzyme design, biosynthesis, biphenyl-derived polymers

INTRODUCTION

Development of sustainable *meta*-selective hydroxylation methods is needed to produce phenolic precursors of pharmaceuticals, agrochemicals, and specialty polymers. However, many aromatic substrates contain *meta* C–H bonds that are intrinsically difficult to activate.¹ The development of highly *meta*-selective monooxygenases would therefore improve the production of functionalized aromatic compounds, while avoiding the use of protecting groups, harsh oxidants, and metal-catalyzed C–H activation.

Addition of 3,3'-dihydroxybiphenyl (33DHBP) residues can be applied to improve the hardness and mechanical resistance of various polymers.² 33DHBP can be synthesized by *meta*-hydroxylation of biphenyl (BP). However, *meta*-selective hydroxylation of BP (Figure 1) is challenging because the *ortho* and *para* positions are more reactive due to their higher electron density.^{3,4} Accordingly, common *meta*-selective hydroxylation techniques require pre-functionalization or the addition of directing groups. For example, *meta*-selective hydroxylation of aromatic rings often

requires bulky directing groups.¹ While *meta*-selective hydroxylation of BP to produce 3-hydroxybiphenyl (3HBP) has been achieved using a complex organic synthesis method,⁵ we are unaware of any report describing hydroxylation of 3HBP to produce valuable 33DHBP (Figure 1).

While regioselective enzymatic conversion of BP to 33DHBP has not previously been reported, other studies have indicated that enzymes for *meta*-hydroxylation of BP should exist in nature.^{6–8} For example, an early report showed that actinomycetes are capable of converting BP to 3HBP;⁶ however, the

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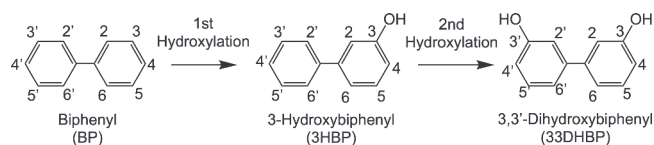


Figure 1. Sequential *meta*-selective hydroxylation cascade to convert BP to 3HBP and 33DHBP.

exact enzyme responsible for *meta*-selective hydroxylation remains unknown. Other studies revealed nonselective hydroxylation of BP at *ortho*, *meta*, and *para* positions catalyzed by an oxygenase from *Hydrogenibacillus* sp.⁷ BP *meta*-hydroxylation has also been shown to be catalyzed by cytochrome P450 (P450); however, the P450 sequences encoding this activity have not been reported.^{8,9} In addition, toluene/*o*-xylene monooxygenase (ToMO) is known to catalyze *meta*-hydroxylation of toluene¹⁰ and naphthalene,¹¹ compounds that are structurally similar to BP. Accordingly, P450 and ToMO are promising scaffolds for engineering novel enzymes capable of catalyzing the *meta*-selective hydroxylation of BP.

In this study, we used rational structure-based approaches to engineer regioselective hydroxylase activity into P450 and ToMO enzyme scaffolds. Our computational workflow enabled the *in silico* screening of predicted binding free energies for target

substrates across several hundred enzyme variants. After multiple iterations of variant design and testing of over 60 ToMO variants, hydroxylation of BP with 100% apparent *meta*-selectivity was achieved using the engineered I100V-E103V-F205G variant, while hydroxylation of 3HBP with over 90% apparent *meta*-selectivity was achieved using the engineered I100V-E103V-F176H, I100V-E103V-I162F-F176H, I100V-E103V-I162Y-F205G, and I100V-E103V-I162F-F205G variants.

RESULTS AND DISCUSSION

Selection of ToMO as an Optimal Monooxygenase Scaffold

Homo sapiens CYP2A13 (UniProt accession Q16696.3) was first selected as a monooxygenase scaffold to attempt the engineering of *meta*-selective BP hydroxylation because it is known to hydroxylate BP, although CYP2A13 is reported to hydroxylate only the *ortho* and *para* positions.¹² For BP hydroxylation, CYP2A13 and designed active-site variants were produced in *Pseudomonas putida* in combination with *H. sapiens* NADPH-cytochrome P450 reductase isoform 1 (HsCPR), as detailed in the [Supporting Information](#). However, only *para*-selective hydroxylation of BP was observed when testing CYP2A13 ([Figure 2A](#)) and its F209L, T305S, and L370V active-site variants ([Figure S1](#)). Therefore, we considered other

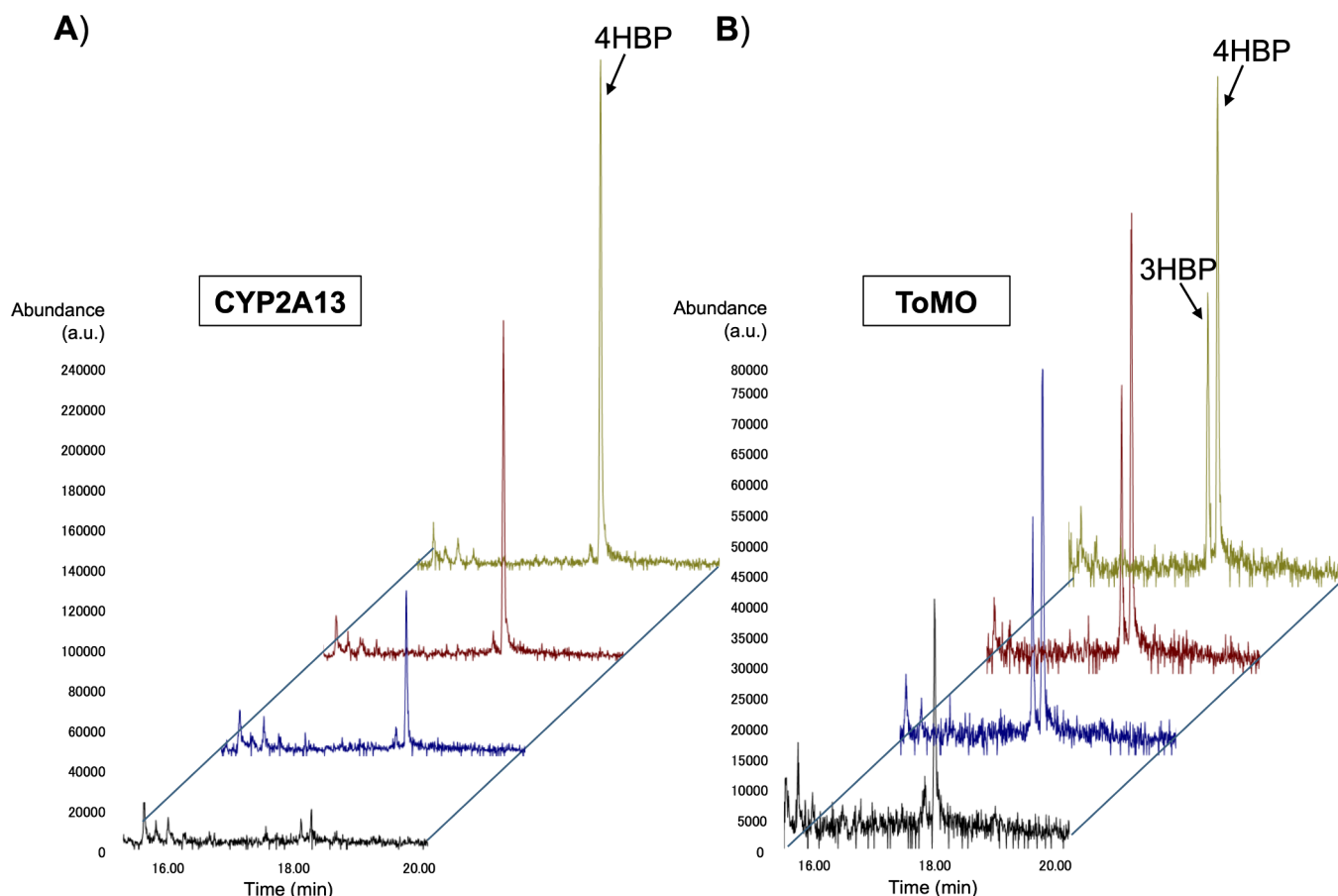


Figure 2. Analysis of enzymatic hydroxylation products of BP. (A) Stacked GC-MS extracted-ion chromatograms (*m/z* 170) show 4-hydroxybiphenyl (4HBP) as the enzymatic product of wild-type (WT) CYP2A13. (B) Stacked GC-MS extracted-ion chromatograms (*m/z* 170) show 3-hydroxybiphenyl (3HBP) and 4HBP as the enzymatic reaction products of the toluene/*o*-xylene monooxygenase (ToMO)-I100V-E103V scaffold; I100V and E103V substitutions were included in the catalytic alpha subunit TouA of our ToMO scaffold. The GC-MS signal is reported as ion abundance in arbitrary units (a.u.). Samples were collected at 0 h (black), 3 h (blue), 10 h (red), and 23 h (yellow) after incubation with BP.

