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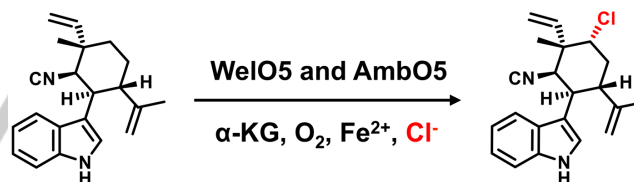
# Evolved Aliphatic Halogenases Enable Regio-complementary C-H Functionalization of an Added-Value Chemical

Takahiro Hayashi,<sup>[a]</sup> Mathieu Ligibel,<sup>[b]</sup> Emine Sager,<sup>[b]</sup> Moritz Voss,<sup>[a]</sup> Jürg Hunziker,<sup>[b]</sup> Kirsten Schroer,<sup>[b]</sup> Radka Snajdrova,<sup>[b]</sup> and Rebecca Buller\*<sup>[a]</sup>

**Abstract:** Non-heme iron halogenases represent synthetically valuable biocatalysts that are capable of halogenating unactivated sp<sup>3</sup>-hybridized carbon centers with exquisite stereo- and regioselectivity. The reported substrate scope of these enzymes, however, is limited primarily to the natural substrates and their analogues. Here we engineered the recently discovered halogenase WelO5\* for chlorination of a pharmaceutically interesting martinelline-derived fragment. Using structure-guided evolution, a halogenase variant with a more than 290-fold higher total turnover number and a 400-fold higher apparent *k*<sub>cat</sub> compared to the wildtype enzyme was generated. Moreover, we identified key positions in the active site which allowed directing the halogen to different positions in our target substrate. This report provides the first example of enzyme engineering to expand the substrate scope of a non-heme iron halogenase beyond the native indole alkaloid-type substrates. The highly evolvable nature of WelO5\* underscores the usefulness of this enzyme family for the late-stage halogenation of value-added chemicals.

The selective installation of a halogen atom into an unactivated, aliphatic sp<sup>3</sup> carbon center is synthetically useful for the production of high-value chemicals, including pharmaceuticals and agrochemicals.<sup>[1]</sup> The substitution of a hydrogen with a halogen atom alters the molecule's properties and may affect the molecule's metabolism and pharmacokinetic profile. In addition, C-X motifs represent useful synthetic handles for the modification of the molecule in further chemical steps. Traditionally, haloalkanes are prepared through synthetic approaches involving functional group transformation using corresponding alcohols,<sup>[2]</sup> alkenes,<sup>[3]</sup> and acids.<sup>[4]</sup> Modern approaches include light-driven<sup>[5]</sup> and rare metal-catalyzed reactions.<sup>[6]</sup> However, these methods generally suffer from lack of stereoselectivity and have specific demands on the substrate structure.<sup>[5c, 6c]</sup> In contrast, (chemo)-enzymatic methods of halogenation need only mild ambient conditions in aqueous reaction media and often exhibit excellent regio- and stereoselectivity, providing new opportunities to overcome the hurdles imposed by the conventional methods.<sup>[7]</sup>

The recently discovered aliphatic halogenases AmbO5, WelO5 and WelO5\* belong to the family of iron/α-ketoglutarate (Fe/α-KG) dependent oxygenases and are the only known members of this enzyme class capable of acting on freestanding substrates.<sup>[8]</sup> These enzymes share an overall high sequence identity (> 79%), whereas WelO5 and WelO5\* stem from different isolates of the same organism named *Hapalosiphon welwitschii* and differ only by 15-amino acids (Figure S1).<sup>[8d, 9]</sup> In nature, these enzymes catalyze the installation of chlorine atoms into several complex indole alkaloids (Scheme 1).<sup>[9]</sup> The catalytic mechanism involves the generation of a high valent Fe<sup>IV</sup>=O intermediate which abstracts a hydrogen atom from the substrate to yield a carbon radical (Figure S2). The carbon radical is then coupled to the iron coordinated chlorine (rather than the hydroxyl), affording the corresponding chlorinated compound in a regio- and stereoselective manner.<sup>[10]</sup> Despite the remarkable catalytic properties of these enzymes, non-heme iron halogenases have not yet been exploited for the chemical transformation of non-indole alkaloid-type substrates. Thus, to expand the biocatalyst toolbox available for aliphatic halogenations, we employed protein engineering approaches to create enzymes capable of catalyzing the chemically challenging sp<sup>3</sup> halogenation of complex molecules beyond the natural substrate scope.



