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Industrializing Biocatalysis

Katrin Hecht and Rebecca Buller[§,*]

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Abstract: Biocatalysis leverages enzymes, nature's catalysts, to enhance essential steps in chemical synthesis, thereby promoting more sustainable and efficient processes. Enzymes, macromolecular proteins, catalyze reactions with precision and efficiency in all living organisms. These biocatalysts have been honed over millenia for their specific roles within a biological system; however, they can be effectively reengineered to address novel challenges through recent advancements in molecular biology and bioinformatics. In this review, we present selected enzyme sourcing and engineering examples from our laboratory demonstrating the transition of enzymatic processes from academic research to application in Swiss industries.

Keywords: Biocatalysis · Bioinformatics · Enzyme discovery · Enzyme engineering · Late-stage functionalization

Dr. Katrin Hecht studied Biology at the University of Regensburg, Germany and obtained her PhD in Physical Biochemistry (Prof. Rainer Jaenicke). Her main interest is in the structure and function of proteins, which she studied previously in various prokaryotic and eukaryotic organisms as well as archaebacteria. In 2016 she joined the Biocatalysis group of Prof. Rebecca Buller, at the Zurich University of Applied Sciences (ZHAW).

Prof. Rebecca Buller is a Biological Chemist and Professor for Biotechnological Methods, Systems and Processes at the Zurich University of Applied Sciences. Rebecca Buller studied Chemistry at the Westfälische – Wilhelms Universität Münster (D) and the University of California Santa Barbara (US). After completing her PhD with a focus on enzyme engineering at ETH Zurich, Rebecca Buller accepted a position as laboratory head at the flavour and fragrance company Firmenich (CH). In 2015, she relocated to the Zurich University of Applied Sciences where she founded the Competence Center for Biocatalysis (CCBIO). Research in Rebecca Buller's laboratory focusses on the expansion of the biocatalytic toolbox by sourcing and engineering enzymes for synthetic applications.

1. Biocatalysis in the Chemical Industry

Synthetic chemistry plays a pivotal role in providing essential goods for daily life, ensuring our well-being, nutrition, and health. The industry's remarkable progress is underpinned by the early work of synthetic chemists who formulated powerful reaction schemes and catalysts, primarily focusing on processing petroleum-based starting materials into valuable new products.[1] However, biological systems may outperform synthetic chemistry in terms of efficiency and selectivity, especially when applied to construct highly complex molecules. Moreover, organic synthetic methods often rely on precious metals or extreme reaction conditions and can produce significant amounts of waste leading to environmental concerns. Taken together, these factors drive the adoption of environmentally benign technologies such as biocatalysis into the chemical industry to enhance the sustainability, energy efficiency, and ultimately, cost-effectiveness of production processes.[2]

Historically, wildtype enzymes have been employed for breaking down oils and proteins, producing semisynthetic antibiotics, and synthesizing simple chiral precursors for the pharmaceutical sector as well as basic chemicals such as acrylamide for polymers.[3] In recent years, significant advancements in experimental and computational technologies have facilitated the creation of tailored enzymes to synthesize complex molecules, modify biological therapeutics and degrade plastic waste.

Against this backdrop, we present recent work from our laboratory conducted in close collaboration with industrial partners across several sectors, including the flavor and fragrance industry (dsm-firmenich, Givaudan),[4,5] pharmaceutical chemistry (Roche, Novartis),[6,7] and agrochemistry (Syngenta).[7]

2. Biocatalytic Manufacture of Flavors, Fragrances and Natural Colorants

2.1 *Flavors: Biocatalytic Reduction of 2E-decenal*

Wildtype enzymes are an important tool for the flavor and fragrance industry to synthesize organoleptically valuable compounds from flavor precursors.[8,9] In this context, ene-reductases (ER) represent an interesting family of enzymes because they catalyze the chemo- and stereoselective hydrogenation of a broad variety of α/β-unsaturated alkenes that are activated by at least one electron withdrawing group.[10]

In collaboration with dsm-firmenich, we set out to harness ene-reductases to develop a biobased alternative to the metal- or organocatalyzed reduction of activated alkenes to form products of organoleptic interest.[4] Toward this goal, we established a library of twenty wildtype ERs[11] expressible in *E. coli* and screened all variants for their ability to reduce 2*E*-decenal and an undisclosed flavor precursor. To recycle the expensive cofactor

