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Account

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A Continuing Career in Biocatalysis: Frances H. Arnold

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Abstract

On the occasion of Professor Frances H. Arnold's recent acceptance of the 2018 Nobel Prize in Chemistry, we honor her numerous contributions to the fields of directed evolution and biocatalysis. Arnold pioneered the development of directed evolution methods for engineering enzymes as biocatalysts. Her highly interdisciplinary research has provided a ground not only for understanding the mechanisms of enzyme evolution but also for developing commercially viable enzyme biocatalysts and biocatalytic processes. In this Account, we highlight some of her notable contributions in the past three decades in the development of foundational directed evolution methods and their applications in the design and engineering of enzymes with desired functions for biocatalysis. Her work has created a paradigm shift in the broad catalysis field.

Keywords: directed evolution, biocatalysis, enzyme engineering, P450s, abiological functions, carbene transfer reactions, nitrene transfer reactions, C–H functionalization

Introduction

Enzymes are the workhorses for nearly all biological processes on earth. Largely thanks to their exquisite specificity and selectivity, enzymes have been increasingly used as catalysts for synthesis of chemicals and materials. However, compared to chemical catalysts, enzymes often have limited stability, substrate scope and/or productivity, which significantly hinders their practical applications in industry. To address these key limitations in biocatalysis, in the early 1990s Frances Arnold took inspiration from nature and developed a simple yet powerful algorithm, called ‘directed evolution’, for evolving enzymes with tailored features.¹ In essence, this algorithm mimics the process of natural Darwinian evolution in the test tube and involves subjecting a gene encoding an enzyme to random mutagenesis, followed by screening or selection of the resulting library for enzyme variants that show improvement of the desired function. The improved enzyme variant is then used as the ‘parent’ for the following round of mutagenesis and screening or selection, and the process is repeated iteratively until the desired goal is attained (**Figure 1**). Around the

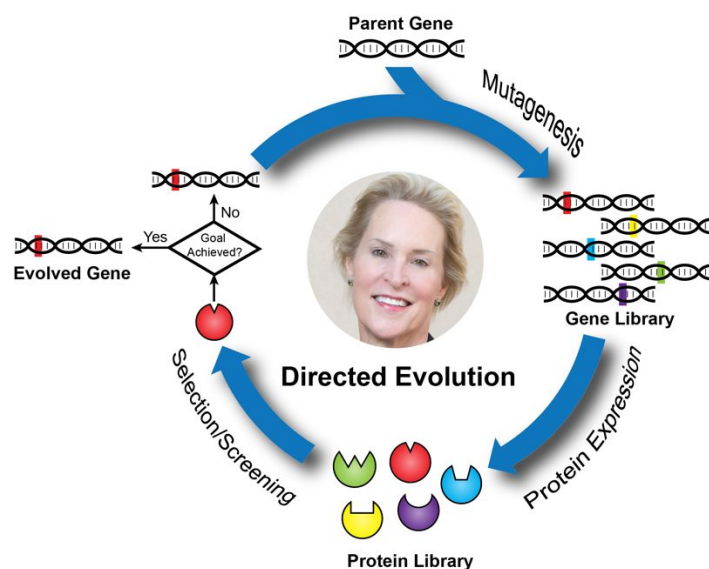


Figure 1. Workflow of a typical directed evolution experiment.

same time as this algorithm was reduced to practice by Arnold,² another pioneer of directed enzyme evolution, Willem P. C. Stemmer at Maxygen, developed and applied DNA shuffling methods to engineer

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3 enzymes with tailored functions.^{3, 4} In the subsequent years, Arnold, Stemmer, and several other protein
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5 engineers around the world, including Donald Hilvert,⁵ Manfred Reetz,⁶ and Uwe Bornscheuer,⁷ have
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7 applied this algorithm in combination with state-of-the-art tools from molecular biology, genetic
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9 engineering, analytical chemistry, and computational biology, to evolve enzymes with improved or novel
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11 functions and investigate the protein structure-function relationships and evolutionary mechanisms,
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13 resulting in a new field of directed evolution.
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16 Arnold is a card-carrying engineer who was trained as a mechanical engineer as an undergraduate
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18 student at Princeton University and a chemical engineer as a graduate student at the University of California
19
20 at Berkeley. Since she started her independent academic career at the Division of Chemistry and Chemical
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22 Engineering at Caltech in the mid-1980s, she has been exploring enzymes for biocatalytic applications and
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24 fundamental research. In this Account, we will first highlight some of her pioneering contributions in the
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26 development and application of directed evolution tools for enzyme engineering in the 1990s, which laid
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28 the foundation for the directed evolution field. We will then survey her work on the synthetically versatile
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30 P450 monooxygenases as an example to illustrate her group's key contributions in engineering enzymes
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32 with improved catalytic efficiency or new substrate specificity, and understanding the evolutionary
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34 mechanisms and protein structure-function relationships. Finally, we will highlight her recent contributions
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36 in evolving enzymes with abiological function, which has contributed to expand the boundaries of
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38 traditional biocatalysis. With the Arnold group continuing to be innovative and highly productive, this
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40 Account only serves as a mid-point review of Frances Arnold's long and illustrious career in biocatalysis.
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45 **I. Early Days in Directed Enzyme Evolution**