**Scheme 1.** Halogenation of 12-*epi*-hapalindole C catalyzed by WelO5 and AmbO5.

Taking a first step in this direction, we report the evolution of the recently described non-heme iron halogenase WelO5\* from *Hapalosiphon welwitschii* IC-52-3<sup>[8d]</sup> for selective halogenation of substrate **1**, an analogue of the martinelline core structure<sup>[11]</sup> (Figure 1a), for use in medicinal chemistry. While martinelline is a potent bradykinin receptor agonist, anticancer activity of the structurally related substrate **1** and some analogues were reported.<sup>[12]</sup>

Exploiting WelO5\*'s promiscuous activity toward the target substrate, we optimized its halogenation activity using structure-guided directed evolution. Within two evolutionary rounds, we engineered a set of two regio-complementary halogenases capable of producing distinct chlorinated compounds (i.e., **1a** and **1b**) with exquisite regio- and stereoselectivity (Figure 1a). Simultaneously, we improved the apparent *K*<sub>cat</sub> and total turnover number (TTN) of the enzyme by more than 400- and 290-fold, respectively, compared to the starting variant. These results demonstrate the highly evolvable nature of the non-heme iron

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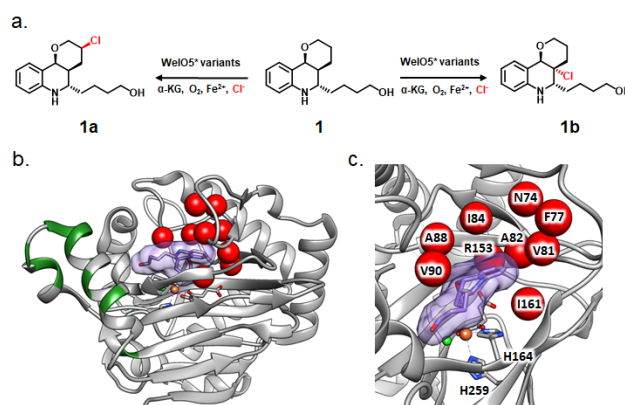
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halogenase WelO5\* and highlight the value of this class of enzymes for selective halogenation of small molecules.

