Directed Evolution of an Fe^{II}-Dependent Halogenase for Asymmetric C(sp³)-H Chlorination

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Supporting Information Placeholder

ABSTRACT: By using structure-guided directed evolution, the substrate scope of the Fe^{II} and α -ketoglutarate dependent halogenase *Wi-WeIO15* from *Westiella intricata* HT-29-1 was engineered to enable chemo-, regio- and diastereoselective chlorination of unactivated C(sp³)-H bonds using NaCl as chlorine source. While Fe^{II} dependent enzymes are often oxygen sensitive, variants of this halogenase could be screened in lysates under aerobic conditions. The new biocatalysts offer a sustainable approach for mild, late-stage chlorination on mg-scale of non-natural hapalindoles containing a ketone instead of an isonitrile functionality, thereby unlocking them for preparative biocatalysis.

Organochlorines play an important role in medicinal chemistry, agrochemicals, and as synthetic intermediates.^{1–5} Selective C-H bond functionalization in the absence of directing groups greatly simplifies synthesis^{6–8} and enables late-stage diversification of complex molecules.^{9–12} Chemo-, regio- and stereoselective C-Cl bond formation under mild and non-hazardous conditions is still a formidable challenge in chemical^{13–17} and enzymatic^{18–24} catalysis, especially direct functionalization of unactivated C(sp³)-H bonds.^{6,12}

Nature has evolved highly selective aliphatic halogenases^{25–27} for performing late-stage halogenations of unactivated C(sp³)-H bonds via radical intermediates (Figure 1a).^{28–30} Remarkably, NaCl is the chlorine source and O₂ and α -ketoglutarate (α KG) activate the catalytic Fe. Two challenges exist with this enzyme class, first, competing pathways of hydroxylation versus halogenation, and second, early characterized members act on substrates covalently bound to a carrier protein.^{31–33} Only recently, aliphatic halogenases acting on free-standing molecules were discovered in the biosynthesis of hapalindoles,^{34–36} a natural product class with a broad range of biological properties from antibacterial, antimycotic to anticancer.³⁷ Although their molecular mode of action and the exact role of the chloride is unknown, it is truly the late stage chlorination performed by Fe^{II} and α KG dependent halogenases that drew our interest from a biocatalytic point-of-view. This reaction was shown for three enzymes from different biosynthetic hapalindole gene clusters, i.e. *WeIO5*, *AmbO5*, and *Hw-WeIO15* (Figure 1b).^{38–40} Due to the novelty of the sequence, homology modeling to predict the active site residues failed until a crystal structure of *WeIO5* was released.^{41,42} By creating chimeras, the natural substrate scope of *WeIO5* was expanded to that of

AmbO5 albeit with reduced activity.³⁹ However, all natural substrates contain an isonitrile functionality, which is a major limitation considering that they are sensitive and rarely to never occur as synthetic intermediates or functional groups in drugs.

Herein, we report the directed evolution of an Fe^{II} and α KG dependent halogenase *Wi-WeIO15* from *Westiella intricata* HT-29-1 for the mild chemo-, regio- and diastereoselective chlorination of unactivated C(sp³)-H bonds of non-natural substrates devoid of any isonitrile functionality and we demonstrate that this enzymatic late-stage C(sp³)-H chlorination can be performed on preparative mg-scale (Figure 1c).

a) Catalytic, asymmetric chlorination of unactivated C(sp³)-H bonds

b) Previous work: Wild type/chimeras of *WeIO5*/*AmbO5* & *Hw-WeIO15* wild type

c) This work: Engineered variants of *Wi-WeIO15* and *Hw-WeIO15*

Figure 1. (a) Asymmetric chlorination of unactivated C(sp³)-H bonds by Fe^{II}/ α KG dependent halogenases. (b) Natural substrates studied by Liu and coworkers, which all contain isonitrile functionalities.^{38–40} (c) Non-natural substrates derived from the structure of 12-*epi*-hapalindole C. Positions of chlorination are highlighted.