*Correspondence: Prof. R. Buller, E-mail: rebecca.buller@zhaw.ch
Institute of Chemistry and Biotechnology, Zurich University of Applied Sciences, CH-8820 Wädenswil, Switzerland

nicotinamide adenine dinucleotide phosphate (NADPH), an *in situ* recycling system consisting of glucose and glucose dehydrogenase (GDH) was implemented. In the initial screening, we identified several enzymes with appreciable reduction capacities of 2*E*-decenal and the second target substrate, even at elevated substrate loads (10 g/L). Considering factors such as thermostability, solubility, and activity at reduced temperature (10 °C), the ene-reductase *PBr*-ER from *Pseudomonas brassicacearum* was selected for further development. Under optimized conditions, the enzyme catalyzed the reduction of 2*E*-decenal at a concentration of 40 g/L to yield >93% of the desaturated product within 24 h, while the other target substrate could be applied at a concentration of 60 g/L yielding > 83% of the desired product within 80 h (100 mL scale). Notably, *PBr*-ER could be stored in the form of cell pellets at –20 °C for at least 14 months without significant loss of activity.[4]

2.2 *Fragrances: Asymmetric Synthesis of Ionones by Engineered SHCs*

Ionones are key fragrance molecules contributing to the scent of many fruits and flowers. Unsurprisingly, the synthesis of racemic ionones from pseudoionones was established as early as the 19th century, making them some of the first synthetic fragrances to be used commercially.[12] Notably, however, ionone enantiomers exhibit distinct organoleptic properties. In the case of γ-dihydroionones, for example, the (*R*)-(-)-isomer (**2**) has a six-fold lower perception threshold than the (*S*)-(+)-isomer (**7**). Additionally, the natural isomer (**7**) contains notes akin to flowers, specifically with an orris-like character, as well as the highly valued animalic aroma reminiscent of aged ambergris.[13]

Thus, to gain access to the enantiopure γ-dihydroionones (**2**, **7**), we teamed up with Givaudan to develop an asymmetric biocatalytic synthesis route.[5] Given the reported methods for accessing enantiomers of γ-dihydroionone,[13] we chose to investigate the most apparent yet previously unexplored approach: the asymmetric cation-olefin cyclization of pseudoionone or one of its derivatives. Selecting the easily accessible (*E*/*Z*)-geranylacetone (**1**, **3**) as our starting material (Fig. 1), we decided to employ squalene-hopene cyclases (SHCs) as the biocatalysts.[5] SHCs catalyze cyclisation reactions of polyene substrates with perfect stereocontrol and were shown to be highly evolvable.[14]

As a first step in our project, we constructed and screened a library of 31 natural SHC homologues expressed in *E. coli,* which we had obtained through genome mining. Among them, *Aci*SHC from the thermophilic bacterium *Acidothermus cellulolyticus* produced a small amount of the desired monocyclic compound (*R*)-γ-dihydroionone (**2**), alongside larger quantities of an unwanted bicyclic side product (**4**) (0.7% versus 2.3%, respectively). Using directed evolution, we improved the performance of the enzyme in two consecutive engineering rounds. The resulting variant, *Aci*SHC_2.3, had acquired four mutations and exhibited a 30-fold increase in conversion toward the monocyclic product when exposed to (*E*/*Z*)-geranylacetone.

Docking studies suggested that the binding modes of the two substrate isomers (*E*-geranylacetone *vs*. *Z*-geranylacetone) within the active site of *Aci*SHC_2.3 dictates whether the reaction yields the desired monocyclic or the alternative bicyclic product. While the (*E*)-isomer (**3**) was exclusively converted into the undesired (*S*,*S*)-linked bicyclic enolether (**4**), the (*Z*)-isomer (**1**) yielded the targeted monocyclic (*R*)-γ-dihydroionone (79% yield in 48h; >99% *ee*). Based on this mechanistic insight, we investigated the synthesis of the enantio complementary (*S*)-γ-dihydroionone, a key precursor to (-)-α-ambrinol (**6**) and a valuable woody-ambery fragrance ingredient. To achieve this, we protected the carbonyl group of (*E*)-geranylacetone (**5**), blocking the second cyclization step. This substrate engineering strategy successfully furnished (*S*)-γ-dihydroionone (**7**) with >99% *ee*.