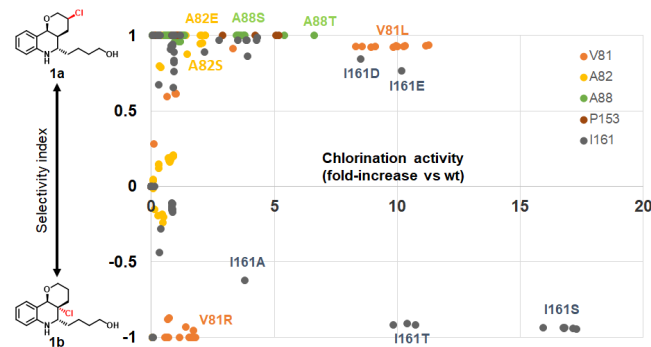
To identify a suitable starting scaffold for evolution, a panel of recombinant carrier-protein independent "stand-alone" aliphatic halogenases (WelO5, AmbO5, WelO5\*, and an engineered SadA variant<sup>[13]</sup>), overexpressed in *E. coli* BL21 (DE3), were used for crude cell-lysate biotransformations of the martinelline-derived fragment **1** in a deep well plate. While no halogenase activity toward the target substrate was detected for WelO5, AmbO5, and the SadA variant, the liquid chromatography coupled to mass spectrometry (LC-MS) analysis of the biotransformation with WelO5\* showed formation of two prominent products with *m/z* ratios of 278.2 and 296.1, consistent with the calculated mass of a hydroxylated and a chlorinated product of **1**, respectively (Figure S3). The structures of the chlorinated product **1a** and hydroxylated product **2a** were solved using nuclear magnetic resonance (NMR) analysis confirming chlorination and hydroxylation of aliphatic carbon centers of **1** (Figure 1a, Table 2). It is noteworthy that although wildtype WelO5\* exhibited promising stereo- and regioselective halogenation activity toward the unnatural substrate **1**, reaction selectivity of the enzyme (i.e., halogenation vs hydroxylation) was low and the hydroxylated compound **2a** was generated in significant amount (~40%, as compared to ~1% yield for the chlorinated product **1a**). Interestingly, the biotransformation of 12-*epi*-hapalindole C, which has been described as a native substrate of WelO5\*,<sup>[8d]</sup> led to the formation of a similar amount of hydroxylated product (~50%) besides the desired chlorinated product 12-*epi*-hapalindole E (~25%, Scheme 1 and Figure S4). This low reaction selectivity displayed by WelO5\* for at least some of its accepted substrates was not unexpected. A previous study on SyrB2, a carrier-protein dependent aliphatic halogenase, demonstrated that the reaction selectivity of this class of enzymes is sensitive to even subtle chemical modifications of their native substrates.<sup>[14]</sup> Based on the characterization of the Fe<sup>IV</sup>=O intermediate in SyrB2 by Mössbauer, Fe K-edge XAS, and EXFAS spectroscopies<sup>[10b]</sup> in combination with computational analysis,<sup>[15]</sup> chlorination versus hydroxylation selectivity was deduced to be governed by the orientation of the substrate with respect to the HO-Fe-Cl plane leading to either the Cl<sup>-</sup> or OH<sup>-</sup> rebound (Figure S2).

To generate a synthetically useful biocatalyst for the late stage-functionalization of the martinelline-derived fragment **1**, we employed structure-guided directed evolution to improve the chlorination activity of WelO5\*. To that end, our target substrate was docked into the iron-binding site of WelO5, a close homologue of WelO5\* with 95% sequence identity (Figure S1), using the Chimera AutoDock Vina tool (Figure 1b).<sup>[16]</sup> This led to the identification of nine residues for individual optimization by site-directed saturation mutagenesis using NNK codons. Each library was evaluated using a crude cell-lysate assay in a 96-well plate format. Out of the nine libraries screened, three libraries (A82, A88, and R153) included variants with a moderate (3 to 5-fold) increase in halogenation activity and two libraries (V81 and I161) comprised variants with significant (10- to 20-fold) improvement in activity compared to the wildtype variant (Figure 2 and 3a). Promising hits were collected and characterized by gene sequencing, identifying mutations beneficial for the halogenation activity (Figure 2).



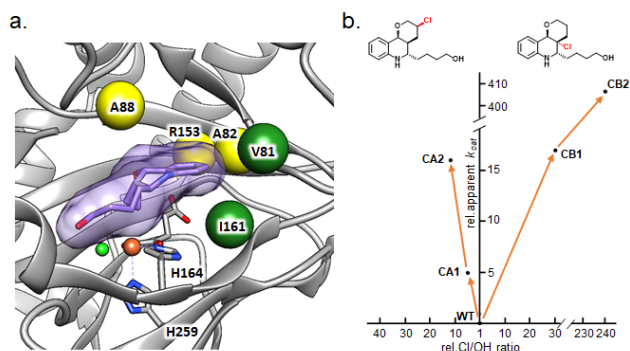
**Figure 1.** a) Regio-complementary chlorination of a martinelline-derived fragment mediated by WelO5\* variants and b) a substrate-docked model of WelO5 (PDB ID: 5iq1). The residues differing between WelO5 and WelO5\* are highlighted in green. The red spheres indicate the residues targeted for randomization. c) Zoom-in view of the active site of the substrate-docked model.

