

# Modulating Chemoselectivity in a Fe(II)/ $\alpha$ -Ketoglutarate-Dependent Dioxygenase for the Oxidative Modification of a Nonproteinogenic Amino Acid

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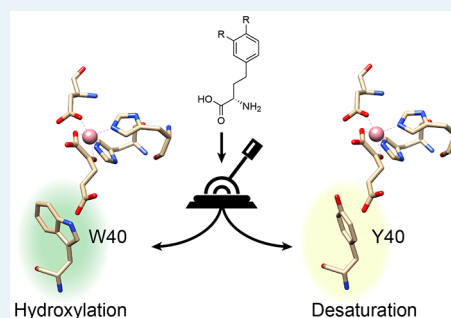
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**ABSTRACT:** Modification of aliphatic C–H bonds in a regio- and stereoselective manner can pose a formidable challenge in organic chemistry. In this context, the use of nonheme iron and  $\alpha$ -ketoglutarate-dependent dioxygenases ( $\alpha$ KGDs) represents an interesting tool for C–H activation as this enzyme family can catalyze a broad set of synthetically valuable reactions including hydroxylations, oxidations, and desaturations. The consensus reaction mechanism of  $\alpha$ KGDs proceeds via the formation of a Fe(IV)-oxo complex capable of hydrogen atom transfer (HAT) from an  $sp^3$ -hybridized substrate carbon center. The resulting substrate radical and Fe(III)–OH cofactor are considered to be the branch point toward the possible reaction outcomes which are determined by the enzyme's active site architecture. To date, the modulation of the reaction fate in Fe(II)/ $\alpha$ -ketoglutarate-dependent dioxygenases via enzyme engineering has been mainly elusive. In this study, we therefore set out to engineer the L-proline *cis*-4-hydroxylase Smp4H from *Sinorhizobium meliloti* for selective oxidative modifications of the nonproteinogenic amino acid L-homophenylalanine (L-hPhe) to produce pharmacological relevant small molecule intermediates. Using structure-guided directed evolution, we improved the total turnover number, the  $k_{cat}$ , as well as the  $k_{cat}/K_m$  of the hydroxylation reaction yielding the desired  $\gamma$ -hydroxylation product by approximately 10-fold, >100-fold, and >300-fold, respectively. Notably, the exchange of only one amino acid in the active site (W40Y) allowed us to reprogram the natural hydroxylase to predominantly act as a desaturase, presumably through tyrosine's capability to serve as a catalytic entity in the reaction mechanism. An investigation of the substrate scope revealed additional acceptance of the noncanonical amino acids L-homotyrosine and (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid by Smp4H variants.

**KEYWORDS:** nonheme Fe(II)/ $\alpha$ -ketoglutarate-dependent dioxygenases, C–H functionalization, enzyme engineering, biocatalysis, nonproteinogenic amino acid, chemoselectivity



## INTRODUCTION

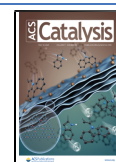
The selective modification of unactivated  $sp^3$ -hybridized carbons poses a considerable challenge for traditional synthetic chemistry often requiring the use of toxic and/or difficult-to-handle chemicals. Particularly, the development of chemo-selective catalysts that can oxidize aliphatic C–H bonds in the presence of more electron-rich functional groups such as olefins, aromatic rings, and nitrogen atoms remains highly desirable.<sup>1</sup> Nonheme iron(II)/ $\alpha$ -ketoglutarate (Fe(II)/ $\alpha$ -KG)-dependent dioxygenases have recently emerged as promising biocatalysts for mild and selective C–H functionalizations.<sup>2–4</sup> The versatile Fe(II)/ $\alpha$ -KG-dependent dioxygenases only require  $\alpha$ -ketoglutarate and  $O_2$  as cosubstrates to catalyze a plethora of different reactions including hydroxylations, epoxidations, halogenations, oxidations, desaturations, endoperoxidations, ring-forming, and rearrangement reactions.<sup>2,3,5,6</sup> The enzymes have been applied in chemoenzymatic natural product synthesis, for example, for the production of

podophyllotoxin, an important lead compound in drug discovery,<sup>7,8</sup> or for the commercial derivatization of amino acids and derivatives such as L-pipecolic acid, L-proline, or L-isoleucine.<sup>9,10</sup> The catalytic mechanism employed by Fe(II)/ $\alpha$ -KG-dependent dioxygenases involves initial formation of a substrate radical by abstraction of an H atom through a high-valent Fe(IV)-oxo species, similar to P450 enzymes (Figure S1).<sup>11</sup> Subsequent events in the enzyme active site determine the fate of the substrate radical and, hence, the different reaction outcomes (Scheme 1). In the case of hydroxylases, for example, the iron-coordinated hydroxyl (Fe(III)–OH) reacts

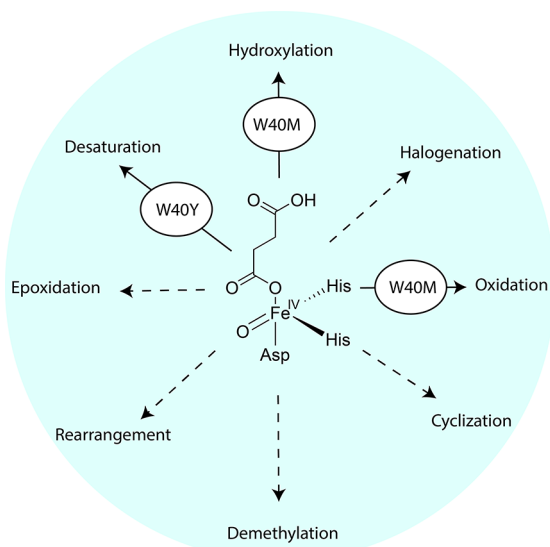
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**Scheme 1. Catalytic Mechanism of Fe(II)/ $\alpha$ -KG-Dependent Enzymes Involves Formation of a High-Valent Fe(IV)-Oxo Species Capable of Formation of a Substrate Radical by H-Atom Abstraction<sup>a</sup>**