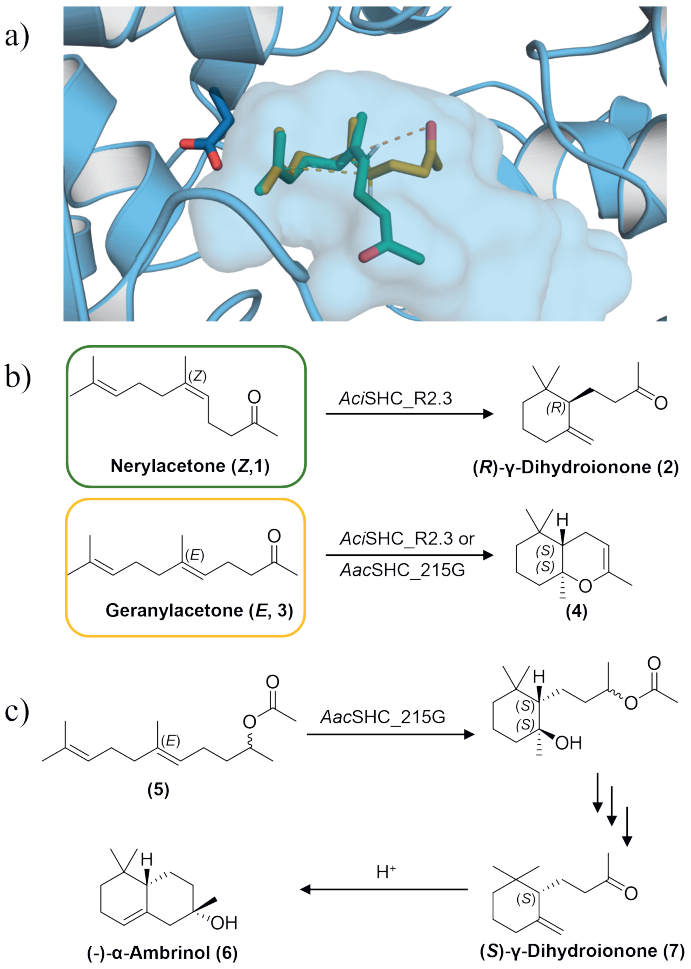

Fig. 1. Harnessing enzyme and substrate engineering allowed synthetic access to both stereoisomers of γ-dihydroionone.[5] a) Snapshot of the active site of *Aci*SHC accommodating *Z*-geranylacetone (green) and *E*-geranylacetone (yellow). The homology model of *Aci*SHC was prepared with SWISS MODEL, docking of the small molecules was performed with AutoDock Vina. b) Production of (*R*)-γ-dihydroionone (**2**) using engineered SHC *Asi*SHC_R2.3. c) Substrate engineering achieves synthesis of a precursor of the complementary (*S*)-γ-dihydroionone (**7**) using the squalene-hopene cyclase *Aac*SHC_215G.

Overall, our study demonstrates that the double-bond geometry of the starting material governs the stereoselectivity of the SHC-catalyzed cyclization and that a selective termination of the cascade after the initial ring formation can be effectively controlled through substrate engineering. These principles might provide a valuable framework for developing future SHC-catalyzed syntheses aimed at producing asymmetric cyclic natural products.

2.3 *Natural Products: Biosynthesis of Anthocyanidins*

The structural complexity of some natural products can render their chemical synthesis difficult. Consequently many natural compounds are traditionally isolated from plant material. However, this approach is limited by factors such as seasonal availability, ecological impact, and environmental variability, all of which affect quality. Moreover, the concentration of complex natural compounds in plant sources is typically very low.[15] As an alternative to directly isolating target compounds from their natural sources, researchers are increasingly turning to engineered prokaryotic or eukaryotic cell factories. These microbial or cell-based systems can be designed to replicate and optimize the natural enzymatic cascades, enabling scalable and controlled synthesis of valuable bioactive molecules.