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47 Thanks to the advances in recombinant DNA technologies and protein crystallography, the
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49 mainstream school of thought in enzyme engineering in the 1980s was to use rational design approaches
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51 based on structural analysis and site-directed mutagenesis. Frances Arnold was among the first chemical
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53 engineers who adopted rational design approaches for enzyme engineering. For example, the Arnold group
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3 introduced rationally designed new metal binding sites into enzymes to improve their stability.^{8,9} However,
4
5 Arnold quickly recognized the limitations of rational design which was mainly due to our limited
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7 understanding of enzyme structure, function, and dynamics as well as catalytic mechanisms. Therefore, in
8
9 the late 1980s, inspired by the early studies of John Maynard Smith¹⁰ and Manfred Eigen¹¹, Arnold decided
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11 to explore molecular evolutionary strategies for engineering enzymes with improved stability and activity.
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14 In her seminal work², Arnold used iterative rounds of random mutagenesis coupled with screening
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16 to improve the activity of subtilisin E in 60% dimethylformamide by 256-fold. This work not only described
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18 the directed evolution algorithm and all the key steps in a directed evolution experiment, but also
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20 demonstrated that beneficial mutations could be accumulated gradually to yield drastic changes in protein
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22 functions and that many of these beneficial mutations were unexpected. This work inspired many other
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24 research groups to use directed evolution as an enzyme engineering strategy. However, one limitation with
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26 this random mutagenesis based directed evolution strategy was that deleterious mutations may be
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28 accumulated too, which resulted in less optimal enzymes. To address this limitation, Arnold group
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30 developed a staggered extension process (StEP) *in vitro* recombination method¹², which not only
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32 accumulates the beneficial mutations but also removes the deleterious mutations by randomly shuffling the
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34 improved genes during each round of directed evolution. In addition to StEP recombination, which was
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36 inspired by Stemmer's 'DNA shuffling' idea^{3,4}, Arnold's group developed a range of directed evolution
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38 tools and strategies, such as the optimized DNA shuffling method¹³, *in vitro* random priming recombination
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40 method¹⁴, digital imaging based on high throughput screening methods for oxygenases^{15,16}, and SCHEMA
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42 structure-guided recombination, which enables extension to more distantly-related genes¹⁷.
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46 Apart from developing new directed evolution tools and strategies, the Arnold group also applied
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48 them to solve some practical challenges in biocatalysis. For example, a *para*-nitrobenzyl esterase was
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50 engineered with improved stability¹⁸, as well as new substrate specificity and improved activity in aqueous-
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52 organic solvents¹⁹. *Para*-nitrobenzyl alcohol is used to protect carboxylic acid functionalities during the
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54 synthesis of cephalosporin-derived antibiotics, and the engineered *para*-nitrobenzyl esterase can be used to
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3 remove the *para*-nitrobenzyl alcohol protecting group during the synthesis of loracarbef, a fourth-
4 generation cephalosporin-derived antibiotic. In addition, the Arnold group engineered a subtilisin E variant
5 with drastically improved stability, which showed nearly identical thermal stability as a naturally occurring
6 thermostable subtilisin, thermitase, yet was highly active at a much broader spectrum of temperatures than
7 the thermitase²⁰.
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13 14 15 16 **II. P450 BM3: a model enzyme for directed evolution**