While screening the individual libraries, we observed the formation of an alternative chlorinated product **1b**, which was evidenced by the shifted retention time in the LC chromatogram compared to product **1a** (Figure S8b). NMR analysis of the new species confirmed installation of the Cl functional group at an alternative sp<sup>3</sup> carbon center (product **1b** in Figure 1a). Interestingly, product **1b** was exclusively generated by variants in which positions V81 and I161 had been mutated, suggesting that these positions serve as regioselectivity switches and dictate formation of either chlorination product **1a** or **1b** (Figure 2) depending on amino acid substitution. By plotting the relationship of halogenation activity and selectivity (formation of product **1a** vs **1b**) of the variants screened (Figure 2), two biocatalysts CA1 (V81L) and CB1 (I161S) were identified which exhibited improved activity and high selectivity to produce halogenated compounds **1a** and **1b**, respectively (Table 1). Notably, the evolved variants showed increased chlorination activity while the hydroxylation activity was not impacted or, in some cases, even diminished, thus simultaneously increasing reaction selectivity (Figure S5).



**Figure 2.** Relationship of chlorination activity and selectivity of variants screened in the first round. The activity was based on crude-cell lysate assays and normalized to the wildtype. The mutations in selected variants are annotated.

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**Figure 3.** a) Mutational “hot spots” identified in this study (yellow: 3- to 5-fold improvement, green: 10- to 20-fold improvement) b) evolutionary trajectory of the evolved variants CA2 and CB2.

Intrigued by these results, the halogenase WelO5\* was separately evolved for the two chlorination reactions. Iterative saturation mutagenesis (ISM)<sup>[17]</sup> was employed to improve the efficiency of variant CA1. Full randomization of residues 88, 153, and 161 by site-directed saturation mutagenesis afforded one variant with improved chlorination activity towards **1a**. Interestingly, variant V81L\_I161M, dubbed CA2, also exhibited shifted hydroxylation regioselectivity and produced two new hydroxylated products, the diastereoisomers **2b** (~31%) and **2c** (~5%), in which the hydroxylation occurred on the same  $sp^3$  carbon as the halogenation (Table 2). The variant CA2 was subsequently produced, purified and analysed *in vitro* showing a further 10-fold improvement in the halogenation activity compared to variant CA1 (Table 1). Despite the achieved overall improvements of reaction selectivity and of the apparent  $k_{cat}$  (12- and 16-fold, respectively), it should be noted that the evolved halogenase variant CA2 still transforms substrate **1** predominantly into hydroxylated products (Table 2). While the amount of halogenated product generated by CA2 is sufficient for medicinal chemistry studies,<sup>[18]</sup> additional optimization of the enzyme will be necessary to further expand its application scope.

A simultaneous two-site randomization was performed to further improve variant CB1 for the selective production of **1b**. A library was constructed using a degenerate SKA codon at position 81 and a VNT codon at position 161. In crude cell-lysate biotransformations, the best variant V81R\_I161S, named CB2, showed nearly complete consumption of substrate **1** and corresponding formation of **1b**, while the undesired hydroxylation reaction was further minimized (<5%). Subsequent *in vitro* analysis of purified CB2 exhibited a significantly improved apparent  $k_{cat}$  of up to 400-fold and a more than 290-fold higher TTN compared to the wildtype (Figure 3b, Table 1). Furthermore, recording of the Michaelis–Menten plot of CB2 showed improved enzyme kinetic parameters for the halogenation of substrate **1** ( $K_M = 0.67$  mM,  $k_{cat} = 3.0$  min<sup>-1</sup>), which are similar to those displayed by the wildtype enzymes acting on the native substrates (Figure S9d).<sup>[9]</sup>

Following our enzyme engineering campaign, we explored the capability of the most active evolved variant (CB2) to install other halogens into **1**. Introduction of alternative halogen salts (500 mM NaBr, NaI, or NaF) instead of NaCl into the reaction mixture was carried out to determine anion promiscuity. While iodine and fluoride were not accepted, presumably due to sterical

and electronic reasons, incubation with the bromide salt generated a product with a  $m/z$  ratio of 340.0 consistent with the calculated mass of the brominated compound (Figure S6). Overall, however, the enzyme prefers the smaller chloride anion (Figure S6a) as evidenced by the predominant formation of **1b** even in the presence of excess NaBr, in line with reported results for the SadA variant<sup>[13]</sup> and WelO5.<sup>[19]</sup>