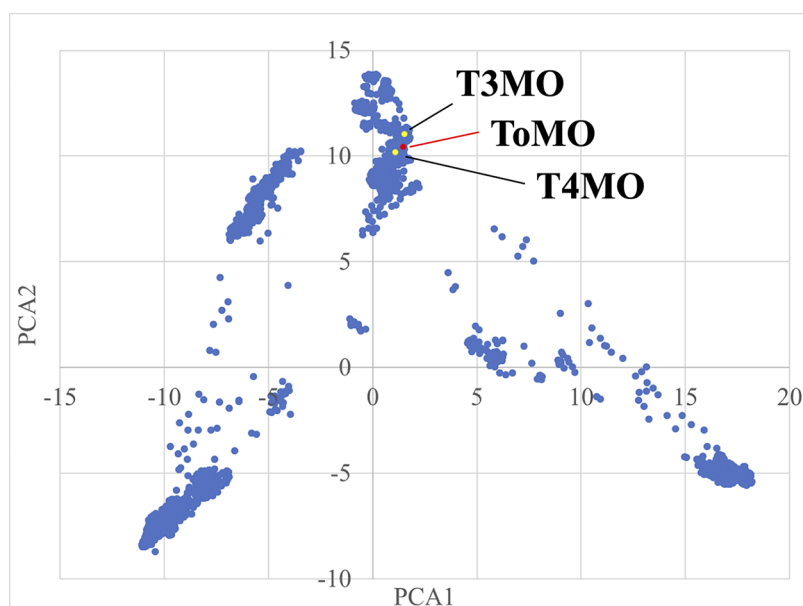


Figure 3. Clustering-based selection of monooxygenase catalytic subunits. The protein sequence of the ToMO catalytic subunit TouA (labeled as ToMO) clustered together with sequences of *Ralstonia pickettii* toluene 3-monooxygenase (T3MO, UniProt accession Q51939) and *Ectopseudomonas mendocina* toluene 4-monooxygenase (T4MO, UniProt accession Q00456) catalytic subunits, according to our principal component analysis (PCA)-based classification method MUSASHI (Multiple-Sequence Alignment-based protein Selection via clustering using High-dimensional analysis). The sample labeled as ToMO corresponds to the sequence of *Pseudomonas* sp. OX1 TouA (GenBank accession AAT40431.1). ToMO, T3MO, and T4MO catalytic subunits share 67–68% sequence identity among each other.

monooxygenases with better potential to catalyze the *meta*-selective hydroxylation of BP.

ToMO is a protein complex consisting of multiple subunits. The ToMO complex is encoded by the gene cluster *touABCDEF* that includes *touA*, which encodes the catalytic alpha subunit TouA; *touC*, which encodes ferredoxin; *touF*, which encodes NADH-ferredoxin oxidoreductase; and other essential genes.¹⁰ *Pseudomonas stutzeri* ToMO was selected as an optimal scaffold for engineering the *meta*-selective hydroxylation of BP based on the previous reports of *meta*-hydroxylation (Figure 2B),^{10,11,13} as well as our recently established clustering method, Multiple-Sequence Alignment-based protein Selection via clustering using High-dimensional analysis (MUSASHI), for enzyme selection (Figure 3).¹⁴ The MUSASHI method was applied to convert multiple-sequence alignments of TouA-related sequences into binary representations, perform principal component analysis (PCA) to reduce dimensionality, and cluster the resulting data to group related enzymes, enabling selection of representative enzymes with common properties within clusters and diversity across clusters.

The selected ToMO, T3MO, and T4MO catalytic subunits were produced in *P. putida* together with TouB–TouF of the ToMO complex, but monooxygenase activity was only detected when using the ToMO catalytic subunit TouA. I100V and E103V substitutions were included in the selected TouA scaffold, based on a report by Notomista et al.¹³ Both T3MO and T4MO catalytic subunits were insoluble when expressed with TouB–TouF (Figure S2), indicating that the TouB–TouF specifically stabilizes the soluble form of TouA. The complete ToMO complex was able to hydroxylate BP to produce 3HBP and 4-hydroxybiphenyl (4HBP) (Figure 2B).

Engineering ToMO for *meta*-Selective Hydroxylation of BP

To rationally select active-site substitutions that may promote the *meta*-selective hydroxylation of BP by ToMO, the catalytic subunit TouA was docked with BP, and eight target active-site residues were identified (Figure 4, Tables 1 and S1). The following TouA substitutions were then designed by considering amino acids with similar sizes and properties, as well as variable residues found at corresponding positions in T3MO and T4MO: I100V, E103V, E103L, E103I, E103Q, Q141C, I162Y, I162F, F196H, T201S, F205I, F205L, F205Y, and F269Y. F205G¹⁰ and F196A¹¹ were selected based on two previous reports. According to a report by Notomista et al., where I100V and E103V substitutions were found to promote the *meta*-selective hydroxylation of toluene,¹³ we decided to generate all substitutions in TouA-I100V-E103V as the enzyme scaffold (Figure S3).

Relative to that of the wild-type TouA and that of the enzyme scaffold TouA-I100V-E103V, the I100V-E103V-F205G variant showed an optimal predicted distance from the BP *meta* position to the Fe cluster, together with a relatively low predicted binding free energy for BP (Table 1); in accordance with the docking results, the I100V-E103V-F205G variant showed 100% apparent *meta*-selective production of 3HBP (Figure 4B and Table 1). The I100V-E103V-I162Y, I100V-E103V-I162F, and I100V-E103V-F205Y variants also showed improvements in the *meta*-selective hydroxylation of BP, relative to that of the TouA-I100V-E103V scaffold. Notably, the I100V-E103V-I162Y variant showed the best overall improvement in BP hydroxylation (Table 1) and naphthalene hydroxylation (Table S1). The I100V-E103V-I162Y variant was able to convert 154.2 μ g BP to 23.5 μ g hydroxybiphenyl in the 5 mL reaction scale of this study.