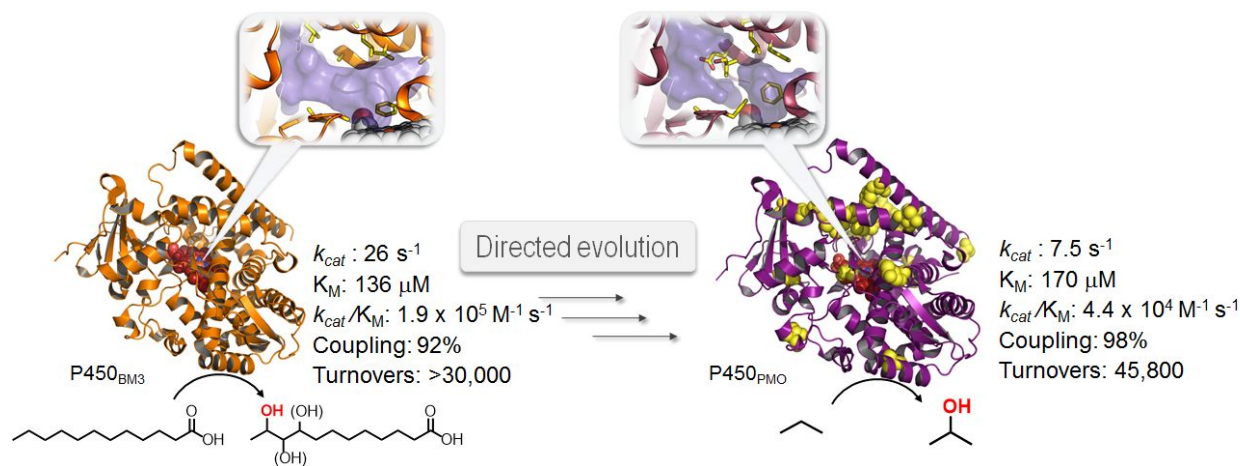
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18 Starting from the late 1990s, protein engineering efforts in the Arnold group began to focus on
19 P450 monooxygenases.^{21, 22} Cytochrome P450 monooxygenases make up a large class of heme-dependent
20 enzymes²³ which have evolved across all kingdoms of life to carry out a myriad of oxidation reactions,
21 while sharing a common three-dimensional fold.²⁴⁻²⁷ This enzyme superfamily provides an impressive
22 example of how nature can generate a broad range of oxidation catalysts from a common protein scaffold.
23
24 Whereas all these enzymes share a cysteine-ligated heme cofactor that is responsible for their activity, the
25 widely diverse substrate specificity of P450s is dictated by their protein sequences, which have diverged as
26 much as 90% over the course of millions of years of natural evolution.²⁴⁻²⁶
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35 Inspired by the functional pliability of natural P450s, the Arnold group identified cytochrome P450
36 from *Bacillus megaterium* (P450 BM3) a particularly attractive target for directed evolution experiments.
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38 At that time, P450 BM3 was one of very few known P450s in which the heme domain is fused to a diflavin
39 (FMN/FAD) reductase domain in a single polypeptide chain.²⁸⁻³⁰ This structural arrangement makes P450
40
41 BM3 catalytically ‘self-sufficient’ and able to support the oxidation of a substrate with the only requirement
42 of air oxygen and a source of reducing equivalents in the form of the nicotinamide adenine dinucleotide
43 phosphate (NADPH) cofactor. In addition, P450 BM3 is soluble, it can be readily expressed in *Escherichia*
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45 *coli*, an excellent host for laboratory evolution experiments, and it is one of the most active hydroxylases
46 known, supporting the hydroxylation of its preferred substrates, long-chain (C12-C20) fatty acids, with
47 rates exceeding 1,000 turnovers per minute.²⁹
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3 While in those years protein engineering efforts with BM3 or other P450s were being pursued using
4 structure-based mutagenesis,³¹⁻³⁶ the Arnold group was interested in applying directed evolution to create
5 P450-based catalysts with altered substrate specificity and/or increased stability. One of the earliest goals
6 in the group was to evolve P450 BM3 to accept linear alkanes as substrates for hydroxylation, including
7 the short-chain gaseous alkenes (propane, ethane, methane) found in natural gas. Such biocatalysts were
8 envisioned to provide a potential approach for the biotransformation of natural gas into liquid alcohols as
9 more convenient and easily transportable fuels. In nature, gaseous alkanes are hydroxylated by multi-
10 component non-heme iron monooxygenase enzymes such as methane monooxygenases. Whereas the
11 structural complexity of these enzymes complicates their use or engineering for biotechnological
12 applications, they are also structurally and mechanistically distinct from P450s, begging the question of
13 whether a P450 could be evolved to mimic their function. A challenge inherent to this goal was the fact the
14 P450 BM3 had no detectable activity on propane or smaller alkanes, providing no obvious path for the
15 improvement of such activity. The Arnold group thus decided to initially improve its hydroxylation activity
16 on octane, which is a poor but viable substrate for this P450,^{22,37} with the underlying idea that BM3 variants
17 with enhanced octane activity would eventually acquire the ability to accept and hydroxylate shorter
18 alkanes. After five generations of random mutagenesis targeted to the heme domain, followed by
19 recombination of beneficial mutations, the group was able to identify a P450 BM3 variant (139-3) with
20 significantly improved octane activity (30 → 1,000 total turnovers (TTN)) as a result of 11 amino acid
21 substitutions accumulated over the course of this process.²² Importantly, such enhancement in octane
22 activity was accompanied by the acquisition of activity on the smaller alkane substrate propane (0 → 500
23 TTN).³⁸ Further rounds of mutagenesis and screening, using first an octane surrogate substrate and then a
24 propane surrogate substrate, led to P450 BM3 variants with progressively increased activity toward the
25 conversion of propane to 2-propanol and even modest activity on ethane (up to 250 TTN with variant
26 35E11).^{38,39} Out of the 17 mutations accumulated in 35E11, only two were found to be located in the active
27 site, illustrating the power of directed evolution in identifying beneficial mutations far from the active site.
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Integral part of this evolutionary process was the development of high-throughput assays for enabling the rapid screening of P450 libraries for activity on chromogenic substrates as surrogates for octane (*p*-nitrophenyl octyl ether and hexyl methyl ether) and propane (dimethyl ether).^{37, 38}

Despite this progress, the catalytic efficiency of 35E11 as an alkane hydroxylase was significantly inferior compared to that of wild-type P450 BM3 on its preferred substrates. The function of this multi-domain P450 relies on finely regulated conformational changes and electron transfer mechanisms, which ensure efficient coupling of fatty acid hydroxylation with NADPH oxidation.²⁹ These mechanisms are disrupted in the presence of non-native substrates and/or as a result of mutations, resulting in an ‘uncoupling’ process and production of reactive oxygen species (ROS) which inactivates the enzyme. To obtain a more efficient biocatalyst for propane hydroxylation, variant 35E11 was further evolved using a ‘domain engineering strategy’, in which beneficial mutations within the heme-, FMN-, and FAD-domain of this P450 were first identified via random and site-saturation mutagenesis and then combined in a final step.⁴⁰ This process generated a proficient propane monooxygenase, named P450_{PMO}, possessing high catalytic efficiency and coupling efficiency for this reaction (**Figure 2**), i.e. catalytic properties comparable to those exhibited by wild-type P450_{BM3} with its preferred substrates.^{40, 41} The laboratory-evolved P450_{PMO} could then be applied for the efficient conversion of propane to 2-propanol in metabolically engineered *E. coli* cells.⁴²