To assess the practical applicability of the evolved variants to synthesize value-added compounds for medicinal chemistry, the biocatalytic reaction was scaled up using an increased substrate load. Chlorination of substrate **1** at 10 mM substrate concentration using crude cell-lysate of the evolved variant CB2 afforded 95% conversion into **1b** (Figure S10). Notably, no major side product (i.e., hydroxylation or other halogenation product) was observed for this variant. The non-optimized preparative experiments with variant CA2 (108 mg substrate **1**, final concentration 2 mM) and CB2 (43.2 mg substrate **1**, final concentration 2 mM) led to isolated yields of 6% (7.7 mg) for product **1a** and 30% (14.4 mg) of product **1b**, respectively, generating sufficient amounts for structure-activity relationship (SAR) studies.<sup>[18]</sup> While further catalyst and process optimization are required, these results demonstrate the possibility to evolve non-heme iron halogenases such as variant CB2 for the synthesis of the chlorinated martinelline fragment **1b**.

**Table 1.** *in vitro* assay results of wildtype and evolved variants of WelO5\*.

Variant (mutation)	TTN	rel. TTN	app. $k_{cat}$ / min <sup>-1</sup>	rel. app. $k_{cat}$
WT	0.11 ± 0.02	1	0.0045 ± 0.0005	1
CA1 (V81L)	0.63 ± 0.08	5	0.024 ± 0.003	5
CA2 (V81L, I161M)	5.6 ± 0.6	51	0.073 ± 0.008	16
CB1 (I161S)	1.6 ± 0.2	14	0.08 ± 0.02	17
CB2 (V81R, I161S)	32.9 ± 1.1	299	1.83 ± 0.09	407

Recent efforts to exploit halogenases for the selective halogenation of unnatural substrates have been focused primarily on flavin-dependent aromatic halogenases.<sup>[20]</sup> In this report, we outline the evolution of WelO5\*, a non-heme iron dependent halogenase, for the selective halogenation of unactivated, aliphatic  $sp^3$  carbon centers in the non-natural substrate **1**. With the introduction of only two mutations, the apparent  $k_{cat}$  and TTN number for halogenation of the martinelline-derived fragment could be improved by two orders of magnitude, highlighting that modulation of substrate scope and regioselectivity in WelO5\* does not necessarily rely on complex, synergistic arrays of functional residues that may limit evolvability. In addition, our results attest to the malleability of non-heme iron halogenases for regiochemical reprogramming while maintaining their high enantioselectivity, suggesting that the construction of an ‘enzyme



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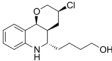
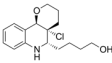
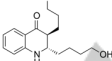
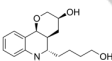
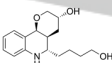
toolbox' for aliphatic halogenations is achievable. Interestingly, the initial low reaction selectivity, i.e. low halogenation versus hydroxylation ratio, could be improved by more than 200-fold during evolution. Our most active variant CB2 showed significantly reduced hydroxylation activity (<5%) indicating the introduction of structural elements that may facilitate the chlorine rebound (over hydroxyl rebound) to the target carbon radical.