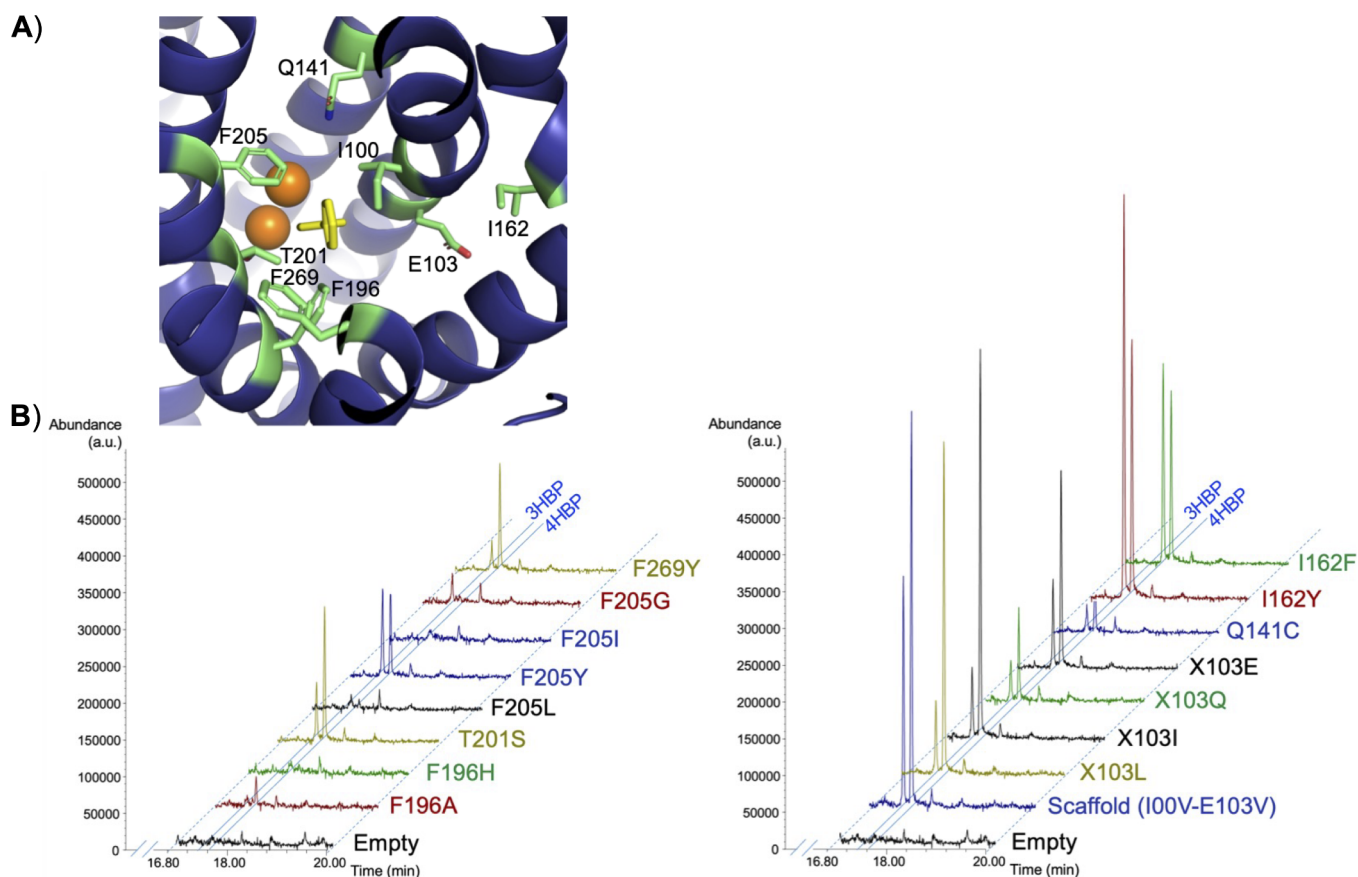


Figure 4. Structure-based engineering of TouA for *meta*-selective hydroxylation of BP. (A) Docked BP in the active site of wild-type TouA (PDB ID 1T0Q), the catalytic subunit of ToMO. The following eight active-site residues (colored lime green) were selected as possible determinants of aromatic hydroxylation regioselectivity: I100, E103, Q141, I162, F196, T201, F205, and F269. (B) GC–MS extracted-ion chromatograms (m/z 170) show 3HBP and 4HBP as the enzymatic reaction products of ToMO variants ($n = 1$). The GC–MS signal is reported as ion abundance in arbitrary units (a.u.). Quantified amounts of 3HBP and 4HBP, based on peak areas, are listed in Table 1. I100V and E103V substitutions were included in our TouA scaffold and all tested TouA variants. For X103L, X103I, X103Q, and X103E substitutions, X represents the amino acid at position 103, which is E in wild-type TouA and V in the scaffold. “Empty” indicates a negative control with an expression vector containing no ToMO genes. The I100V-E103V-F196H variant could not be produced as a soluble protein, as shown in Figure S3.

While 100% apparent *meta*-selectivity could be achieved using the I100V-E103V-F205G variant, this was accompanied by reduced activity. In a previous study,¹³ the *meta*-selective I100V-E103V variant also showed significantly reduced (3- to 9-fold) activity for phenol, benzene, toluene, and *o*-xylene substrates. These results indicate that there is a tradeoff between *meta*-selective hydroxylation and overall catalytic activity.

To provide insight into the structural basis of the observed regioselectivity of the I100V-E103V scaffold and the highly *meta*-selective I100V-E103V-F205G variant, molecular dynamics (MD) simulations of wild-type TouA, the I100V-E103V scaffold, and the I100V-E103V-F205G variant, all in complex with BP, were performed (Figure S4). Analysis of the MD trajectory of the I100V-E103V-F205G variant showed conformations with more favorable *meta*-to-*para* distance relationships during the early stages of the simulation, particularly within the first 50 ns (Table S2). Although BP gradually adopted conformations further from the diiron center after 100 ns, the I100V-E103V-F205G variant maintained

more favorable *meta*-selective distance metrics than wild-type TouA throughout the 200 ns simulations (Table S2).

To further investigate the structural basis of the *meta*-selectivity, the position of BP relative to residue 205 was analyzed. BP adopted conformations substantially closer to residue 205 in both the I100V-E103V scaffold and the I100V-E103V-F205G variant compared to wild-type TouA, with the closest distances observed in the I100V-E103V-F205G variant, which lacks the bulky F205 side chain (Table S2). In addition, BP adopted distinct orientations relative to the diiron center, as shown by differences in the active-site orientation angle of BP among the three systems (Table S2). Together, these results indicate that the F205G substitution influences both the position and orientation of BP within the active site, leading to the adoption of conformations that are favorable for *meta*-hydroxylation.

Despite the improved *meta*-selectivity of the engineered variants, overall catalytic activity remained reduced. Therefore, engineering a hydrophobic active-site access channel was next explored to further improve the activity of the *meta*-selective TouA variants.

Table 1. Predicted Binding Parameters and Experimental Hydroxylation Activity TouA Active-Site Variants with BP Substrate^a

enzyme / variant	Vina affinity (kcal/mol)	meta-Fe dist. (Å)	para dist. / meta dist.	[HBP] (3 h, μM)	3HBP fraction (%)
wild-type (I100Q)	−5.83	4.3	0.99	N.T.	N.T.
I100V	−6.85	5.8	0.94	9.83 ± 5.11	32
I100V-E103V (scaffold)	−6.33	6.0	0.95	7.78 ± 1.07	38
I100V-E103I	−6.65	6.0	0.93	18.49 ± 12.59	17
I100V-E103L	−6.95	5.8	0.94	12.57 ± 3.93	19
I100V-E103Q	−6.82	5.8	0.94	15.14 ± 14.31	36
I100V-E103V-Q141C	−6.92	5.8	0.95	7.15 ± 5.49	35
I100V-E103V-I162F	−6.96	5.8	0.94	5.15 ± 1.10	56
I100V-E103V-I162Y	−7.23	5.8	0.95	27.61 ± 8.25	61
I100V-E103V-F196A	−7.05	3.3	1.07	0	N.T.
I100V-E103V-F196H	−5.87	6.3	0.93	N.T. ^b	N.T. ^b
I100V-E103V-T201S	−7.62	6.5	0.93	4.53 ± 2.67	35
I100V-E103V-F205G	−7.18	4.0	1.10	1.13 ± 0.22	100
I100V-E103V-F205I	−7.60	4.0	1.11	0	N.T.
I100V-E103V-F205L	−5.66	6.0	0.94	0	N.T.
I100V-E103V-F205Y	−5.81	5.7	0.96	4.90 ± 1.56	50
I100V-E103V-F269Y	−7.12	6.0	0.95	3.06 ± 1.07	22

^aAverage distances are listed. Abbreviations: Vina, AutoDock Vina; BP, biphenyl; 3HBP, 3-hydroxybiphenyl; 4HBP, 4-hydroxybiphenyl; HBP, hydroxybiphenyl (3HBP and 4HBP); dist., distance; h, hour; N.T., not tested. ^bI100V-E103V-F196H could not be produced as a soluble protein (Figure S3).