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3 **Figure 2. Directed evolution of a cytochrome P450 propane monooxygenase.** The long-chain fatty acid
4 hydroxylase P450 BM3 from *Bacillus megaterium* was converted into a highly efficient propane
5 monooxygenase (P450_{PMO}) over 13 rounds of directed evolution.^{22, 38, 40} The evolved enzyme accumulated
6 3 beneficial mutations in the reductase domain and 20 in the heme domain (yellow, sphere models), many
7 of which are distant from the heme cofactor (red, sphere model). Refinement of catalytic efficiency for
8 propane oxidation, an activity not exhibited by wild-type P450 BM3, also involved a profound reshaping
9 and partitioning of the substrate access pathway (inserts).⁴¹
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20 **Lessons from directed evolution experiments**

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22 The conversion of P450 BM3 into an efficient propane monooxygenase not only demonstrated the
23 remarkable plasticity of P450s toward adopting new functions but also the effectiveness of directed
24 evolution in progressively adapting an enzyme to execute a new function. This ‘substrate walk’ strategy
25 has proven effective in the laboratory evolution of a variety of other enzymes and proteins.⁴³⁻⁴⁸ In addition
26 to altered substrate specificity, the Arnold group demonstrated over the years how a variety of other
27 properties of P450s, including the ability to operate as peroxygenases, i.e., catalyze hydroxylation reactions
28 driven by hydrogen peroxide,⁴⁹ and their stability against thermal⁵⁰ and chemical denaturation⁵¹ could be
29 improved through directed evolution. These studies, along with a growing number of examples from other
30 groups, notably in the case of enantioselective transformations,^{52, 53} contributed to formulate the now
31 broadly accepted notion that the fitness of a protein with respect to a given functional or structural property
32 can be gradually enhanced in the laboratory, often through a series of small mutational steps,⁵⁴ as long as
33 the desired property remains feasible and properly chosen screening/selection tools are applied.
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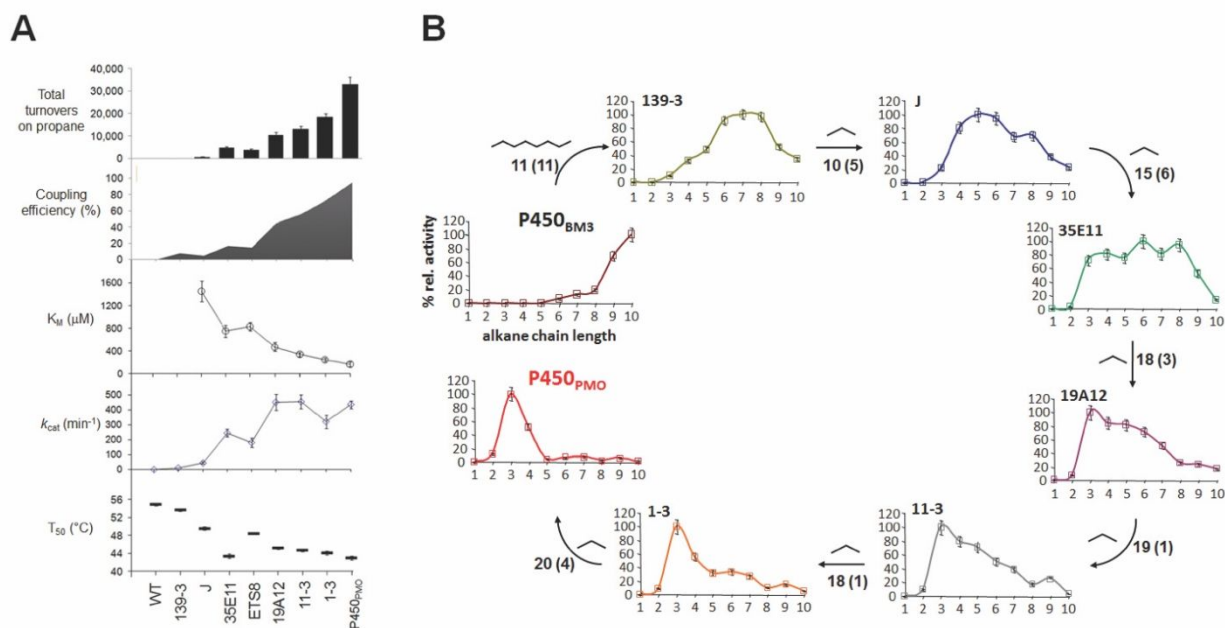


Figure 3. Catalytic and stability properties (a) and substrate profile (b) of selected variants along the P450_{PMO} lineage.⁴¹ Total turnover numbers (TTN), coupling efficiency (mol oxidized product/mol oxidized NADPH), and kinetic properties (K_M , k_{cat}) refer to propane oxidation. Thermal stability is shown as T_{50} (half-maximal inactivation temperature after 10-min incubation). Substrate profile is reported as relative activity on C1–C10 alkane series. Adapted with permission from ref. 41. Copyright 2008, Elsevier.