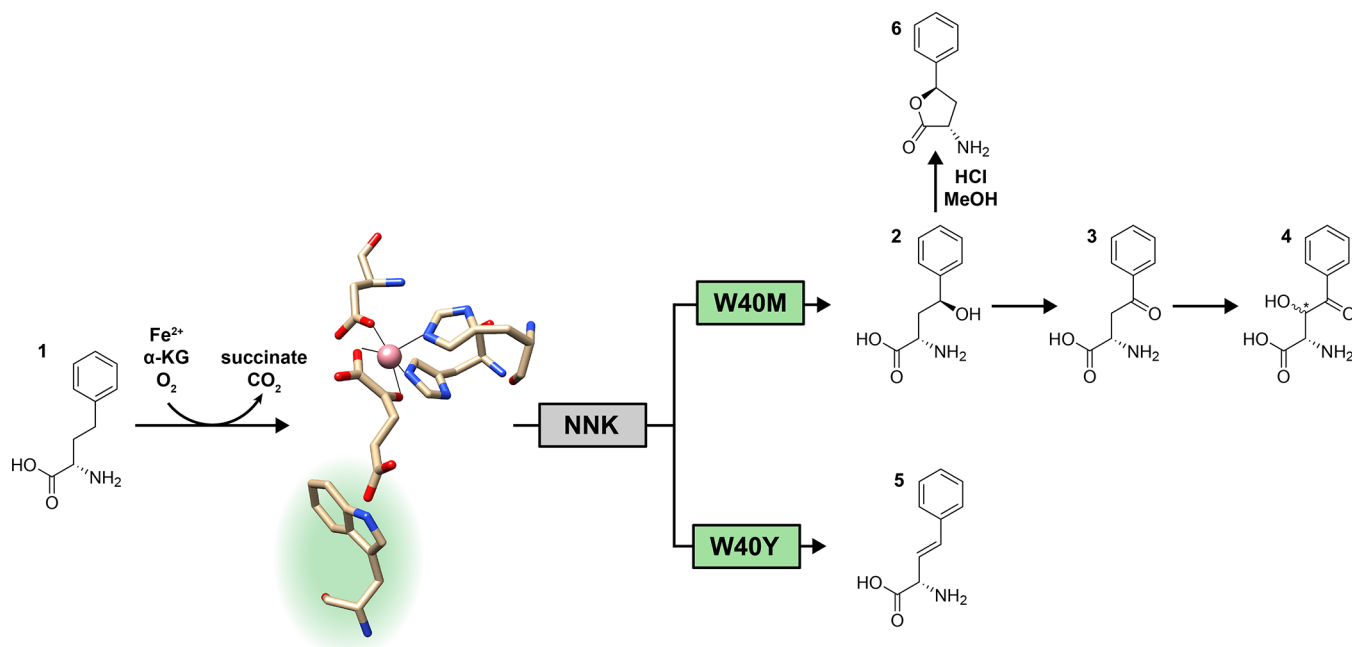


<sup>a</sup>Subsequent catalytic steps define the different reaction outcomes in dependence of the active site environment of the corresponding enzyme. In the case of SmP4H, the position W40 represents a functional junction, and its selective substitution allows fine-tuning of the chemoselectivity of the enzyme.

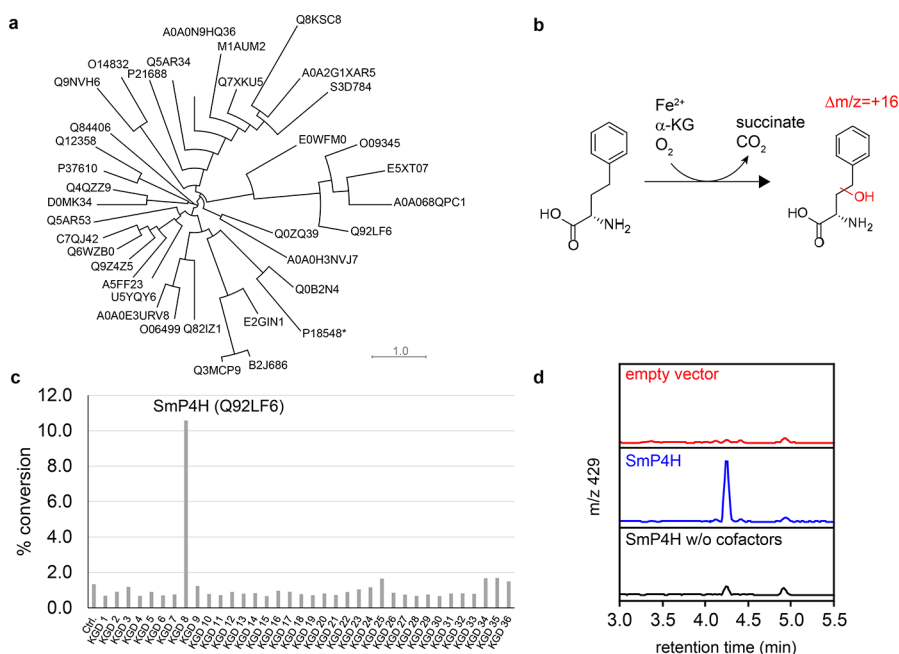
with the carbon substrate radical leading to the formation of a new C–O bond.<sup>11</sup> In reaction outcomes other than hydroxylation, rebound of the hydroxyl group is often suppressed by the enzyme, thereby enabling a different fate of the substrate radical, such as rebound of a halogen<sup>12,13</sup> or olefin formation in the case of desaturases.<sup>14</sup> Further processing of the hydroxylated intermediate by the Fe(II)/ $\alpha$ -