We initiated our study by synthesizing a simplified derivative of 12-*epi*-hapalindole C, which is a natural substrate of

Fe^{II}/αKG dependent halogenases. The vinyl group was replaced by a methyl (**1**) and, more importantly, the isonitrile group was replaced with a keto moiety (**2**). These changes simplified the synthesis of screening compound **2** by several steps, while additionally the ketone functionality opens new routes to novel chemical diversification of this alkaloid class.

Initially, we cloned *Hw*-WelO15 from *Hapalosiphon wel-witschii* IC-52-3 and a new fourth homolog, *Wi*-WelO15 from *Westiella intricata* HT-29-1. The two chlorinases have 94 and 99% sequence identity to the characterized WelO5, respectively. Transformation of compound **1** yielded 91-93% chlorinated *R*-**1a** with both recombinantly expressed enzymes, *Hw*-WelO15 and *Wi*-WelO15, revealing that the vinyl to methyl exchange appears to have little effect on substrate recognition. The isonitrile group was suggested to play a major role in native substrate recognition⁴¹ which we replaced with a carbonyl group in **2**, keeping the H-bond acceptor feature. This functional group change abolished the activity of *Hw*-WelO15 and *Wi*-WelO15, confirming the importance of the isonitrile for substrate binding in the wild type enzymes. By mass spectrometry, we could exclude enzyme inactivation by self-oxidation, a common problem for many related Fe^{II}/αKG dependent dioxygenases.⁴³⁻⁴⁵

With the goal to create a collection of engineered enzymes for mild and asymmetric chlorination in organic synthesis, compound **2** was selected as our lead structure. To gain structural insights, a variant of *Wi*-WelO15 (*Wi*-0)⁴⁶ was crystallized, the structure solved by molecular replacement with a recently published structure of WelO5, and compound **2** was docked into the active site (Figure 2). The new information about structural flexibility obtained from the open and closed forms of *Wi*-0 and WelO5 created a suitable starting point for structure guided enzyme evolution.⁴⁷⁻⁵⁵

Figure 2. Lead structure **2** (yellow) docked⁵⁶ in the open conformation of *Wi*-0. Residues selected for mutagenesis are shown in green (1st sphere, generation 1-3) and cyan (2nd sphere, 4th generation). Grey residues did not yield active variants in the 1st generation.

Next, we implemented an aerobic and lysate-based screening protocol for HPLC screening (see Supporting Information for details), and started a structure-guided directed evolution campaign using strategies reported by Reetz and Bäckvall.⁵⁷⁻⁵⁹ Based on their vicinity to the docked compound **2**, seven active site residues were initially selected for single-site saturation

mutagenesis (1st generation, Figure 3). As a result, obtained hits from the HPLC assay showed primarily hydrophobic or small amino acids as new residues. In the 2nd generation, the active site was reshaped by multi-residue libraries, allowing for cooperative effects.^{57,59} Small libraries were designed by using smart amino acid alphabets compiled from the single-site saturation results of generation 1. We used the DC analyzer⁶⁰ to reduce codon redundancy. The library sizes (screening effort)⁶¹ were designed using statistical guidance allowing for a 95% chance to find 1 out of the top 3 variants.^{62,63} *Wi*-5 (A82L/V90P) and *Wi*-7 (V81T/A82M/A88V/V90P) were identified as the best variants in the 2nd generation. Meanwhile, we found N74R at the end of the active site tunnel having subtle positive effects on production of **2a**. Hence, 1st and 2nd generation hits were rationally fine-tuned by adding the mutation N74R, creating the 3rd generation. Variants showed a broad spectrum of positive and negative effects on the production of **2a**, with *Wi*-8 (N74R/V81T/A82M/A88V/V90P) being the best hit. After screening generation 1-3, 12 hits were characterized as lysates obtained from OD₆₀₀ normalized cell suspensions (Table S5). We tested screening compound **2** and derivatives **3-5** with substituents introduced into the indole part of the substrate. Under these controlled conditions, *Wi*-5 and *Wi*-8 were identified as the most promising variants producing up to 60% of the chlorination products **2a-5a** with >99.9% regio- and stereoselectivity.