Engineering the Hydrophobic Active-Site Access Channel of ToMO

TouA, the catalytic subunit of ToMO, contains a long hydrophobic active-site access channel partially comprising hydrophobic residues W167, A171, L268, L272, W338, W344, L393, and L402 (Figure 5A). TouA-I100V-E103V scaffold variants with increased active-site access channel size were designed by substituting channel residues with smaller hydrophobic residues. As a result, the I100V-E103V-W167F, I100V-E103V-W167L, I100V-E103V-L268A, I100V-E103V-L268G, I100V-E103V-L272V, I100V-E103V-W338F, and I100V-E103V-W338L variants showed higher activity compared to that of TouA-I100V-E103V for hydroxylation of BP (Figure 5B), and 7-ethoxycoumarin, but not for naphthalene (Figure 5S). The I100V-E103V-W167L variant was able to convert 154.2 μg BP to 83.4 μg hydroxybiphenyl (49.0% yield) in the 5 mL reaction scale of this study. When combined with the *meta*-selective substitution F205G in the TouA-I100V-E103V scaffold, W167F and W167L did not lead to an improvement in overall activity (Figure 5C). Fortunately, L402A and L268A substitutions led to significant improvements in *meta*-selective BP hydroxylation activity, when combined with F205G in the TouA-I100V-E103V scaffold (Figure 5C,D). In the 5 mL reaction scale, the highly *meta*-selective I100V-E103V-F205G-L402A variant was able to convert 154.2 μg BP to 6.4 μg hydroxybiphenyl, a production level over 2-fold higher than that of the I100V-E103V-F205G variant.

Engineering ToMO for 33DHBP Production

To select optimal substitutions that may promote *meta*-selective hydroxylation of 3HBP to produce the target product 33DHBP, 437 single amino acid substitutions of the TouA active site were explored based on predicted binding to 3HBP (Supporting Information). In this virtual screening, binding free energy was estimated for 3HBP bound in two correct (with the 3-OH group far from the iron cluster) and two incorrect (with

the 3-OH group close to the iron cluster) conformations (Table 2 and Figure 6A). The following 12 substitutions were then selected based on favorable binding free energies for the correct conformations of 3HBP: V100H, E104Q, E134I, E134L, F176D, F176C, F176G, F176H, F176I, F176M, L192R, and L192G (Table 2 and Supporting Information). Selected substitutions were tested in the TouA-I100V-E103V scaffold for the ability to hydroxylate 3HBP; as a result, *meta*-selectivity for 3HBP could be improved (Figure 6B and Table 3). The I100V-E103V-F176H variant showed over 90% apparent *meta*-selectivity for hydroxylation of 3HBP (Figure 6B and Table 3), with no observed activity toward BP (Table S3). F176P was also selected based on predicted binding free energy (Supporting Information); however, TouA-I100V-E103V-F176P could not be produced as a soluble protein (Figure S6).

The I100V-E103V-F205G variant was also found to hydroxylate 3HBP with *meta*-selectivity (Figure 7 and Table 4), although the structure-based residue scan did not indicate an increased affinity for 3HBP with the F205G substitution (Supporting Information). The loss of favorable interactions with the BP substrate and the aromatic F205 side chain may explain why predicted binding free energy for F205G was higher than that of wild-type TouA; however, the correct conformations still showed lower predicted binding free energies compared to the incorrect conformations for the F205G substitution (Supporting Information).

The following pairs of substitutions were next designed by combining successful *meta*-selective substitutions in the TouA-I100V-E103V scaffold: F176H-F205G, I162Y-F176H, I162F-F176H, I162Y-F205G, and I162F-F205G. The I162Y-F176H, I162F-F176H, I162Y-F205G, and I162F-F205G substitution pairs resulted in highly *meta*-selective hydroxylation of 3HBP to produce 33DHBP (Figure 7 and Table 4). Compared to that of the TouA-I100V-E103V scaffold, the I100V-E103V-I162Y-F205G and I100V-E103V-I162F-F205G variants produced higher amounts of the target product 33DHBP, with over

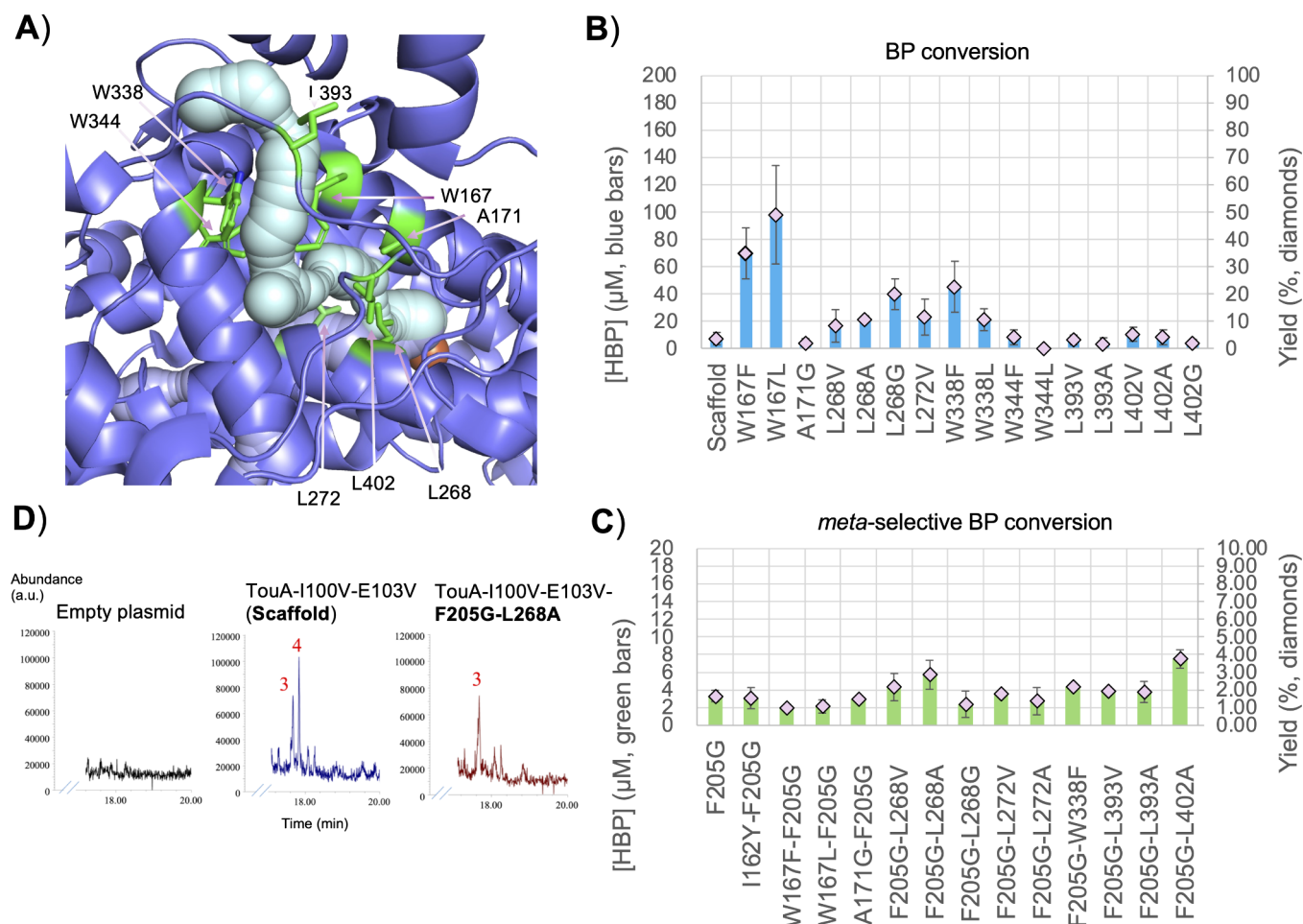


Figure 5. Engineering the hydrophobic active-site access channel of TouA for improved BP hydroxylation. (A) Structure of TouA with the hydrophobic active-site access channel visualized using CAVER and key tunnel residues shown in green. (B) Quantitative analysis of BP hydroxylation based on product fluorescence; here all TouA variants contain the I100V and E103V substitutions of the TouA scaffold. (C) Quantitative analysis of BP hydroxylation based on product fluorescence; here all TouA variants contain the I100V and E103V substitutions of the TouA scaffold, as well as the F205G *meta*-selective substitution. BP hydroxylation was quantified based on product fluorescence from three independent samples for each condition ($n = 3$). (D) TouA-I100V-E103V-F205G-L268A *meta*-selective production of 3HBP ($n = 1$); “3” indicates 3HBP, and “4” indicates 4HBP, as determined by GC-MS; the GC-MS signal is reported as ion abundance in arbitrary units (a.u.).