Important lessons on how proteins can evolve were also derived from a detailed characterization of the evolutionary intermediates along the lineage of P450_{PMO} (Figure 3A).⁴¹ These studies revealed that the adaptive walk from the long-chain fatty acid hydroxylase P450_{BM3} to the propane monooxygenase P450_{PMO} proceeded through a series of ‘promiscuous’ variants (e.g. 139-3, 35E11) that are characterized by a broad substrate profile, as determined based on their activity profile across the series of C1–C10 alkanes and other substrates.⁴¹ However, further optimization of propane activity was accompanied by ‘re-specialization’ of the P450 for the new function (Figure 3B), resulting in a variant (P450_{PMO}) that features a very narrow substrate profile centered around propane and has no residual activity on fatty acids, i.e. no remnants of the enzyme native function.⁴¹ On the one hand, these experiments showed that, starting from a

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3 specialized enzyme, the acquisition of a new substrate activity can proceed via a ‘generalist’ enzyme, with
4 modest activity on multiple substrates in addition to the native one(s). On the other hand, given that no
5 negative selection was applied throughout the directed evolution of P450_{PMO}, these studies also showed that
6 positive selection alone (i.e. propane activity) was sufficient for evolving a ‘specialist’ enzyme from another
7 specialized one. Prior to this work, it was generally assumed that a combination of positive and negative
8 selection pressure was necessary to reinforce the desired phenotype in an enzyme/protein while removing
9 undesirable native (or non-native) activities, as supported by a number of protein engineering
10 experiments.⁵⁵⁻⁵⁷

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12 The P450_{PMO} ‘story’ also illustrated the importance of addressing the activity/stability trade-off
13 often observed in directed evolution experiments. Indeed, at a point during the evolution of P450_{PMO} –
14 specifically at the level of 35E11 (**Figure 3A**), thermostabilization of the protein (via function-neutral
15 mutations) was necessary to enable the accumulation of additional beneficial mutations to enhance propane
16 activity.⁴¹ This finding reiterated the important relationship between protein stability and evolvability, a
17 notion nicely outlined in a previous study of the Arnold group.⁵⁸ Via a comparative study with a marginally
18 stable vs. a highly stable variant of BM3, they indeed demonstrated how the thermostable P450 showed a
19 significantly higher propensity to acquire activity on new substrates upon random mutations when
20 compared to the less stable enzyme. This study thus illustrated the key role of stability-based epistasis in
21 adaptive evolution,⁵⁹ an important implication of which is that, in the realm of natural proteins, function-
22 neutral yet stabilizing mutations may accumulate by genetic drift to permit the evolution of new functions.⁶⁰
23 In another study involving P450 BM3 as a model enzyme, Arnold and coworkers further demonstrated that
24 promiscuous enzymatic activities (e.g. activity on a new substrate) can arise via a neutral path, i.e., through
25 mutations that are neutral to the property under selective pressure,⁶¹ and these can provide the starting points
26 for the evolution of new functional proteins.

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28 The Arnold group’s interest toward investigating the impact of mutational events on emergence of
29 new functions has also extended to recombination, a key mechanism occurring in the context of natural
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3 protein evolution. In particular, the group introduced a new strategy for directed evolution via
4 recombination of homologous enzymes, called SCHEMA, which relies on the structure-based identification
5 of crossover points for minimizing structural disruption in the resulting chimeras. Using the β -lactamase
6 scaffold as a testbed, a large set of chimeric proteins was generated by SCHEMA-guided recombination of
7 the homologous β -lactamase enzymes TEM-1 and PSE-2 and analyzed for retention of function (**Figure**
8 **4A**). This study showed that recombination is conservative (much more so than random mutagenesis) and
9 results in a higher frequency of folded and functional variants despite of the introduction of multiple amino
10 acid mutations compared to the parent enzymes (**Figure 4B**).⁶² To address the question of whether these
11 mutational changes are associated with the acquisition of new functional traits, similar recombination
12 experiments were conducted using three homologs of P450 BM3 sharing ~65% sequence identity.⁶³ This
13 process generated a new family of ~6,000 chimeric P450s carrying an average of 70 mutations from the
14 closet parent, approximately half of which folded properly and a third of which display enzymatic activity.
15 Characterization of these chimeric P450s revealed that functional members of these libraries acquired
16 significant functional diversity, including the ability to accept substrates not accepted by the parent
17 enzymes.⁶⁴ In addition, a fraction of the chimeric P450s were found to exhibit higher stability compared to
18 the parent proteins (**Figure 4C**) and, based on structure-stability correlations, highly thermostable variants
19 could be generated through the targeted recombination of stabilizing fragments.⁶⁵ The same overall
20 approach later proved valuable toward generating thermostabilized and functional variants of fungal
21 cellobiohydrolases, which find utility in the degradation of cellulose into fermentable sugars for biofuel
22 applications.⁶⁶ Overall, these studies contributed to demonstrate the value of structure-based recombination
23 as a strategy to navigate the functional space in enzymes and provide valuable starting points for further
24 functional evolution.
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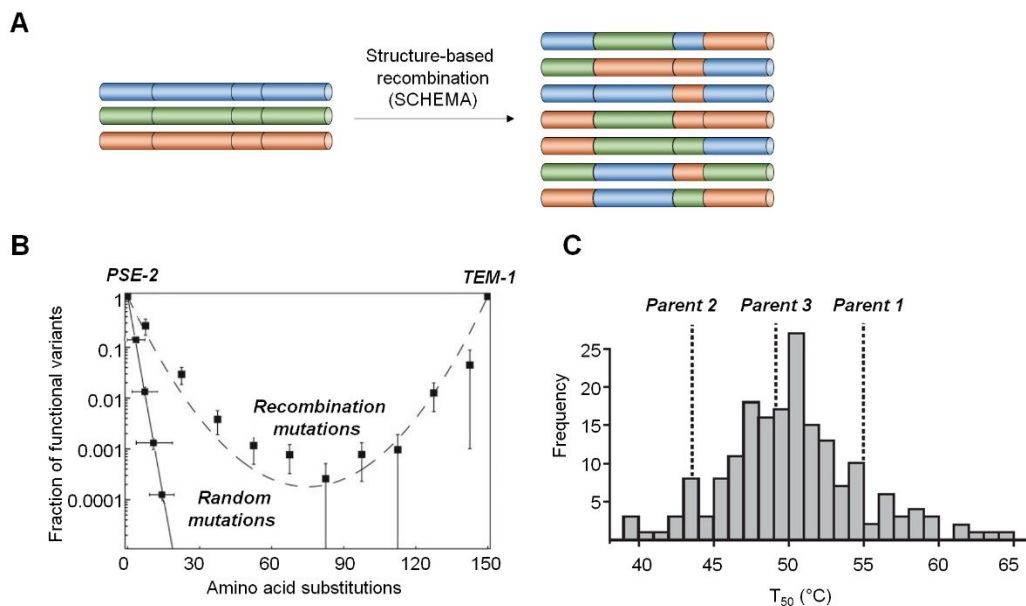