KG-dependent dioxygenases is another way to generate diversity.<sup>15</sup> Often, these functional differences are governed by subtle structural dissimilarities in the enzyme active sites, affecting the positioning of the substrate in relation to the catalytic residues and cofactors, and are not readily predictable by inspection of the enzyme structure alone.<sup>16,17</sup> Directed evolution approaches could, therefore, be a promising strategy to not only improve the dominant Fe(II)/ $\alpha$ -KG-dependent dioxygenase activity for non-native substrates but also access new reactivities altogether. Intrigued by the idea to engineer variants of the same parental enzyme capable of carrying out multiple types of  $sp^3$ -carbon functionalizations, we set out to build a Fe(II)/ $\alpha$ -KG-dependent dioxygenase toolbox for the derivatization of non-native substrates with the aim to create valuable building blocks for the chemical and pharmaceutical industry.

Taking a first step in this direction, we describe the setup and screening of a library consisting of wild-type Fe(II)/ $\alpha$ -KG-dependent dioxygenases and the subsequent use of a semirational protein engineering approach to adapt a proline hydroxylase from *Sinorhizobium meliloti* (SmP4H)<sup>18</sup> for efficient and selective oxidative modifications of the non-proteinogenic amino acid L-homophenylalanine (L-hPhe, **1**).  $\gamma$ -Hydroxylated  $\alpha$ -amino acids are interesting synthons in the manufacture of valuable intermediates and building blocks. While access to  $\beta$ -hydroxylated  $\alpha$ -amino acids can be obtained via, e.g., threonine aldolases,<sup>19</sup> we describe here the use Fe(II)/ $\alpha$ -KG-dependent dioxygenases as a novel method to access  $\gamma$ -hydroxylated  $\alpha$ -amino acids and therefore further expand the enzymatic toolbox for the synthesis of highly functionalized nonproteinogenic amino acids. The targeted product, the  $\gamma$ -hydroxylated L-hPhe (**2**), is a valuable intermediate in the synthesis of potential kynureninase or kynurenine monooxygenase inhibitors.<sup>20–22</sup> Kynureninases and kynurenine monooxygenase are a group of enzymes involved in the kynurenine pathway which is the major pathway for tryptophan catabolism in mammals. Several



**Figure 1.** A minimal set of mutations leads to altered chemoselectivity in SmP4H resulting in different modifications of L-homophenylalanine.



**Figure 2.** Screening approach to identify a suitable Fe(II)/α-KG-dependent dioxygenase with hydroxylase activity toward L-homophenylalanine. a) Fe(II)/α-KG-dependent dioxygenase library consisting of 36 enzymes was selected to represent a diverse phylogenetic set of functional enzymes. b) Target reaction for oxidative modification of L-homophenylalanine. c) Fe(II)/α-KG-dependent dioxygenase library screen led to the identification of L-homophenylalanine hydroxylation activity by the enzyme SmP4H from *Sinorhizobium meliloti* (Table S1). d) Selective ion monitoring (SIM) of dansyl-derivatized γ-hydroxylated L-hPhe; red, empty vector control; blue, expressed SmP4H in the reaction mix; black, expressed SmP4H in the reaction mix but without cofactors.

intermediates of this pathway are potentially toxic and have been implicated in the progression of neurodegenerative disorders, psychiatric disorders, and cancer, triggering the search for potent inhibitors of the responsible enzymes.<sup>22,23</sup>

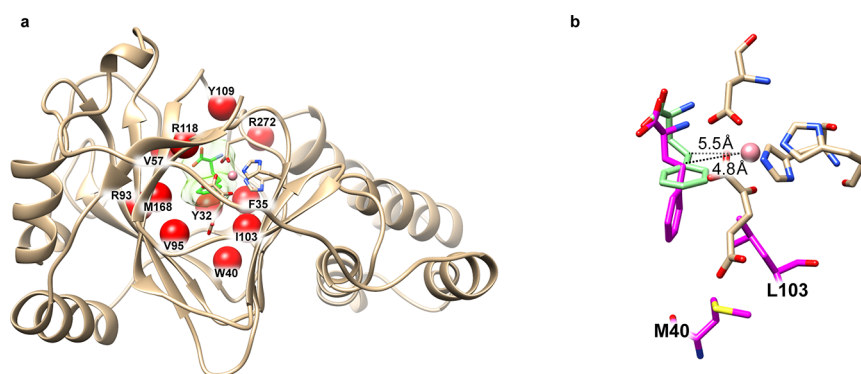
While the utility of Fe(II)/α-KG-dependent amino acid hydroxylases has been demonstrated for the biocatalytic production of other noncanonical amino acids, the substrate scope in these examples often remained limited to the native amino acid substrate or structurally close congeners.<sup>9,24,25</sup> Starting from a small promiscuous hydroxylation activity of SmP4H, a natural proline hydroxylase, toward L-hPhe (**1**), we identified enzyme variants with significantly improved hydroxylation efficiency, enabling the biocatalytic production of γ-hydroxylated L-hPhe (**2**), a versatile precursor molecule that can be further transformed chemically and enzymatically. Applying acidic conditions, one-pot self-lactonization of the obtained product was possible to give the lactone product, (3*S*,5*R*)-3-amino-5-phenyldihydrofuran-2(3*H*)-one (**6**). Furthermore, close inspection of the biocatalytic reaction revealed an enzymatic cascade leading to two additional products, the corresponding γ-ketone **3** ((*S*)-2-amino-4-oxo-4-phenylbutanoic acid) and β-hydroxy-γ-ketone **4** ((2*S*)-2-amino-3-hydroxy-4-oxo-4-phenylbutanoic acid). Importantly, the introduction of a new catalytic entity, a tyrosine (W40Y), allowed us to tune the chemistry taking place in the active site turning SmP4H into a predominant desaturase, giving almost exclusively 3,4-desaturated L-hPhe (**5**) (Figure 1).