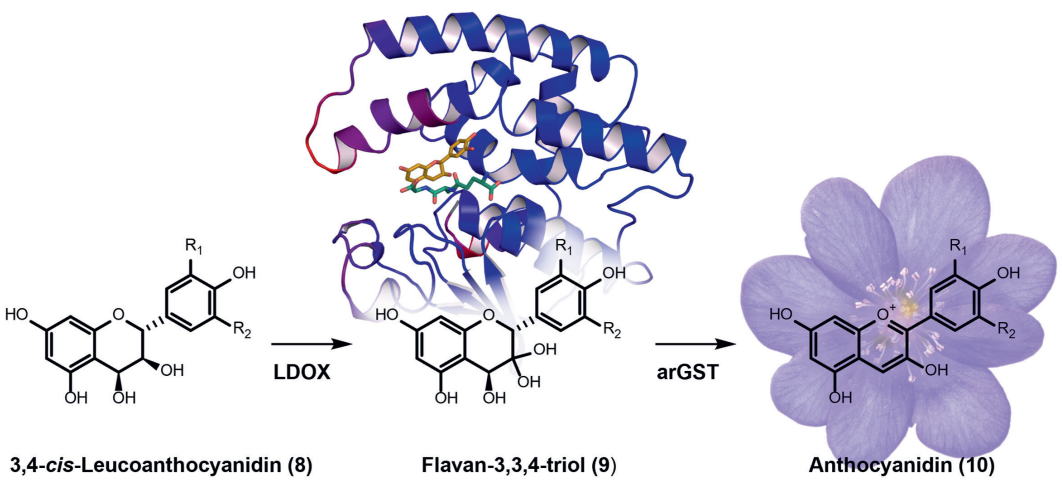

Fig. 2. Anthocyanin-related glutathione transferases (arGST) catalyze the last step in the biosynthesis of anthocyanins. The presence of arGST, here *Pt*GSTF8 from poplar, improves anthocyanidin synthesis from flavan-3,3,4-triol.[16] *Pt*GSTF8 (PDB: 8AGQ) is shown as a tertiary complex with (-)-catechin (orange) and GSH (green). Substitution at R_1 and R_2 defines the color of the product pigments: Cyanidin (R_1 = OH, R_2 = H) is reddish-purple to magenta, pelargonidin (R_1 = H, R_2 = H) is orange-reddish and delphinidin (R_1 = OH, R_2 = OH) appears purple to blue.

One long-standing goal in the development of cell factories has been the efficient production of anthocyanins. Anthocyanins are water-soluble pigments responsible for red, blue, and purple colors in flowering plants, fruits, and leaves. They attract interest as natural colorants, antioxidants, and bioactive compounds used in food, nutraceuticals, and cosmetics.[15] Efforts to produce anthocyanins in microbial cell factories, however, have so far resulted in only low yields of the colored products. Intrigued by the discrepancies between available *in vivo* and *in planta* data, we reconstructed the anthocyanin biosynthetic pathway in yeast and identified a crucial missing step: anthocyanin-related glutathione transferases (arGSTs), previously recognized mainly as anthocyanin transporters, also catalyze an essential step in the late anthocyanin pathway, the dehydration of flavan-3,3,4-triols (**9**) to anthocyanidins (**10**). Incorporating arGSTs into the yeast pathway increased the production titers of the colored end product by over 35-fold when starting from glucose (Fig. 2).[16]

3. Biocatalysis to Produce APIs and to Accelerate Medicinal Chemistry

3.1 *Active Pharmaceutical Ingredients: Asymmetric Reduction of an Ipatasertib Precursor*

Ipatasertib (**13**), a selective Akt inhibitor for metastatic prostate and triple-negative breast cancer, is synthesized in a 10-step sequence involving both chemical and enzymatic transformations to establish three stereocenters.[17,18] A key step in the synthesis is the KRED-catalyzed asymmetric reduction of a prochiral ketone (**11**) to the (*R*,*R*)-*trans* alcohol intermediate (**12**). To enhance the efficiency of this transformation we engineered a second generation high-performance ketoreductase (KRED) in collaboration with F. Hoffmann-La Roche Ltd (Fig. 3).[6,19]

In an initial screen, the NADPH-dependent reductase *Ssal*-KRED was selected from an in-house panel of 63 wildtype enzymes[20] for its absolute stereoselectivity. Through a combination of single-site and combinatorial saturation mutagenesis targeting active site and tunnel residues, and subsequent machine learning (Gaussian process)-guided library design, variant M3 with a 22-fold activity improvement over the wildtype enzyme was identified. Further rounds of rational and iterative mutagenesis targeting cofactor- and substrate-interacting residues yielded variant M6, harboring ten mutations, with a 64-fold increase in apparent k_{cat} and improved scaffold robustness. Preparative-scale reactions using M6 achieved ≥98% conversion and 99.7% diastereomeric excess at 100 g/L substrate loading, establishing a scalable and commercially viable biocatalytic step in ipatasertib (**13**) synthesis.