Figure 4. Directed evolution of enzymes using structure-based recombination. (a) Generation of chimeric enzymes via SCHEMA-guided recombination of fragments derived from homologous parent sequences and chosen to minimize structural disruption.¹⁷ (b) SCHEMA-guided recombination of β -lactamase PSE-2 and TEM-1 produces a higher fraction of functional chimeras compared to random mutagenesis.⁶² (c) Chimeric P450s obtained via SCHEMA-guided recombination of P450 BM3 (CYP102A1) with its homologous enzymes CYP102A2 and CYP102A3 can exhibit higher stability against thermal denaturation compared to any of the parent P450s.⁶⁵ Adapted with permission from ref. 62. Copyright 2005, National Academy of Sciences.

Laboratory-evolved P450s for biocatalytic oxidations

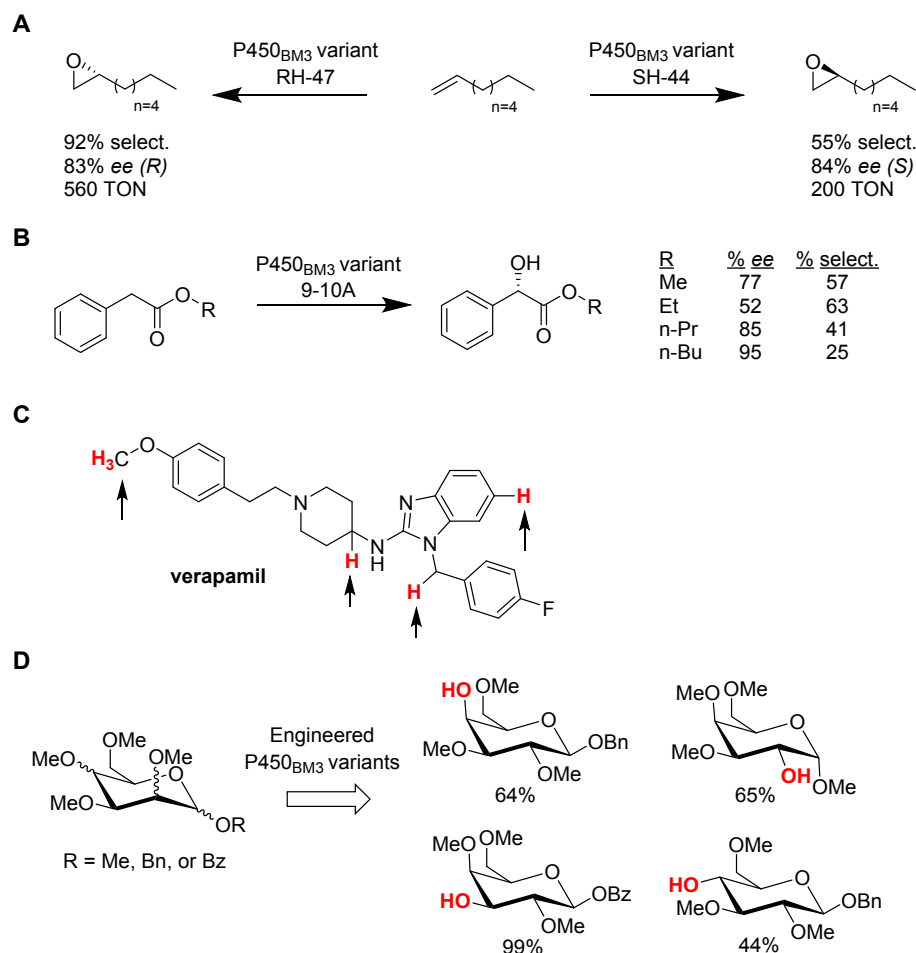
Oxidation catalysts are valuable tools for a variety of synthetic applications,⁶⁷ and the synthetic potential of (engineered) cytochrome P450s in this regard has not been overlooked by Frances Arnold, along with a number of other groups.⁶⁸⁻⁷¹ In particular, Arnold and coworkers demonstrated how substrate promiscuous P450 BM3 variants isolated during the evolution of alkane oxidases could be useful for the selective hydroxylation of C_6 - C_{10} alkanes at the subterminal (ω -1) position (up to 86% regioselectivity and