While the detailed mechanism behind the improved regio- and reaction selectivity of the evolved variants remains unclear, we attempted to shed light on the selectivity governing factors by carrying out a docking study using the available crystal structure of WelO5 (PDB ID: 5IQT), a close homologue of WelO5\*, with substrate **1**. Taking into consideration available literature data,<sup>[10b, 14]</sup> our docking study suggests that substrate positioning with respect to the iron-oxo and the Cl/OH ligands dictates both, regioselectivity and reaction selectivity. Two major substrate binding modes were observed: While wildtype halogenation at C12 is well predicted by binding mode A, hydroxylation by WelO5\* seems more likely to occur in binding mode B (Figure S11). The set of mutations acquired in CA2 presumably improved regioselectivity by favoring binding mode A and slightly shifting the substrate toward the iron center, allowing H-abstraction from a single C-H bond, namely C12, instead of at two different positions as observed for the wildtype enzyme. In fact, both the chlorinated product **1a** and the major hydroxylated product **2b** show the same regio- and stereoselectivity (Table 2). CB variants, on the other hand, show chlorination activity consistent with the binding mode B (Figure S11c). The key mutation I161S, which was acquired during the first round of evolution, is assumed to stabilize this binding mode facilitating the efficient H-atom abstraction on the tertiary carbon center C9. The high reaction selectivity of variant CB2 can potentially be ascribed to a combination of the substrate positioning and the inherent reactivity of Cl over OH.<sup>[21]</sup>

To gain further insight into the improved activity of the evolved variants, we additionally performed homology modelling using I-TASSER.<sup>[22]</sup> Comparison of the starting variant with the evolved variant CB2 (V81R\_I161S) showed that the replacement of isoleucine with serine at position 161 significantly widened the active site pocket, presumably facilitating substrate access (Figure S12).

Total turnover numbers of non-heme iron halogenases are reportedly low due to auto-oxidative inactivation. The carrier-protein dependent SyrB2, for example, has been shown to only catalyze  $7 \pm 2$  turnovers on its native substrate L-Thr-S-SyrB1 prior to inactivation,<sup>[23]</sup> CmaB is active for  $16 \pm 6$  turnovers on its substrate L-*allo*-Ile-S-CmaD,<sup>[24]</sup> while the free-standing halogenase WelO5 exhibits approximately 75 turnovers in the biotransformation of its native substrate.<sup>[6a]</sup> In this study we present that enzyme engineering allows to create halogenase variants with "natural" catalytic performance albeit for non-natural substrates (CA2:  $5.6 \pm 0.1$  turnovers, CB2:  $32.9 \pm 1.1$  turnovers, Table 1). During only two rounds of evolution the total turnover numbers of CA2 and CB2 could be increased by 50- and 300-fold, respectively, boding well for the expansion of the halogenases' application scope as straightforward reshaping of the active site was sufficient to enhance the precise functioning of the catalytic machinery.

**Table 2.** Distribution of the hydroxylated and halogenated biotransformation products from the wildtype and the evolved variants based on their isolated yields.

Compound/variant	WT <sup>[a]</sup>	CA2	CB2 <sup>[b]</sup>
 <b>1a</b>	1%	6%	
 <b>1b</b>			30%
 <b>2a</b>	40%		
 <b>2b</b>		31%	
 <b>2c</b>		5%	

[a] conversion values for WT WelO5\* were determined by LC-MS analysis.

[b] variant CB2 produced < 5% hydroxylated product

Future efforts to understand the underlying mechanisms for halogenation of non-native substrates will focus on the structural analysis of the evolved variants which display improved activity toward selective halogenation reactions. Such efforts could lead to general engineering principles for this emerging class of enzymes and thus enable selective late-stage halogenation of traditionally difficult-to-derivatize target substrates.

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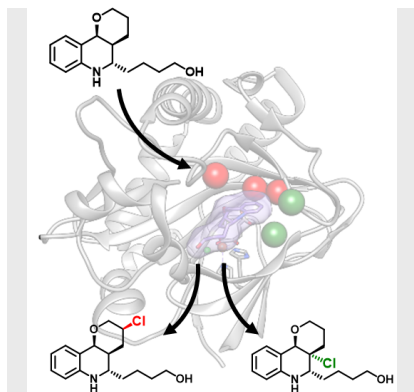
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## COMMUNICATION

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Beyond natural substrates: A protein catalyst was engineered to perform selective halogenation of a value-added chemical. Directed evolution of non-heme iron halogenase WelO5\* afforded a variant with an up to 400-fold higher apparent  $k_{cat}$  and a 290-fold higher TTN than the wildtype enzyme while achieving high stereo- and regioselectivity (>99%).



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