Table 2. Structure-Based Scoring of 12 Optimal Active-Site Variants for *meta*-Selective 3HBP Hydroxylation^a

substitution	correct 1 dAffinity	correct 2 dAffinity	incorrect 1 dAffinity	incorrect 2 dAffinity
I100H	−0.28	−0.30	0.04	0.03
E104Q	−0.05	−0.68	0.24	0.29
E134I	−0.36	−0.34	0.20	0.14
E134L	−0.46	−0.51	−0.21	0.02
F176D	−0.39	−0.38	0.14	0.24
F176C	−0.45	−0.45	0.08	0.14
F176G	−0.38	−0.41	0.08	0.14
F176H	−0.49	−0.57	0.10	0.14
F176I	−0.57	−0.53	−0.07	0.02
F176M	−0.76	−0.54	−0.09	0.05
L192R	−0.31	−0.19	0.11	0.05
L192G	−0.18	−0.28	0.01	0.29

^a“Correct 1”, “Correct 2”, “Incorrect 1”, and “Incorrect 2” 3HBP conformations are shown in Figure 6A.

90% apparent *meta*-selectivity based on LC-MS peak areas, and the I100V-E103V-I162Y-F205G variant also showed improved hydroxylation of BP (Table 4). Therefore, the I100V-E103V-I162Y-F205G variant can be used to efficiently catalyze both steps in the conversion of BP to 33DHBP.

CONCLUSIONS

This study demonstrates the rational design of a bacterial monooxygenase to catalyze sequential, *meta*-selective hydroxylation of non-natural biaryl substrates. While *meta*-selectivity could not be engineered into a P450 scaffold, ToMO proved to be a robust scaffold for engineering regioselective catalytic promiscuity through control of BP and 3HBP substrate positioning within the active site. Automated substrate docking and structure-based residue scanning across hundreds of TouA variants successfully guided the engineering of distinct enzymes to catalyze highly *meta*-selective hydroxylation of BP and 3HBP in *P. putida*. As a result, the TouA-I100V-E103V-F205G variant hydroxylated BP with 100% apparent

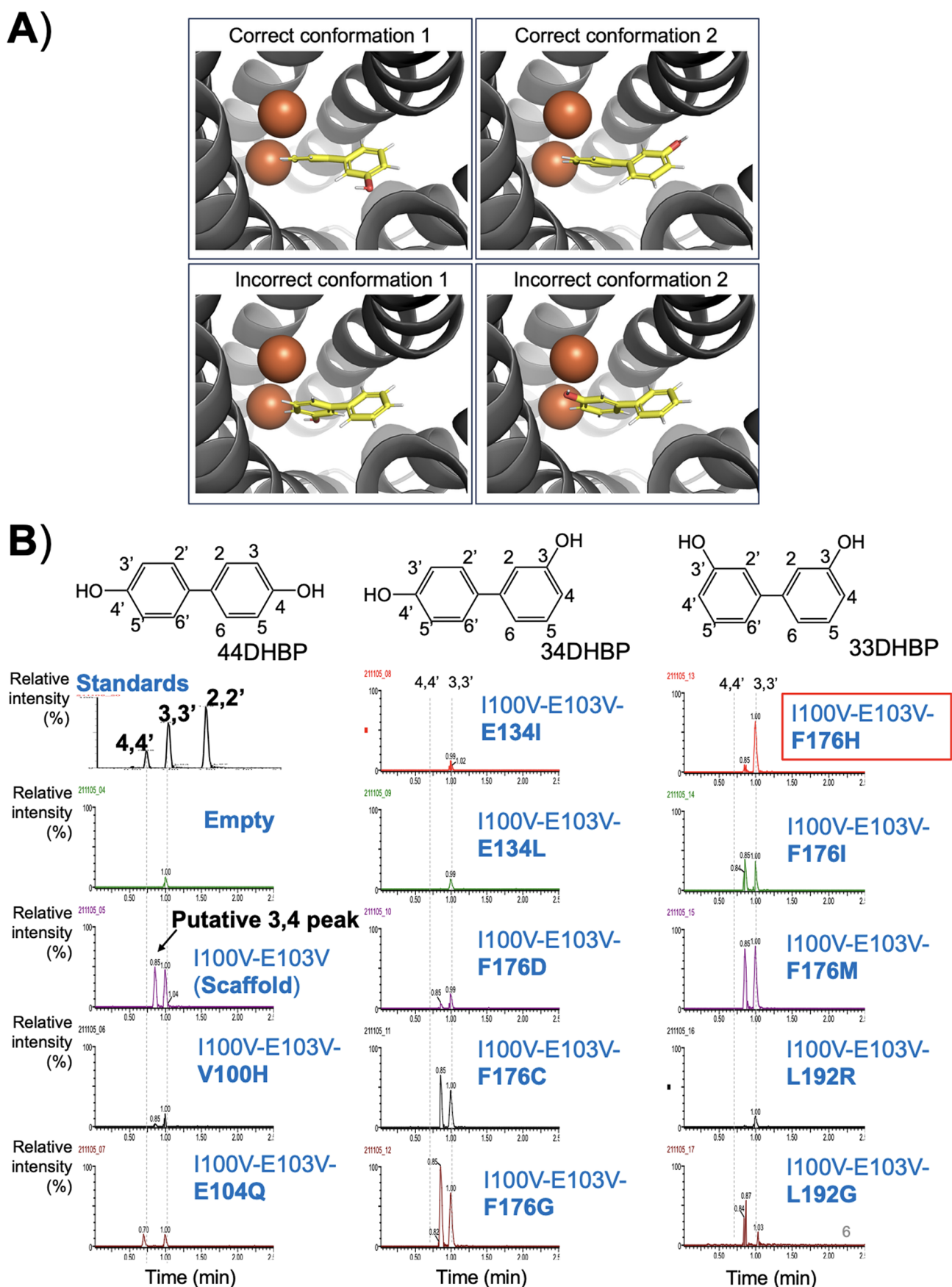


Figure 6. Hydroxylation of 3HBP by TouA active-site variants. (A) Four conformations of docked 3HBP in the active site of wild-type TouA (PDB ID 1T0Q), the catalytic subunit of ToMo. (B) Liquid chromatography–mass spectrometry (LC–MS) extracted-ion chromatograms ($[M-H]^-$, m/z

Figure 6. continued

185.061) indicate 33DHBP (indicated by “3,3'”) and 34DHBP (indicated by “Putative 3,4 peak”) as the enzymatic reaction products of ToMO ($n = 1$); I100V and E103V substitutions were included in the TouA scaffold. Retention times are indicated for each detected peak.