## RESULTS AND DISCUSSION

We embarked on our enzyme engineering campaign by assembling a panel of 36 functionally characterized Fe(II)/α-KG-dependent dioxygenases (Figure 2a and Table S1). To identify a suitable starting scaffold for evolution, we screened

our wild-type enzyme library, overexpressed in *E. coli* BL21(DE3), for hydroxylase activity toward L-hPhe in a 96-well plate crude lysate assay using liquid chromatography coupled to mass spectrometry (LC-MS) to detect product formation (Figure 2b). While most enzymes did not show any activity toward the target molecule, the biocatalytic reactions carried out with proline hydroxylase SmP4H led to the formation of a small amount of a product with Δ*m/z* = +16 to the substrate, indicating modification of L-hPhe with a hydroxyl group (Figure 2c). Enzymatic origin of this activity was confirmed by running reactions with an empty vector and in the absence of the cofactor α-KG, in which only traces of hydroxylated product were detected (Figure 2d). Isolation of the putative hydroxylation product from upscaled biocatalytic reactions allowed confirmation of the structure using nuclear magnetic resonance (NMR) analysis and revealed hydroxylation to occur regio- and stereoselectively at the aliphatic sp<sup>3</sup>-γ-carbon (Figure 1). Enzymatic functionalization at this position complements existing chemical methods to access unactivated aliphatic C–H bonds in the presence of electron-rich functional groups, like aromatic rings.<sup>1,26</sup> For example, the chemical derivatization method of L-hPhe using a glycine imine cobalt(III) complex with acetophenone followed by reduction developed by Laval et al. led to a racemic mixture of γ-hydroxylated L-hPhe.<sup>27</sup>

While SmP4H showed high regioselectivity, hydroxylation activity was low (approximately 10% conversion under assay conditions). We, therefore, opted for a structure-guided semirational protein engineering approach to improve hydroxylation efficiency and generate an enzyme for the preparative biocatalytic production of hydroxylated L-hPhe. In the absence of a crystal structure for SmP4H, a homology model based on the structure of a homologous proline hydroxylase (MIP4H) (66% sequence identity, Figure S2)<sup>25</sup>



**Figure 3.** a) A substrate-docked homology model of Smp4H wild-type. The red spheres indicate the residues targeted for site directed mutagenesis using NNK libraries. b) Active site of improved variant W40M I103L showing the original docking solution for L-hPhe in the parent enzyme (green) and the new docking solution found in the double mutant (magenta). The acquired mutations W40M and I103L enable a new position of the substrate presumably shortening the distance of the  $\gamma$ -carbon atom to the Fe(II) center from 5.5 to 4.8 Å.

**Table 1. Total Turnover Number and Apparent  $K_m$ ,  $k_{cat}$ , and  $k_{cat}/K_m$  of Engineered Smp4H Variants for the Hydroxylation of L-hPhe<sup>a</sup>**

variant	$K_m$ [mM]	$k_{cat}$ [ $\text{min}^{-1}$ ]	rel $k_{cat}$	$k_{cat}/K_m$ [ $\text{M}^{-1} \text{s}^{-1}$ ]	rel. $k_{cat}/K_m$	TTN	rel TTN
WT	$1.10 \pm 0.24$	$0.015 \pm 0.001$	1	$0.2 \pm 0.5$	1	$4.1 \pm 0.6$	1.0
W40M	$0.24 \pm 0.04$	$0.124 \pm 0.003$	8	$8.6 \pm 0.2$	38	$10.6 \pm 1.1$	2.6
W40M I103L	$0.40 \pm 0.08$	$1.680 \pm 0.068$	112	$70.0 \pm 14.0$	308	$38.7 \pm 3.5$	9.4

<sup>a</sup>Mean values ( $n = 3$  biological replicates) are shown. Reaction conditions: 2 mM L-hPhe, 10 mM ascorbic acid, 1  $\mu\text{M}$  enzyme, 2 h at 20 °C.

was built using SwissModel,<sup>28</sup> and the target molecule L-hPhe was docked in the active site (Figure 3). In one of the docking solutions, the molecule is located such that its carboxyl and amino groups were directed toward Arg118 and Asp108, respectively. A similar positioning of the native substrate L-proline is observed in the crystal structure of MIP4H and enables electrostatic and H-bonding interactions to lock the amino acid substrate near the catalytically active Fe(II) species.<sup>25</sup> Using this docking model, we identified 11 sites in the vicinity of the target molecule. For each of the 11 sites, we individually created site-saturated mutagenesis libraries using NNK degenerate codons encoding all 20 amino acids.