3.2 *Late-stage Functionalization: Enzymatic Halogenation of Small Molecules*

Late-stage functionalization is a powerful strategy in synthetic chemistry that enables the modification of complex molecules at advanced stages of their synthesis. By selectively introducing functional groups or modifying existing ones, late-stage func-

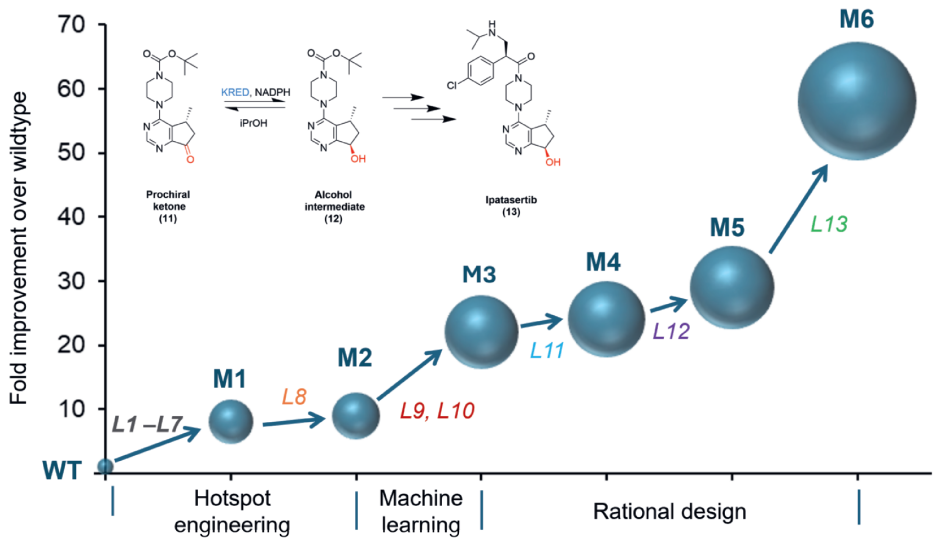

Fig. 3. Through the combination of mutational scanning, machine learning and structure-guided rational design a KRED variant was engineered that catalyzes a key step in the synthesis of ipatasertib, namely the reduction of a prochiral ketone (**11**) to the (*R*,*R*)-*trans* alcohol (**12**). The optimized variant M6 accumulated 10 amino acid substitutions and showed a 64-fold improved k_{cat} compared to the wildtype enzyme, with a conversion rate ≥ 98% and a diastereomeric excess of 99.7% (*R*,*R*-*trans*) at 100 g/L substrate load.[19]

tionalization facilitates structure-activity relationship studies, optimization of pharmacological properties, and efficient production of analogs, ultimately accelerating drug discovery and development.[21,22] In this context, halogenation is a particularly valuable strategy as the incorporation of a halogen atom influences a molecule's physical, chemical, and biological properties. In addition, halogens serve as versatile synthetic handles, enabling subsequent transformations such as cross-coupling reactions, which facilitate rapid further diversification.[23]

The asymmetric halogenation of C-H bonds is of particular interest as this reaction is not easily performed using synthetic chemistry.[24] Among known enzyme families, α-ketoglutarate dependent halogenases (α-KGHs) stand out as they can halogenate non-activated sp^3 C-H bonds with high stereo- and regioselectivity.[24] The enzyme family comprises halogenases, which act on protein-tethered substrates, but also a handful of enzymes such as WelO5/ WelO5* from *Hapalosiphon welwitschii* that are capable of halogenating free-standing molecules and therefore are more easily applied to synthetic chemistry challenges.[25]