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3 83% *ee*)³⁸, the epoxidation of terminal C₅-C₈ alkenes (up to 94% regioselectivity and 83% *ee*; **Scheme**
4 **1A**)⁷², and the α -hydroxylation of 2-aryl-acetic acid esters to give valuable mandelic acid derivatives (up
5 to 99% regioselectivity and 94% *ee*; **Scheme 1B**)⁷³. In another contribution, it was shown that P450-
6 catalyzed hydroxylation could be combined with chemical fluorination to enable the rapid and site-selective
7 installation of one or more fluorine substituents in a variety of target small-molecule drugs and building
8 blocks.⁷⁴ As the C-H sites targeted by engineered bacterial P450s often overlap with those oxidized by
9 human liver P450s, this strategy is valuable toward improving the metabolic stability of drugs or other
10 bioactive molecules.^{74, 75}

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12 Since hepatic P450s account for the majority of Phase I metabolic transformations of drugs *in vivo*,
13 an important application of cytochrome P450s (human and non-human) lies in the production of the
14 authentic human metabolites of drugs and drug candidates,⁷⁶⁻⁷⁹ as characterization of the pharmacological
15 activity and toxicity of these compounds represents a critical step toward their approval for use as
16 pharmaceuticals. Along with others,^{80, 81} the Arnold group showed that laboratory-evolved variants of P450
17 BM3, including substrate-promiscuous variants from the aforementioned P450_{PMO} lineage and
18 recombination libraries, can prove useful to generate human metabolites of marketed drugs.^{82, 83} In
19 collaborative effort with Eli Lilly, Arnold and coworkers showed the successful application of a small
20 collection of these engineered P450_{BM3} variants (~120) for producing nearly all of the human metabolites
21 (12/13) of the drugs verapamil and astemizole.⁸³ They also reported the directed evolution of a P450_{BM3}
22 variant for the oxidation of acidic drugs such as naproxen, which are typically metabolized by human
23 CYP2C9.⁸⁴

24
25 In another important synthetic application, P450 BM3 variants were engineered by the Arnold
26 group to enable the regioselective deprotection of single methyl groups from permethylated
27 monosaccharides.⁸⁵ The deprotected monosaccharides could be then converted in a chemoenzymatic
28 process to deoxy- and fluorinated monosaccharides and disaccharides, thus providing a new approach to
29 access valuable building blocks for the synthesis of polysaccharides and other sugar-functionalized
30

molecules. While P450-mediated chemoenzymatic synthesis has recently emerged as an attractive strategy for the late-stage functionalization of complex bioactive molecules,^{75, 86, 87} a collaboration between the Arnold and Stoltz groups recently demonstrated the utility of engineered P450s for the total synthesis of the natural product nigelladine A.⁸⁸ In this case, a key allylic oxidation step, which had proven inefficient using chemical methods, could be realized using an engineered P450 BM3 variant, enabling further elaboration of the synthetic intermediate to the desired natural product.



Scheme 1. Laboratory-evolved P450 BM3 variants for synthetic applications: (a) selective epoxidation of terminal alkenes;⁷² (b) stereoselective synthesis of mandelic acid derivatives;⁷³ (c) generation of human drug metabolites (sites hydroxylated by the engineered P450 BM3 variants are indicated by arrows);⁸³ and

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3 (d) regioselective demethylation of permethylated monosaccharides.⁸⁵ TON = turnover number; *ee* =
4 enantiomeric excess; select. = selectivity.
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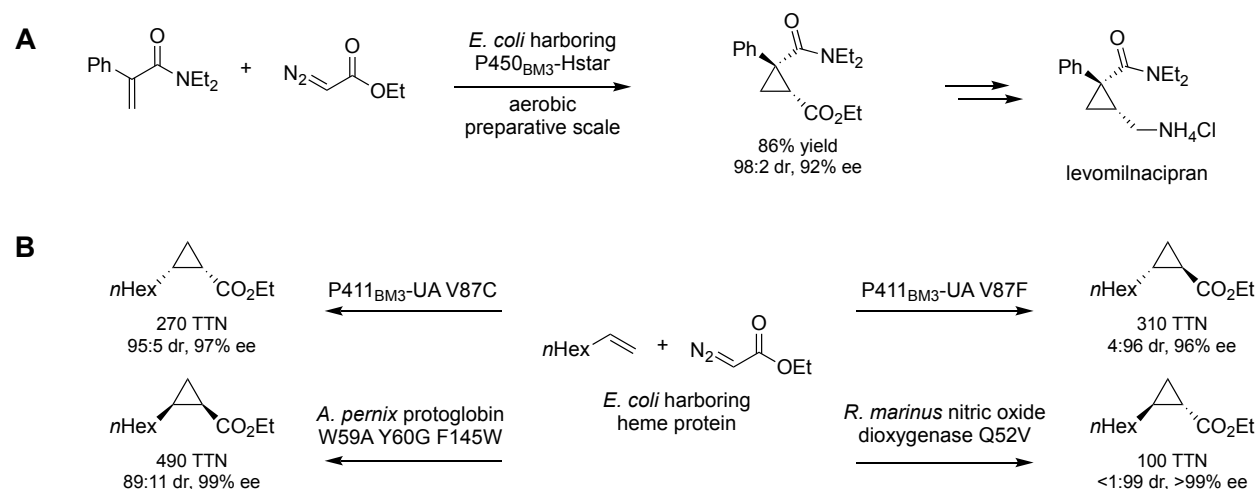
9 III. Evolving enzymes for new-to-nature biotransformations