Screening of the 1100 variants was performed using the LC-MS based crude cell lysate assay. Mutations at two positions, W40 and I103, were identified that gave up to a 4.5-fold improved conversion (Figure S3). Particularly, smaller amino acid residues at these positions seemed to improve hydroxylation, putatively by enabling better accommodation of the phenyl moiety of L-hPhe (Table S4). The best variant in this first round of engineering, W40M, was subsequently characterized as a purified enzyme and showed an approximately 8-fold higher apparent  $k_{cat}$  compared to the wild-type (Table 1). In order to find a potentially more beneficial combination of mutations, we created a two-site randomized library using degenerate codons DBK<sup>29</sup> and DYA at positions W40 and I103, respectively (Figure S4). The best variant emerging from the second round of engineering, W40M I103L, showed an additional ca. 2-fold increased conversion to the hydroxylated product compared to its parent W40M in the crude lysate screen which was verified by kinetic analysis with purified enzymes. Overall, the double variant (W40M I103L) exhibited a >100-fold improved  $k_{cat}$  and a >300-fold improved  $k_{cat}/K_m$  compared to the wild-type enzyme (Table 1 and Figure S5). Additionally, the total turnover number (TTN) of the double variant (W40M I103L) for the hydroxylation of L-hPhe was increased by 10-fold to a value of approximately 39 during

the course of the evolution. For comparison, the total turnover number of the wild-type Smp4H for its native substrate proline was found to be  $257 \pm 10$ .

Besides its utility as a potential precursor for the synthesis of kynurenine monooxygenase inhibitors,  $\gamma$ -hydroxylated L-hPhe can also undergo self-lactonization under acidic conditions to give lactone 6, a structural analogue of pharmacological relevant compounds.<sup>30</sup> Using 20  $\mu\text{M}$  W40M I103L and 0.5 mM L-hPhe, lactone 6 could be generated from  $\gamma$ -hydroxylated L-hPhe in a one-pot reaction by replacing water with an organic solvent under acidic conditions. Isolation of lactone 6 from upscaled chemoenzymatic reactions allowed confirmation of the structure using nuclear magnetic resonance (NMR). Under these conditions, a yield of 94% lactonization was achieved further underscoring the chemoenzymatic utility of C–H functionalization by the W40M I103L variant.

Conversion of L-hPhe with the evolved Smp4H variants, however, did not exclusively yield  $\gamma$ -hydroxylated L-hPhe. Both in the crude lysate and *in vitro* experiments, we observed two side products,  $\gamma$ -ketone 3 and  $\beta$ -hydroxy- $\gamma$ -ketone 4 (Figure 1). The identity of  $\gamma$ -ketone 3 was confirmed by comparison to a commercially available standard by LC-MS, whereas the structure of  $\beta$ -hydroxy- $\gamma$ -ketone 4 was solved by NMR analysis. Our experiments suggest that these products result from further oxidation of the  $\gamma$ -hydroxylated product 2 (Figure S6 and Figure S7). Using the commercially available standard for ketone 3 we could show that this molecule is a well-accepted substrate for the engineered Smp4H variants and is hydroxylated surprisingly efficiently by W40M I103L to give  $\beta$ -hydroxy- $\gamma$ -ketone 4 (Figure S8). All enzymatically functionalized compounds can easily be separated chromatographically and serve as valuable synthons in medicinal chemistry.

Based on these findings, we reevaluated the oxidative modification of L-hPhe and the conversion into the different products using purified enzymes (Table 2). Comparison of the different Smp4H variants shows that we were able to improve



production of  $\gamma$ -hydroxylated L-hPhe in a significant manner from 1.6% to 35.3% (W40M I103L). Improved conversion was achieved as well in the case of the subsequent oxidation to product 3 and formation of  $\beta$ -hydroxy- $\gamma$ -ketone 4. While in the case of W40M, accumulation of  $\gamma$ -ketone 3 was observed, the W40M I103L variant showed increased conversion toward  $\beta$ -hydroxy- $\gamma$ -ketone 4. Michaelis–Menten analysis confirmed that the additional mutation of I103L leads to an increased catalytic efficiency toward the  $\beta$ -hydroxylation of  $\gamma$ -ketone 3 (Figure S8). This multifunctional character of Smp4H is not uncommon for this class of enzymes. Several examples of Fe(II)/ $\alpha$ -KG-dependent dioxygenases exist that catalyze a hydroxylation and sequential oxidation to the ketone, the aldehyde, or the carboxylic acid<sup>15,31,32</sup> or possess multiple activities including hydroxylation, desaturation, and epoxidation such as PtlD<sup>33</sup> involved in the neopentalenoketolactone biosynthesis.<sup>34</sup>

Intrigued by the catalytic promiscuity of the Fe(II)/ $\alpha$ -KG-dependent dioxygenase Smp4H, we searched the enzyme library screening data for alternative products, hoping to identify Smp4H variants capable of chemistries other than L-hPhe hydroxylation. Interestingly, we found a variant, W40Y, which gave a product (>80% selectivity) corresponding to a loss of 2 *m/z* units (Figure S9). Using NMR, we could solve the structure of the obtained product and identified the compound as 3,4-desaturated L-hPhe 5 (Figure 1). Kinetic analysis of variant W40Y allowed us to determine  $k_{\text{cat}}$  ( $0.83 \pm 0.02 \text{ min}^{-1}$ ) and  $K_{\text{m}}$  ( $0.12 \pm 0.02 \text{ mM}$ ) values, which are in a similar range as seen for the hydroxylase variants (Figure S13). This report therefore is, to our knowledge, the first example of remodeling a Fe(II)/ $\alpha$ -KG-dependent hydroxylase into a desaturase by enzyme engineering. Notably, our results additionally highlight that the here-described engineered Smp4H variant represents a rare case of a desaturase that acts on the C–C unit without an activating  $\alpha$ -heteroatom,<sup>14</sup> a synthetically very desirable reaction. To date, only PrhA,<sup>35</sup> BcmF,<sup>36</sup> and KabC<sup>37</sup> have been described to desaturate entirely unactivated C–C units in their natural substrates, whereas native VioC<sup>14</sup> and CarC<sup>38</sup> have been reported to exhibit non- $\alpha$ -heteroatom assisted desaturation activity in addition to hydroxylation activity when acting on non-native compounds.