Table 3. Evaluation of 12 Selected Active-Site Variants for *meta*-Selective Hydroxylation of 3HBP^a

sample	[33DHBP] (8 h, μ M)	33DHBP fraction (%)
empty	<0.02	N.A.
I100V-E103V (Scaffold)	0.1	49
I100V-E103V-V100H	0	N.A.
I100V-E103V-E104Q	0.02	100
I100V-E103V-E134I	0	N.A.
I100V-E103V-E134L	0.02	100
I100V-E103V-F176D	0.02	80
I100V-E103V-F176C	0.22	61
I100V-E103V-F176G	0.29	37
I100V-E103V-F176H	0.26	94
I100V-E103V-F176I	0.08	44
I100V-E103V-F176M	0.34	50
I100V-E103V-L192R	<0.02	N.A.
I100V-E103V-L192G	0	22

^aAbbreviation: 33DHBP, 3,3'-dihydroxybiphenyl; h, hour; N.A., not applicable.

meta-selectivity, the TouA-I100V-E103V-F176H variant hydroxylated 3HBP with over 90% apparent *meta*-selectivity and no activity toward BP, and the TouA I100V-E103V-I162Y-F205G variant hydroxylated 3HBP with over 90% apparent *meta*-selectivity together with improved hydroxylation of BP. In the future, metabolic engineering and bioprocess optimization studies should be pursued to enable preparative- and industrial-scale production of 3HBP and 33DHBP.

In summary, this work establishes effective computational monooxygenase engineering strategies that can realize new-to-nature regioselective reactions and provides a sustainable biosynthetic route to valuable biaryl polymer precursors.

MATERIALS AND METHODS

Molecular Modeling, Docking, and Scoring

BP was built and energy minimized in the active site of a CYP2A13 crystal structure (PDB ID 2P85) using Molecular Operating Environment (MOE) (Figure S1A).¹⁵

To guide the structure-based engineering of TouA for *meta*-selective hydroxylation of BP (Figure 5), AutoDock Vina was used to dock BP into homology models of TouA variants. Because AlphaFold could not position Fe-coordinating residues properly, TouA variants were modeled using SWISS-MODEL.¹⁶ A common TouA template structure (PDB ID 3N20) was used as the template for all variants, except for the Ile100Val-Glu103Val-Thr201Ser variant, which was modeled using a second automatically selected TouA template (PDB ID 3RNF). Receptor structures were prepared for docking by aligning all structures to the 3N20 template using PyMOL. Protonation states of polar residues were assigned

at pH 7.0 using PDB2PQR,¹⁷ and Fe atoms were reinserted from the template structure. The protonated structures were then converted to PDBQT format with assigned partial charges using the *prepare_receptor4.py* script from AutoDock,¹⁸ after which the Fe atoms were assigned partial charges of +1.3. To prepare the BP ligand for docking, the 3D structure of BP was downloaded from PubChem, converted to PDB format using PyMOL, and then converted to PDBQT format with assigned partial charges using the *prepare_ligand4.py* script from AutoDock. BP was docked into the active sites of wild-type TouA and variants using AutoDock Vina.¹⁸ The distances between BP *meta* position carbons and *para* position carbons to the iron center were evaluated using a custom Python script and verified by checking each structure in PyMOL.

To design TouA active-site variants for the hydroxylation of 3HBP (Figure 6), four conformations of 3HBP were modeled and energy minimized in the active site of a TouA crystal structure (PDB ID 1T0Q), using MOE. MOE was then used to perform a residue scan of active-site residues, using each of the four 3HBP-TouA complex structures; change in binding free energy to 3HBP (Δ Affinity) was compared for 437 amino acid substitutions (Table 2 and Supporting Information). Δ Affinity represents the change in predicted binding affinity for the target ligand upon single amino acid substitution, relative to the wild-type TouA reference. Negative Δ Affinity values indicate improved predicted binding affinity, whereas positive values indicate reduced predicted binding affinity (Table 2 and Supporting Information).

All modeled complex structures were analyzed in PyMOL, and reported distances were measured between heavy atoms. The TouA active-site access tunnel was visualized using the CAVER 3.0.3 PyMOL plugin.¹⁹

Molecular Dynamics

Molecular dynamics simulations were performed using the 1T0Q crystal structure²⁰ as the initial model. Protein chains B and C, sulfate ion, and sulfanilacetic acid were removed. The crystallographic diiron center with the bridging hydroxide and crystallographic water molecules was retained. The structure of BP with a binding conformation obtained from AutoDock Vina was inserted into the active site. For TouA variants, amino acid substitutions were introduced in the active site prior to structure preparation. Protein structures were then prepared by assigning bond orders, adding missing hydrogen atoms, optimizing hydrogen-bonding networks, assigning protonation states corresponding to pH 7.0, and performing restrained energy minimization. For variant structures, substituted residues were additionally subjected to energy minimization.

The prepared systems were solvated using the TIP3P (transferable intermolecular potential 3-point) water molecules in an orthorhombic periodic box with a 12 Å solvent buffer in each dimension, and Na⁺ ions were included for neutralization. The systems were then relaxed using a modified relaxation protocol. Molecular dynamics simulations were performed using Desmond,²¹ with the OPLS4 force field,²² under NPT (constant number of atoms, pressure, and temperature) conditions at 300 K and 1.01325 bar. Simulations were performed for 200 ns, and trajectory frames were recorded every 200 ps.

The shortest *meta* carbon–Fe distance minus the shortest *para* carbon–Fe distance was calculated as “*meta* - *para* Δ ” (Table S2).