Martinelline is a known antagonist of the bradykinin receptor, and structurally related analogues have demonstrated anti-cancer properties.[26–28] In collaboration with Novartis, we set out to analyze the structure-activity relationship of small molecules of this type. Toward this end, we engineered variants of the halogenase WelO5* to enable the selective chlorination of a martinelline-derived fragment.[7,29,30] In detail, we first constructed a wildtype library of α-ketoglutarate-dependent halogenases (α-KGHs) and screened them against the target molecule (**16**).[29] Among the wildtype enzymes tested, only WelO5* catalyzed chlorination of this compound, albeit with low initial activity. Guided by substrate docking studies, nine active-site residues were selected for site-saturation mutagenesis. Corresponding libraries were generated and screened, leading to the identification of chlorination at two distinct sites of the martinelline-derived fragment (site A: product (**14**) and site B: product (**15**)). The final optimized variant for halogenated product (**14**) was dubbed CA2 (V81L, I161M) and exhibited a 51-fold increase in total turnover number (TTN) and a 16-fold improvement in relative apparent k_{cat} compared to the parent WelO5*. Variant CB2 (V81R, I161S), engineered to produce product (**15**), demonstrated even greater performance improvements, with a 300-fold increase in TTN and a 400-fold improvement in relative apparent k_{cat}, while achieving near-exclusive chlorination at site B of the target molecule (Fig. 4).

Building on this initial study, which represented one of the first reported cases of expanding the substrate scope of freestanding α-ketoglutarate dependent halogenases, we evaluated whether we could selectively halogenate the target molecule (**16**) directly from its synthesis mixture, *i.e.* in the presence of its exo- and endoisomers. Satisfyingly, application of CB2 to this mixture resulted in selective chlorination of the isomer (**16**) to produce the desired product (**15**). In addition, we could construct a new WelO5* variant, MGA (V81M, A88G, I161A), capable of stereoselectively chlorinating the enantiocomplementary exoisomer (**17**). Remarkably, the inversion of substrate stereo-preference was achieved by mutating as little as three residues in the enzyme's active site.[30]

To explore the accessible halogenase substrate scope further, we teamed up with Syngenta to evolve a panel of halogenases capable of modifying the macrolide soraphen A (**19**), a mycobacterial polyketide characterized by an 18-membered macrocyclic ring. Soraphen A is of interest to agrochemistry as it exhibits potent antifungal activity through inhibition of acetyl-CoA carboxylase.[31]

When screening our in-house halogenase library, consisting of wildtype enzymes and engineered variants, we identified one enzyme, WelO5* (V81G, I161P), with low but detectable chlorination and hydroxylation activity on soraphen A at two positions

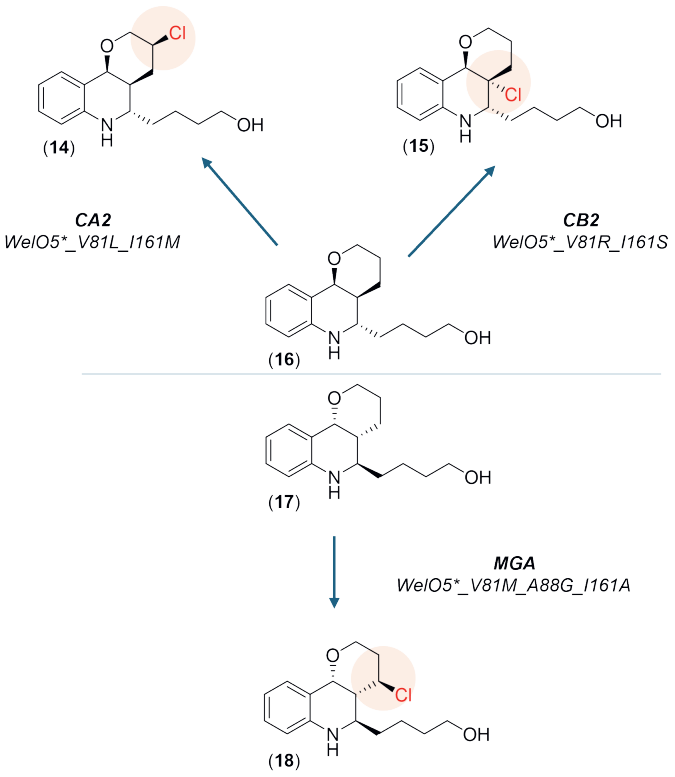

Fig. 4. Engineered variants of WelO5* selectively halogenate the exo-isomers (**16** and **17**) of a martinelline-derived fragment furnishing a set of uniquely derivatized and enantiopure products (**14**,**15**, and **18**).[29,30]