To better understand the role of the introduced tyrosine residue in the context of Smp4H-catalyzed L-hPhe desaturation, we carried out in-depth docking analyses. These studies revealed that the tyrosine residue may cause the aromatic ring of the substrate to shift slightly leading to a conformation that exposes both  $\beta$ - and  $\gamma$ -H atoms to the Fe(II) center (Figure S10). Similar substrate placements—i.e., proximity of multiple substrate carbons to the active iron center—have also been found in Fe(II)/ $\alpha$ -KG-dependent dioxygenases which have been described to carry out multiple reaction chemistries such as VioC, capable of hydroxylation and desaturation.<sup>14,39,40</sup> As a consequence, the newly introduced tyrosine residue might lead to a substrate placement relative to the active Fe-center in which two sequential H-atom transfers (HAT) are favored over rebound of a hydroxyl group allowing diradical dimerization to give the desaturated product (Figure 4a). In this scenario, however, the intermediate Fe(III)–OH, expected to be a modest oxidant, would be capable of catalyzing the second HAT step at the carbon adjacent to the radical center. In addition, the Smp4H variant W40Y would be strikingly

efficient in favoring the second HAT step over hydroxyl rebound (Table 2).

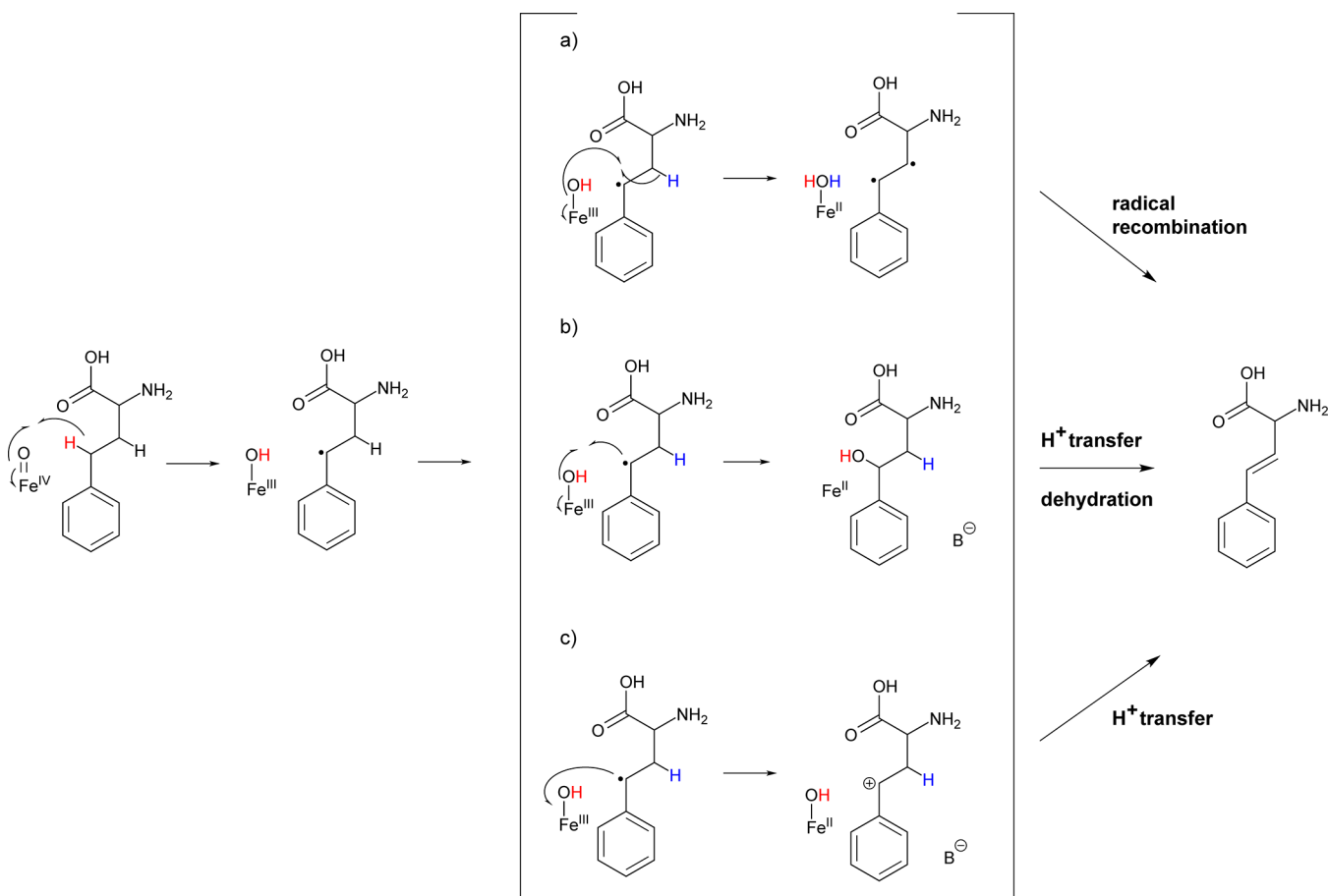
**Table 2. Product Distribution of Oxidatively Modified L-Homophenylalanine Generated by Wild-Type and Evolved Variants Based on Extracted Ion Chromatograms<sup>a</sup>**

Product	WT	W40M	W40M I103L	W40Y	W40F
2	1.6%	17.8%	35.3%	5.9%	21.8%
3	4.3%	17.0%	4.1%	5.1%	4.8%
4	0.3%	7.3%	29.8%	6.8%	26.9%
5	0.1%	1.9%	0.7%	50.7%	0.8%

<sup>a</sup>Reaction conditions: 1 mM L-hPhe, 10 mM ascorbic acid, 20  $\mu\text{M}$  enzyme, 60 min at 20 °C. Mean values ( $n = 3$  biological replicates) are shown.