Figure 3. Engineering workflow of the halogenase *Wi-WelO15*. Screening effort given in brackets. Activity information is provided in Tables S5 and S6.

To further increase activity, *Wi-5* and *Wi-8* were selected as templates for another round of evolution (4th generation). Learning from the effect of N74, we selected 2nd sphere residues N74, S93, S103 and A175 (Figure 2) for a multi-site library and again used tailored smart alphabets. Screening yielded variant *Wi-11* (N74R/V81T/A82M/A88V/V90P/S93L/S103A) and *Wi-12* (N74R/A82L/V90P/S93D) as top variants producing chlorinated **2a**.

Next, we verified the catalytic activities of the obtained hits with isolated and purified variants. Selected chlorinases were tested with compounds **1-8**. Reaction optimization included the substitution of dithiothreitol (DTT) with ascorbic acid for Fe^{II} recycling^{64,65} and using higher sodium chloride (100 mM) concentration. First, we decided to probe the impact of the isonitrile/ketone substitution. As a result, the engineered *Wi-WelO15* variants still chlorinate the isonitrile **1** with an expected reduced efficiency (Table S6). *Wi-0* hardly transformed the ketones **2-5** (<1% yields), whereas *Hw-WelO15* produced moderate amounts of chlorinated ketones **2a-5a**, with **3** being the best substrate obtaining **3a** in 33% yield. All natural hapalindoles contain either isonitriles or isothiocyanates, but our work demonstrates that some of the wild types, *i.e.* *Hw-WelO15*, might be more versatile than anticipated.

Using 2 mol% purified *Wi-11* or *Wi-12*, 91% of **2a** was obtained (Figure 4). When exploring the tolerance of indole ring substitutions, we found that the simple methylation at C5 or C6 reduced chlorination to 14% or 26% production at the best for **3a** or **5a** with purified variants, respectively. It is often recognized that certain enzymes/processes work better in lysate than as purified enzymes, benefiting from the presence of various cell components. Using lysates instead, containing a comparable amount of catalyst, production improved to 34% (**3a**) and 50% (**5a**).

Introducing the H-bond acceptor OMe at C5 recovered activity giving 42% **4a** with purified enzymes and 54% with lysates. Products were found to be chlorinated on C13 with *R*-configuration, showing identical absolute stereochemistry as related 12-*epi*-hapalindole E. In summary, our chlorinase panel allows late-stage chlorination and access to **1a-5a** with regio- and diastereoselectivities >99.9%.

Small structural changes in the terpene ring of **6-8** proved to be more challenging. Elimination of a methyl group at C12 (**6**) or sp² hybridization (**8**) abolished detectable chlorination, deducing that C12 position requires a quaternary carbon center for corrected substrate positioning prior to chlorination. Nevertheless, substrates **6-8** are transformed with moderate activity into several new products. LC-MS analysis assigned most of them to be hydroxylated compounds. Hydroxylation was not observed during screening, but the higher activities under optimized reaction conditions revealed minor hydroxylation of **2-5**, too. Yet, our panel allows chlorination of **2-5** with excellent chemoselectivities of 96-99% (Tables S5 and S6). Achieving such a high selectivity is exceptional because non-natural substrates for Fe^{II}/αKG dependent halogenases are predominantly reported to be hydroxylated.^{66,67} Interestingly, despite their low

activity, *Hw-WelO15* wild type and *Wi-WelO15* variants carrying I84H start shifting chemoselectivity towards 30% hydroxylation of **2** (Figure S16), an indication that chemoselectivity can also be controlled solely by substrate binding site mutations.

Figure 4. Substrate scope of *Wi-WelO15* variants. Best product formation (% HPLC) is given for 8 h and 30 °C in 200 μL with 0.5 mM **1-8** with either [a] 2 mol% (10 μM) purified enzyme or [b] cell lysate (prior to lysis normalized to OD600 = 5.0). Absolute stereoconformation was assigned by NOESY and crystal structures of **1-3** (CCDC 1839575-1839577). A detailed overview is provided in Tables S5 and S6. [c] Yield after HPLC purification. [d] Total conversion, <1% chlorination.