(**20** and **21**). Using a combination of rational enzyme library design and machine learning-guided directed evolution, we evolved an optimized variant, WelO5* (V81S, A88I, I161A). This enzyme exhibited a 100-fold improvement in TTN at site (**20**) compared to the parent enzyme, with a k_{cat} on par with those reported for wildtype halogenases acting on their native substrates. Notably, the resulting chlorinated derivatives of soraphen A retained antifungal activity as shown in phenotypic tests[7,32] and, in this way, the enzymatic late-stage functionalization helped pinpoint regions of the molecule critical for biological function, providing valuable insights for future structure-activity relationship (SAR) studies (Fig. 5).

3.3 *Expanding the Biocatalytic CH Activation Toolbox*

Despite the above-enumerated discovery and engineering efforts, the number of natural and engineered halogenases that act on freestanding substrates remains limited.[25] Most wildtype halogenases, such as WelO5,[33] AmbO5,[34] and BesD,[35] were identified through the analysis of biosynthetic pathways. Sequences from these initial hits were then used in bioinformatic screens to uncover additional halogenases; however, only a few enzymes could be identified in this way leaving a limited set of enzymes that can be further engineered.[29,36]

A possible strategy to supplement the biocatalytic halogenase toolbox is to start with an enzyme known to bind a desired substrate and reprogram it to catalyze a different reaction. This is a promising avenue forward in the halogenation case due to the structural and mechanistic similarities between Fe/αKG-dependent hydroxylases and halogenases.[37] A pioneering study, for example, highlighted the feasibility of reprogramming hydroxylase SadA into a halogenase. By mutating a single residue in the conserved His-X-(Asp/Glu)-Xn-His metal-binding motif, a variant (SadA_D157G) was generated that gained halogenation activity on its natural substrate.[38] Building on this concept, our group reprogrammed the L-proline *cis*-4-hydroxylase SmP4H from *Sinorhizobium meliloti* into a *cis*-3-halogenase.[39]

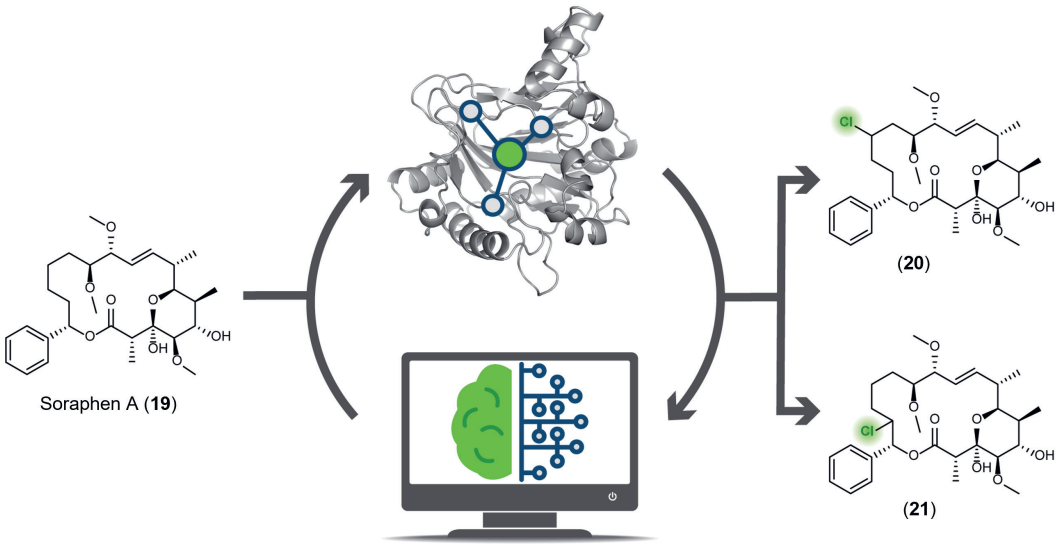

Fig. 5. WelO5* variants, which were engineered using a combination of smart library design and machine learning, stereo- and regioselectively halogenate soraphen A (**19**).[32]