More likely, the introduced tyrosine not only influences the interaction of substrate and iron center sterically but also actively contributes to the desaturation mechanism: The product distribution of Smp4H variant W40F, in which position 40 was substituted with a phenylalanine instead of a tyrosine, supports this hypothesis as it showed almost no desaturated product (Table 2). Even though a structural effect of the tyrosine substitution cannot be excluded, the predominant hydroxylation activity of variant W40F makes it plausible that the additional hydroxyl group of the tyrosine residue is mechanistically relevant. Consequently, the desaturation reaction could take place via an intermediate hydroxylation of L-hPhe followed by a deprotonation event in which tyrosine acts as the potential base (Figure 4b). However, using  $\beta$ - or  $\gamma$ -hydroxylated L-hPhe as a substrate in biocatalytic reactions with variant W40Y did not yield any desaturated product suggesting that a cation-triggered desaturation pathway might be a more likely scenario (Figure 4c). Here, the conversion of the intermittent L-hPhe radical to 3,4-desaturated L-hPhe would involve electron transfer from the radical to the Fe(III)–OH species followed by deprotonation via tyrosine, acting as a base. A precedent for the required unusual low  $\text{p}K_{\text{a}}$  of the phenolic hydroxyl to perform general base catalysis has been suggested, for example, in the case of nitrile hydratases,<sup>41,42</sup> alanine racemases,<sup>43,44</sup> and 2-((R)-2-hydroxypropylthio)ethanesulfonate dehydrogenase.<sup>45</sup> In this reaction pathway, the phenyl moiety of the substrate would stabilize the formed carbocation, mechanistically resembling the  $\alpha$ -heteroatom effect described in natural desaturases.

In our quest to synthesize even more potent kynurenine monooxygenase inhibitors such as (S)-2-amino-4-(3,4-dichlorophenyl)-4-oxobutanoic acid ( $\text{IC}_{50}$  of 0.2  $\mu\text{M}$  compared to compound 3 with an  $\text{IC}_{50}$  of 7.6  $\mu\text{M}$ ),<sup>22,46</sup> we probed the substrate promiscuity of our evolved Fe(II)/ $\alpha$ -KG-dependent dioxygenase Smp4H with the noncanonical amino acids L-homotyrosine and (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid. The substitutions present on the aryl rings of the molecules were additionally expected to modulate the properties of any arising radical at the  $\gamma$ -position, with the corresponding L-homotyrosine radicals being more nucleophilic and the (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid radicals being more electrophilic than the respective L-homophenyl radical. Strikingly, we were able to detect formation of products with  $\Delta m/z = +16$  indicating the installation of a hydroxyl group into both additional non-native substrates. Whereas in Smp4H W40M I103L-catalyzed



**Figure 4.** Proposed pathways for the Smp4H W40Y-catalyzed desaturation. After the C(sp<sup>3</sup>)-H abstraction, the reaction can proceed through a) a second hydrogen abstraction (2-HAT) and a subsequent diradical recombination, b) hydroxylation and dehydration, or c) carbocation formation and subsequent H<sup>+</sup> removal by a base to generate the C=C bond.

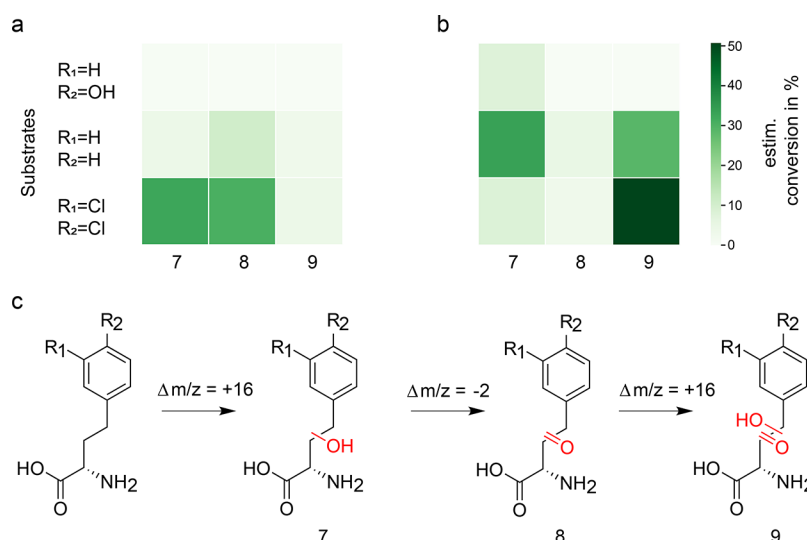
reactions of L-homotyrosine the biocatalytic cascade halted at the first hydroxylation step (compound 7, Figure 5), the respective reactions with (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid predominantly led to the formation of a product with  $\Delta m/z = +30$  to the substrate (Figure 5). In addition, we found that wild-type Smp4H only very poorly accepts L-homotyrosine as a substrate (conversion 0.5%) but readily oxidizes (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid leading to the prevalent formation of products with  $\Delta m/z = +16$  (compound 7, Figure 5) and  $\Delta m/z = +14$  (compound 8, Figure 5), similarly reflecting the intrinsic reactivity of the substrate (Table S5, Table S6, and Figure S14).