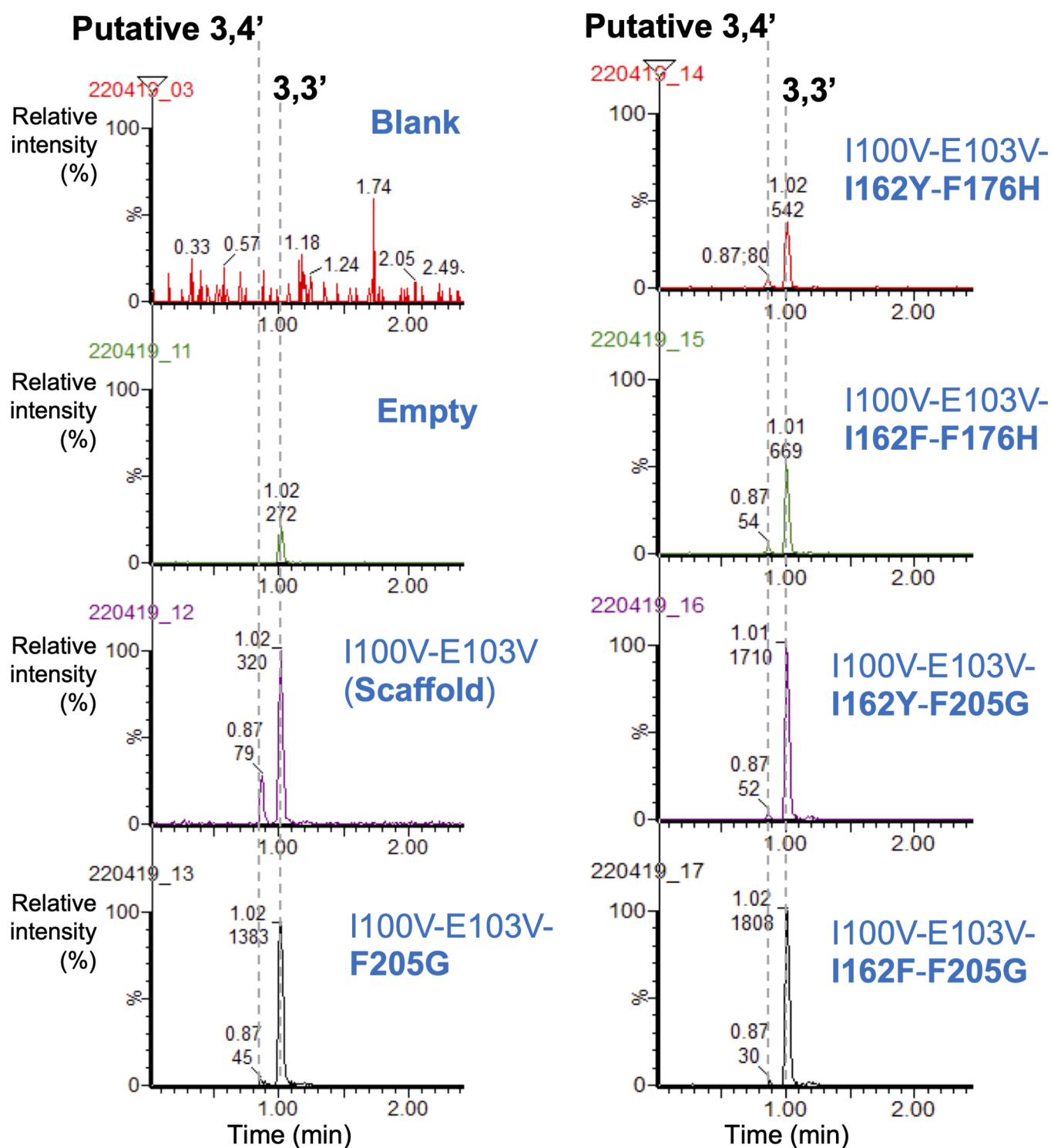


Figure 7. Analysis of 3HBP hydroxylation by combined TouA active-site variants. LC-MS extracted-ion chromatograms ($[M-H]^-$, m/z 185.061) show 33DHBP (indicated by “3,3’”) and 34DHBP (indicated by Putative “3,4’”) as the enzymatic reaction products of ToMO, using the TouA-I100V-E103V scaffold ($n = 1$). Retention times and peak areas are listed for each detected peak.

Negative *meta* - *para* Δ values indicate that the *meta* position is closer to the active site Fe than the *para* position. The percentage of simulation frames in which the *meta* position was closer than the *para* position was defined as “*meta* frames (%)” (Table S2). BP1-205 and BP2-205 distances were measured from the centroids of biphenyl rings

1 and 2, respectively, to the C α atom of residue 205 (Table S2). The active-site orientation angle of BP (BP1-AC-BP2; AC denotes the active center) was measured using the *para* carbon atoms of both BP rings, with the oxygen atom of the bridging hydroxide in the diiron center as the vertex (Table S2).

Table 4. Hydroxylation Activities of Combined TouA Active-Site Variants^a

enzyme variant	[HBP] (3 h, μ M)	[33DHBP] (8 h, μ M)	33DHBP fraction (%)
I100V-E103V (scaffold)	4.76 $\pm$ 0.89	0.050	80
I100V-E103V-F205G	2.43 $\pm$ 0.55	0.373	97
I100V-E103V-I162Y-F176H	3.01 $\pm$ 1.17	0.118	87
I100V-E103V-I162F-F176H	1.54 $\pm$ 0.73	0.156	93
I100V-E103V-I162Y-F205G	4.18 $\pm$ 1.37	0.472	97
I100V-E103V-I162F-F205G	1.45 $\pm$ 0.30	0.502	98
I100V-E103V-F176H-F205G	0	N.T.	N.T.

^aAbbreviations: HBP, hydroxybiphenyl (3-hydroxybiphenyl and 4-hydroxybiphenyl); 33DHBP, 3,3'-dihydroxybiphenyl; 34DHBP, 3,4-dihydroxybiphenyl; h, hour; N.T., not tested.

MULTiple-Sequence Alignment-based protein Selection via clustering using Hlgh-dimensional analysis (MUSASHI)

TouA, the catalytic subunit of ToMO, encoded by *Pseudomonas* sp. OX1 (GenBank accession AAT40431.1), was used as the query sequence for an NCBI BLAST search. The BLASTP search was performed against the ClusteredNR database using the following General Parameters: Max target sequences, 5000; Expect threshold, 0.05; and the following Scoring Parameters: Matrix, BLOSUM62; Gap Costs, Existence: 11, Extension: 1; Compositional adjustments, Conditional compositional score matrix adjustment. Redundant sequences were removed from the collected 5,000 sequences using CD-HIT²³ at a threshold value of 1.0, resulting in 3866 nonredundant sequences. The 3866 amino acid sequences were aligned using multiple alignment with fast Fourier transform (MAFFT) with the following parameters: strategy, FFT-NS-1; scoring matrix, BLOSUM62; gap opening penalty, 1.53; offset value, 0.0.²⁴

The resulting sequence alignment was converted into a binary vector matrix to construct a mean-centered variance-covariance matrix for principal component analysis (PCA).¹⁴ PCA was then used to project the multidimensional sequence information onto two dimensions.

Preparation of Genes for Recombinant Expression in *P. putida*

Genes encoding the main oxidoreductases (Table S4), as well as the *R. pickettii* T3MO catalytic subunit (UniProt accession Q51939) and the *E. mendocina* T4MO catalytic subunit (UniProt accession Q00456), were codon-optimized for optimal expression in *P. putida*. *TouB-F* from *P. stutzeri* were not codon-optimized. Codon-optimized sequences of the active oxidoreductase genes of this study are provided in the Supporting Information. All genes used in this study were artificially synthesized by Fasmac Co., Ltd. DNA encoding the N-terminal transmembrane domains of CYP2A13 and HsCPR were not included in our constructs. All gene fragments were cloned into pBBR122 for constitutive expression in *P. putida*. Additional constructs were designed to produce P450 and CPR as a fusion protein via a peptide linker, SGRTTPTTPTTP (Figure S7).

For TouA, the catalytic hydroxylase subunit of ToMO, the I100V-E103V variant, which enhances *meta*-selective hydroxylation of toluene, was used as the enzyme engineering scaffold.¹³ Mutant sequences of CYP2A13 and *TouA* were produced using inverse PCR with overlapping primer sets. Sets of primers with an overlap of 18 bases, including the mutation sites, were used for PCR amplification, starting from the template plasmid. PCR was performed using PrimeSTAR[®] Max DNA Polymerase (Takara Bio). After treating the mutated PCR products with the restriction enzyme DpnI, the products were transformed into DH5a *E. coli* to produce each desired plasmid.

Growth and Transformation of *P. putida*

P. putida KT2440 was obtained from the NITE Biological Resource Center (NBRC). Recombinant *P. putida* was generated by transforming each pBBR122 plasmid, according to the methods of Iwasaki

et al.²⁵ Glycerol stocks of *P. putida* were inoculated into LB liquid medium containing 100 mg/L kanamycin and cultured at 30 °C with shaking at 180 rpm. Secondary cultures were initiated by transferring 600 μ L of initial culture to 5 mL of fresh LB medium, followed by incubation at 30 °C with shaking at 180 rpm until the optical density at 600 nm (OD₆₀₀) reached approximately 0.4. 4 mL of culture was centrifuged at 5800 g (8000 rpm) for 1 min to collect the cells. All centrifugation steps were performed using a TOMY MX-307 centrifuge. The pelleted cells were resuspended in 4 mL of 300 mM sucrose at room temperature. This washing step was repeated once more to clean the cells. The cells were then pelleted again by centrifugation at 5800 g (8000 rpm) for 1 min and resuspended in 0.4 mL of 300 mM sucrose.

For electroporation, 100 μ L of the *P. putida* cell suspension and 120 ng of plasmid DNA (2 μ L of 60 ng/ μ L solution) were added to a 0.2 cm wide electroporation cuvette (Bio-Rad), gently mixed by tapping, and incubated on ice for 10 min. An electric pulse was applied at 2.5 kV and 600 Ω , after which 900 μ L of LB medium was immediately added. The cell suspension was transferred to a sterilized 2 mL tube and incubated at 30 °C with shaking at 180 rpm for 3.5 h. The culture was then centrifuged at 5800 g (8000 rpm) for 1 min, and approximately 700 μ L of the supernatant was discarded. The remaining cells were resuspended in the residual LB medium. The suspension was plated in duplicate onto LB agar plates containing either 100 mg/L kanamycin and incubated overnight at 30 °C.