Chemical synthesis of chlorinated hapalindoles and their ketone precursors involve early-stage chlorination.⁶⁸⁻⁷⁰ In sharp contrast, using lysates of our *Wi-5* variant, we here demonstrated late-stage chlorination of **2-5** on mg-scale in a single step, producing 4.5 to 8.5 mg after HPLC purification of the new hapalindole derivatives. This late-stage chlorination opens new synthetic routes to structurally diverse chlorinated hapalindoles and will accelerate their exploration in pharmacology.

Finally, kinetics for the chlorination of **2** were performed (Table 1). The 4th generation variants *Wi-11* and *Wi-12* show the best v_{max} , with a 131 to 276-fold improvement compared to *Wi-*

1. The mutations affect turnover and binding differently. Despite having a similar catalytic efficiency, Wi-11 is the fastest, but Wi-12 binds compound **2** more strongly. Variant Wi-5, which proved to be a valuable variant for scale up (Table S7), was found to show highest catalytic efficiency with **2**. The kinetic data reveals that our evolved chlorinases are as efficient biocatalysts for preparative synthesis as the engineered and mechanistically related oxygenase TauD^{71,72} and the engineered flavin dependent halogenases PrnA⁷³ and RebH⁷⁴ with non-natural substrates.

Table 1. Selected kinetics for the chlorination of **2** with Wi-WeIO15 variants.

Enzyme ^[a]	v_{max} / $\mu M \min^{-1}$	K_m / μM	k_{cat} / $\min^{-1}$	k_{cat}/K_m / $\min^{-1} mM^{-1}$
Wi-0	--[b]	--[b]	--[b]	--
Wi-1	0.10	95.24	2.40	25.15
Wi-5	7.16	26.44	2.86	108.34
Wi-11	27.62	137.10	11.05	80.57
Wi-12	13.11	70.46	5.24	74.43

[a] Wi-1: A82L, Wi-5: A82L/V90P, Wi-11: N74R/V81T/A82M/A88V/V90P/S93L/S103A, Wi-12: N74R/A82L/V90P/S93D. All enzymes contain V61/D284N. [b] no detectable chlorination.

In conclusion, the power of structure-guided directed evolution was demonstrated by evolving a member of the challenging class of Fe^{II}/αKG dependent chlorinases. It was possible to exchange the isonitrile, present in natural substrates, by a common carbonyl group. This mild, late-stage chlorination using NaCl as halogen source provides the unique possibility to functionalize unactivated, aliphatic C(sp³)-H bonds in the presence of more electron-rich functional groups, which is a remaining challenge in today's synthesis toolbox.^{6,12} We also demonstrated that our WeIO15 variant panel can be used for late-stage chlorination on mg-scale with perfect regio- and stereoselectivity (>99.9%) and excellent chemoselectivity (96-99%) under mild reaction conditions. With our findings, further engineering projects can be pursued towards substrates that have broader purposes. While this manuscript was under review, Hayashi et al.⁷⁵ demonstrated comparable evolutionary success with a martinelline-derived fragment, and Neugebauer et al.⁷⁶ identified a new family member acting on amino acids. Hayashi et al.⁷⁵ confirmed the importance of V81, A82 and A88 for the adaptation of this enzyme class towards non-natural substrates. Their and our residues might be considered as an important starting point for future engineering studies to improve the turnover number and binding constant of new non-natural substrates. We expect that structural investigations with our variants will reveal the ketone binding mode and ultimately lead to a deeper insight into the structure-function relationship for this enzyme class.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

- Experimental details and analytical data (PDF)
- X-ray data for compound **1-3** (CIF, CIF, CIF) and Wi-0, (PDB 6GEM).

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Notes

The authors declare no competing financial interests.

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