## CONCLUSION

The plethora of reactions accessible via Fe(II)/ $\alpha$ -KG-dependent dioxygenases makes them highly interesting catalysts for the production of difficult-to-synthesize products. By setting up and screening a wild-type library of functionally characterized Fe(II)/ $\alpha$ -KG-dependent dioxygenases, we identified an enzyme capable of performing oxidative modifications on the nonproteinogenic amino acid L-homophenylalanine at the  $\beta$ - and  $\gamma$ -positions as well as accepting L-homotyrosine and (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid as substrates leading to valuable kynurenine monooxygenase inhibitors and their building blocks. Functionalization of unactivated C(sp<sup>3</sup>)-H bonds in the presence of more electron-rich functional groups, like aromatic rings, remains a challenge in organic chemistry—

however, as we show here, this can be overcome by means of a suitable biocatalyst.<sup>1,26</sup>

In addition, we showcase a rare example of engineering a Fe(II)/ $\alpha$ -KG dioxygenase toward higher activity.<sup>47</sup> Not only did we successfully identify enzyme variants with a >100-fold improved apparent  $k_{cat}$ , a >300-fold improved  $k_{cat}/K_m$ , and a 10-fold increased total turnover number for the hydroxylation of L-hPhe, but also we revealed the importance of position W40 in Smp4H which serves as a functional junction for the reaction outcome. By substituting position W40 with specific amino acids, we were able to improve L-hPhe hydroxylation activity while almost completely abolishing acceptance of the native substrate L-proline (W40M) (Figure S11), or we could trigger the formation of an alternative, desaturated product (W40Y). Notably, the functional switch between a hydroxylase and a desaturase was achieved by exchanging a single amino acid supporting the possibility to deliberately access the chemical versatility of  $\alpha$ -ketoglutarate-dependent dioxygenases through a redesign of the active site architecture. This finding is underscored by further recent studies on Fe(II)/ $\alpha$ -KG-dependent dioxygenases in which it was shown that only a small number of mutations was necessary to change the reaction outcome or to increase substrate promiscuity.<sup>24,48–51</sup> Identifying such hotspots is an important step to provide starting points for further engineering studies and acts as a basis to obtain deeper insight into the structure–function relationship of this enzyme class.



**Figure 5.** Substrate screen with wild-type Smp4H and engineered variant W40M I103L investigating conversion of L-homotyrosine, L-homophenylalanine, and (S)- $\alpha$ -amino-3,4-dichlorobenzenebutanoic acid. a) Conversions with wild-type Smp4H. b) Conversions with engineered variant Smp4H W40M I103L. c) Proposed reaction cascade based on  $\Delta m/z$  values in analogy with confirmed structures from L-homophenylalanine ( $R_1 = H$ ,  $R_2 = H$ ). Conversions are estimated through comparison with the negative control omitting the enzyme, and the calculations assume similar ionization efficiency of products originating from the same substrate.

At present, the mechanism of olefin installing desaturation catalyzed by Fe(II)/ $\alpha$ -KG-dependent dioxygenases remains poorly understood.<sup>14,52</sup> As discussed above, the engineered Smp4H variant W40Y may follow one of several hypothetical reaction mechanisms similar to what has been described for the VioC-catalyzed desaturation of L-homoarginine.<sup>14</sup> Notably, in several recent mechanistic analyses of Fe(II)/ $\alpha$ -KG-dependent dioxygenases, strong evidence for a cation mechanism was presented, such as, for example, in an enzyme-catalyzed 1,2-phosphono-migration reaction<sup>53</sup> or the desaturation of cyclopeptin by AsqJ.<sup>54</sup> In addition, recent insight into the pyrrolidine and olefin formation mechanism of Fe(II)/ $\alpha$ -KG-dependent dioxygenase KabC in the biosynthesis of kainic acid further alludes to the possibility that a cation-triggered desaturation may be a general reaction principle used by these enzymes.<sup>37</sup>

To further elucidate the underlying mechanistic motifs allowing for olefin insertion between unactivated carbons, we aligned the structures of literature-known desaturases (PrhA (5YBN), VioC (6DAX), and CarC (4OJ8)) with the model of our engineered Smp4H variant. Strikingly, in the case of CarC, a similar relative placement of tryptophan 40 in Smp4H and the mechanistically important tyrosine 165 in CarC was observed (Figure S12). CarC catalyzes two subsequent reactions, a stereoinversion reaction turning (3S,5S)-carbapenam to (3S,5R)-carbapenam and a subsequent desaturation reaction to (5R)-carbapenam<sup>39</sup> (Figure S12). In CarC, tyrosine 165 has been shown to be involved in the stereoinversion reaction which is not catalytic but proceeds stoichiometrically. Since multiple turnovers of desaturation are possible in the case of the Smp4H variant, however, the tyrosyl residue in our Smp4H variant probably does not act as a hydrogen atom donor to quench the substrate radical as proposed in the CarC stereoinversion.<sup>39</sup>

At this point, we can therefore only speculate if position W40 might be of similar relevance in other  $\alpha$ -ketoglutarate-dependent dioxygenases. It is noteworthy, though, that in our wild-type dioxygenase library position W40 is conserved in five

out of 36 enzymes, indicating that the elucidation of this position's exact mechanistic or structural function in future enzyme engineering studies might be, in fact, a worthwhile pursuit.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscatal.1c00678>.

Experimental section, additional data, and figures including NMR elucidation of product structures, uniprot numbers of enzyme wild-type library, primer sequences, sequencing results of evolution rounds, sequence alignments, product distribution tables, biocatalysis results with L-proline, kinetic characterization of Smp4H variants, raw MS spectra of biotransformations, and docking studies with engineered variants (PDF)

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## Notes

The authors declare no competing financial interest.

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