Bioconversion Assays

Recombinant *P. putida* was cultured in LB medium containing 100 mg/L kanamycin, 1 mM aminolevulinic acid (ALA), and 0.1 mM FeSO₄ at 30 °C with shaking at 180 rpm for 24 h. 500 μ L of cultures at OD₆₀₀ = 1 were centrifuged at 5800 g (8000 rpm) and resuspended in 5 mL of reaction solution (50 g/L glucose, 50 mM MES-KOH, pH 7.0).

For bioconversion of BP, naphthalene, or 7-ethoxycoumarin, 5 μ L of 200 mM BP, naphthalene, or 7-ethoxycoumarin in DMSO was added to each sample for a final concentration of 0.2 mM. For bioconversion of 3HBP, 5 μ L of 400 mM 3HBP in DMSO was added to each sample for a final concentration of 0.4 mM. For quantitative bioconversion experiments, after incubating the samples for 3 h with BP, naphthalene, or 7-ethoxycoumarin, or for 8 h with 3HBP, 300 μ L of each sample was collected and centrifuged at 20,400 g (15,000 rpm) for 10 min, and the supernatants were recovered. For the BP time-course experiments shown in Figure 2, samples were additionally collected after 0, 10, and 23 h of incubation.

Fluorescence-Based Quantification of Hydroxylated Products

Fluorescence of monohydroxylated products was measured in 384-well plates (Greiner Bio-One, Item No. 781080) using a plate reader. Conditions for the fluorescence-based hydroxylation assay are shown in Table S5.^{26–28}

BP hydroxylation by ToMO was quantified based on product fluorescence from three independent samples for each condition

($n = 3$) (Tables 1 and 4), with the exception of three inactive variants from Table 1 and the variants in Table S3 ($n = 1$). BP hydroxylation by CYP2A13 was quantified based on product fluorescence from one to three independent samples for each condition ($n = 1$ –3). 3HBP and 4HBP standards were analyzed under matching conditions to generate calibration curves for quantitative analysis. Concentrations of monohydroxybiphenyl products (3HBP and 4HBP) are reported as mean $\pm$ standard deviation (SD) (Tables 1 and 4).

Hydroxylation of naphthalene and 7-ethoxycoumarin was determined based on product fluorescence from one to three independent samples for each condition ($n = 1$ –3).

Mass Spectrometric Analysis of Hydroxylation Products

Monohydroxybiphenyls (3HBP and 4HBP) were analyzed by GC–MS, and dihydroxybiphenyls (33DHBP and 34DHBP) were analyzed by LC–MS operated in negative ion mode. The 3HBP fraction (%) was estimated from the relative GC–MS peak areas of 3HBP and 4HBP ($n = 1$) (Table 1). LC–MS was used to quantify 33DHBP produced from 3HBP ($n = 1$) (Table 3 and Table 4). LC–MS peak areas of 33DHBP and 34DHBP were used to estimate the 33DHBP fraction (%) ($n = 1$) (Tables 3 and 4).

GC–MS and LC–MS parameters are given below.

GC–MS: GC–MS, Agilent 7890B/5977A GC/MSD; column, DB-35MS (30 m, 0.25 mm ID, 0.25 μ m); injection port temperature, 300 $^{\circ}$ C; split ratio, 10:1; injection volume, 1 μ L; oven temperature, 50 $^{\circ}$ C (0 min) $\rightarrow$ 10 $^{\circ}$ C/min $\rightarrow$ 320 $^{\circ}$ C (0 min); flow rate, 0.68 mL/min; carrier gas, He; m/z range, 35–500.

LC–MS: LC, Waters ACQUITY UPLC H-Class; MS, Waters Xevo G2-XS QToF; column, UPLC BEH C18 2.1 mm $\times$ 50 mm $\times$ 1.7 μ m; column temperature, 40 $^{\circ}$ C; injection volume 1 μ L.

3HBP and 4HBP were identified by GC–MS analysis of standard compounds (Tokyo Chemical Industry Co., Ltd. [TCI]) as extracted ions at m/z 170 with elution times of 17.71 and 17.55 min, respectively. 44DHBP, 33DHBP, and 2,2'-dihydroxybiphenyl (22DHBP) were identified by LC–MS analysis of standard compounds (44DHBP, TCI; 33DHBP, BLD Pharmatech Ltd.; 22DHBP, TCI) as $[M-H]^-$ ions with m/z 185.061 and elution times of 0.70–0.75, 0.99–1.03, and 1.60 min, respectively. As the 34DHBP standard was not available, the m/z 185.061 peak eluting at 0.85–0.87 min was assigned to 34DHBP. Standard curves were used to estimate product concentrations of 3HBP and 33DHBP from GC–MS and LC–MS data, respectively. BP and 3HBP *meta*-selectivity (%) were estimated from 3HBP and 33DHBP fractions, respectively.

Western Blotting for Detection of Hexahistidine-Tagged Proteins

500 μ L of culture medium from each sample was centrifuged, and the supernatant was removed. The pellets were treated with BugBuster[®] (Merck). After centrifugation at 20,400 g (15,000 rpm) for 15 min at 4 $^{\circ}$ C, soluble and insoluble fractions were collected. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (EzApply, ATTO) was added to each sample, and the samples were heated at 95 $^{\circ}$ C for 15 min to denature and solubilize the proteins in SDS. SDS-PAGE was performed using an electrophoresis cell (Criterion Cell, Bio-Rad) and a Criterion TGX gel (Bio-Rad). The electrophoresis buffer (Tris/Glycine/SDS buffer, Bio-Rad) was added to the system, and 10 μ L of each sample was loaded into each well. Electrophoresis was carried out at a constant voltage of 200 V for 40 min.

After electrophoresis, protein transfer was performed using a Trans-Blot transfer pack and a Trans-Blot Turbo System (Bio-Rad). The membrane was immersed in a blocking solution (TBS-based, pH 7.2; Nacalai Tesque) and shaken at room temperature for 1 h. The membrane was then washed three times with TBS-T (137 mM NaCl, 2.68 mM KCl, 1% polyoxyethylene sorbitan monolaurate, 25 mM Tris-HCl, pH 7.4) for 5 min each at room temperature. A rabbit

anti-6X His tag antibody (Abcam, ab9108) was diluted 1:20,000 in TBS-T and used as the primary antibody. The membrane was incubated with the primary antibody for 2 h at room temperature, followed by three washes with TBS-T for 5 min each. An anti-rabbit IgG, AP-linked secondary antibody (Cell Signaling, #7054) diluted 1:3000 in TBS-T was then used. The membrane was immersed in the secondary antibody solution and shaken for 1 h at room temperature, followed by three washes with TBS-T. Color development with alkaline phosphatase was performed by immersing the membrane in a substrate solution (0.1 M NaCl, 5 mM MgCl₂, 0.33 mg/mL nitro blue tetrazolium, 0.33 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, 0.1 M Tris-HCl, pH 9.5) at room temperature until color appeared.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.6c03624>.

P450 engineering results, Western blot analysis of ToMO, T3MO, and T4MO catalytic subunit production, additional Western blot analyses of TouA variants, hydroxylation parameters for TouA, molecular dynamics simulations, distance and angle metrics from molecular dynamics, additional activities of TouA variants, BP hydroxylation activity of TouA variants, oxidoreductase sequence information, constructs for P450, CPR, and ToMO expression, and fluorescent detection of hydroxylated products (DOCX)

Supplementary dataset of predicted parameters for 3-hydroxybiphenyl (3HBP) binding to TouA (XLSX)

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Author Contributions

*C.J.V. and T.M. contributed equally. The study was designed by CJV, TM, RH, AK, and TH. Laboratory experiments were performed by TM. Computational enzyme design was performed by CJV. Mass spectrometry analyses were performed by HI. Data were analyzed by CJV, TM, SY, and RH. CJV, TM, and SY wrote the manuscript.

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Notes

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