To achieve this, we compared twenty literature-reported αKG hydroxylases with the reprogrammed SadA_D157G and the natural halogenase WelO5, and selected the five most similar enzymes in terms of sequence and structure. After introducing the critical aspartate/glutamate to glycine substitution into the active site, evaluation of the resulting enzyme variants revealed SmP4H_D108G to display chlorination activity at the C3 position of L-proline, while retaining C4 hydroxylation (Fig. 6).[39] Through four rounds of directed evolution, we achieved a nearly 100-fold improvement in the apparent k_{cat}/K_M for chlorination when using variant SmP4H-7 (V57L, S107T, D108G, D113E, T115P, R274H).

Using the same principle of reaction pathway reprogramming, we further engineered SmP4H to selectively desaturate the nonproteinogenic amino acid L-homophenylalanine (**24**) together with Novartis. Importantly, a single active site mutation (W40Y) switched the enzyme's function from hydroxylase to desaturase, likely due to tyrosine's role in the mechanism (Fig. 6). The engineered variants also showed expanded substrate scope, accepting other noncanonical amino acids like L-homotyrosine and (*S*)-α-amino-3,4-dichlorobenzenebutanoic acid.[40]

4. Discussion and Outlook

Our studies demonstrate that enzymes can be effectively tailored to meet the demands of specific tasks and reaction conditions in industrial settings. To accelerate their broader adoption, however, enzyme discovery and engineering must become more efficient. Promising strategies in this respect are the implementation of machine learning–powered enzyme discovery[41,42] and engineering,[43–45] including the construction of information-enriched enzyme libraries[46,47] and automated high-throughput screening,[48] as well as *de novo* enzyme design.[49]

Looking ahead, these technologies will not only accelerate the pace at which enzymes can be engineered but will also expand their catalytic capabilities beyond nature's repertoire. Integrating tailored and designed enzymes with transition metal catalysis, photocatalysis, or electrocatalysis, for example, is already becoming a reality in academia and will considerably broaden the chemical space accessible to biocatalysis.

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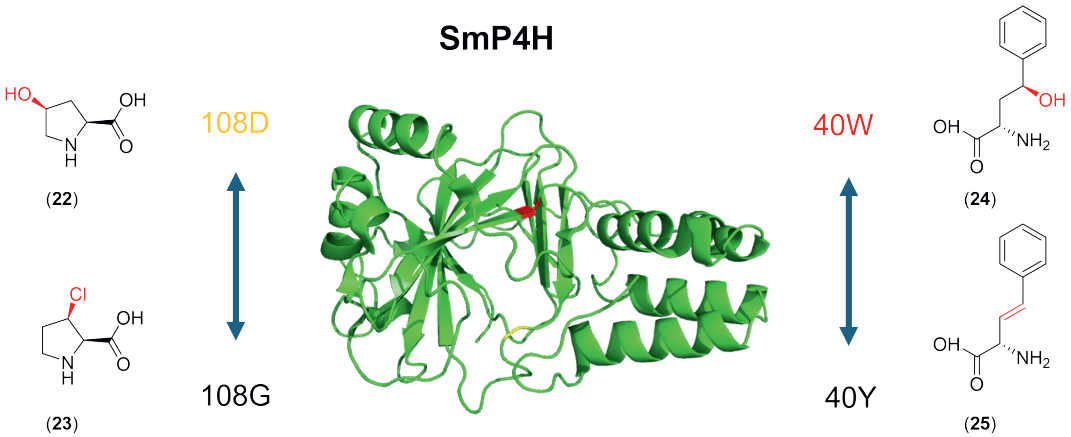

Fig. 6. Hydroxylase SmP4H from *Sinorhizobium meliloti* can be reprogrammed to carry out different reactions, such as hydroxylation, halogenation and desaturation of natural substrates (L-Pro (**22**)) and non-natural substrates (L-homophenylalanine (**24**)). Notably, a single amino acid change induces the reaction pathway switch from hydroxylation to desaturation[40] or from hydroxylation to halogenation.[39]

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