11 Starting in 2010, protein engineering efforts in the Arnold group began to focus on the evolution
12 of cytochrome P450s for abiological functions such as carbene and nitrene transfer
13 reactions. These are synthetically valuable transformations not known
14 in naturally-occurring enzymes. The contributions of the Arnold group
15 and others⁸⁹⁻¹⁰⁰ in this area have greatly expanded the repertoire of enzymes
16 as biocatalysts for synthetic applications.
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24 The preparation of cyclopropanes by carbene transfer to olefins, though well known in transition
25 metal catalysis,^{101, 102} is novel to the biological world. In 2013, the Arnold group showed that heme proteins
26 such as engineered cytochrome P450 BM3 enzymes could function as cyclopropanation biocatalysts,
27 promoting the cyclopropanation of styrenyl olefins in water.¹⁰³ By mutating the proximal axial ligand from
28 cysteine to serine, variants of P450_{BM3} (termed P411_{BM3} because of the signature ferrous CO Soret peak at
29 411 nm rather than 450 nm for cysteine-ligated P450) also function efficiently in whole-cell *E. coli* and
30 catalyze carbene transfer styrene cyclopropanation in *vivo* with up to 67,800 turnovers.¹⁰⁴
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39 Enzymes are sustainable catalysts for the preparation of pharmaceuticals and other high-value
40 compounds,^{105, 106} and new-to-nature enzymatic activities can significantly expand the biocatalytic
41 repertoire available for this purpose.¹⁰⁷ Taking advantage of the versatility of engineered cytochromes
42 P450^{98, 100, 108, 109} as cyclopropanation enzymes, the Arnold group showed their potential for producing
43 industrially important pharmaceutical building blocks such as the cyclopropane core of the antidepressant
44 levomilnacipran, which was prepared on a preparative scale using P450_{BM3}-Hstar, an evolved His-ligated
45 cytochrome P450_{BM3} variant (**Scheme 2A**).¹¹⁰ Stereocomplementary biocatalysts are key assets for the
46 synthesis of drugs and complex molecules.^{94, 111-119} Upon exploring a collection of heme enzymes, the
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Arnold group was able to identify a set of biocatalysts useful for producing the four possible stereoisomers of a cyclopropanation reaction with ethyl diazoacetate and an unactivated alkene (**Scheme 2B**).¹²⁰ Notably, no iron-based catalyst was previously known for this transformation and heme alone only catalyzed this reaction with < 1 turnover.

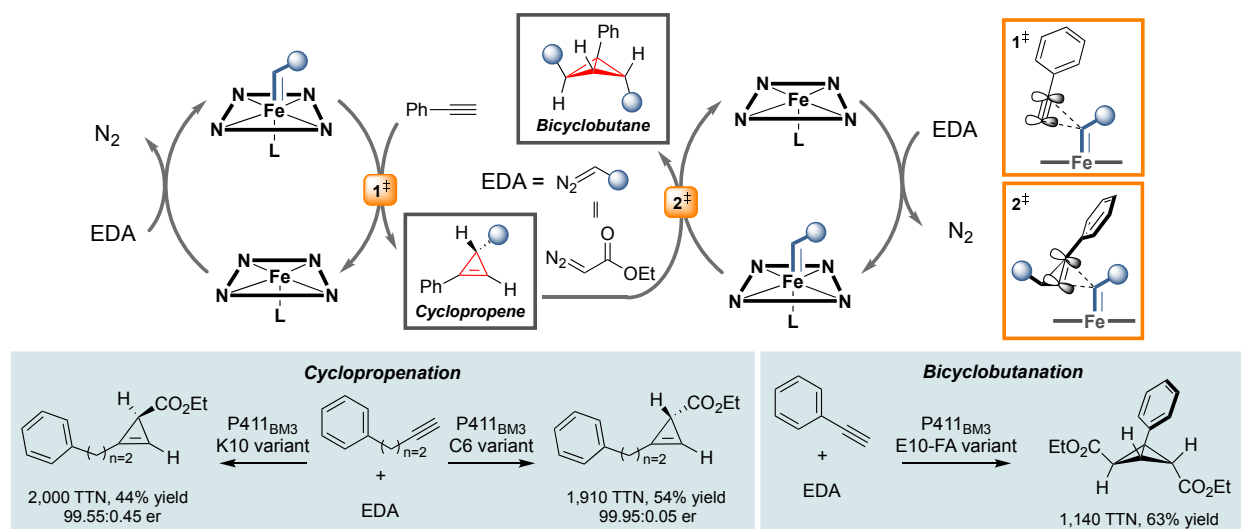


Scheme 2. Engineered heme protein-catalyzed new-to-nature transformations: (a) synthesis of the cyclopropane core of antidepressant levomilnacipran;¹¹⁰ (b) enzymatic stereodivergent cyclopropanation of octene catalyzed by a suite of engineered cytochrome P411 proteins and globins.¹²⁰

Highly strained rings

Bicyclobutanes are puckered carbocycles that are difficult to assemble owing to their high ring strain. Once synthesized, however, they manifest interesting properties that make them uniquely suited for a variety of applications, such as strain-release chemistry¹²¹ and stimulus-responsive materials.¹²² In a recent study, the Arnold group described the directed evolution of a P411 variant, called P411_{BM3}-E10-FA, capable of accepting phenylacetylene and ethyl diazoacetate as substrates to make bicyclobutane product as a single stereoisomer (**Scheme 3**).¹²³ Key to this success is the enzyme's ability to stabilize the putative reactive cyclopropene intermediate and exert precise stereocontrol over two carbene transfer events. P411_{BM3} variants that yield cyclopropenes as the final products were also identified, such that each enantiomer of a

cyclopropene can be made enzymatically with thousands of turnovers and high enantioselectivity (**Scheme 3**).



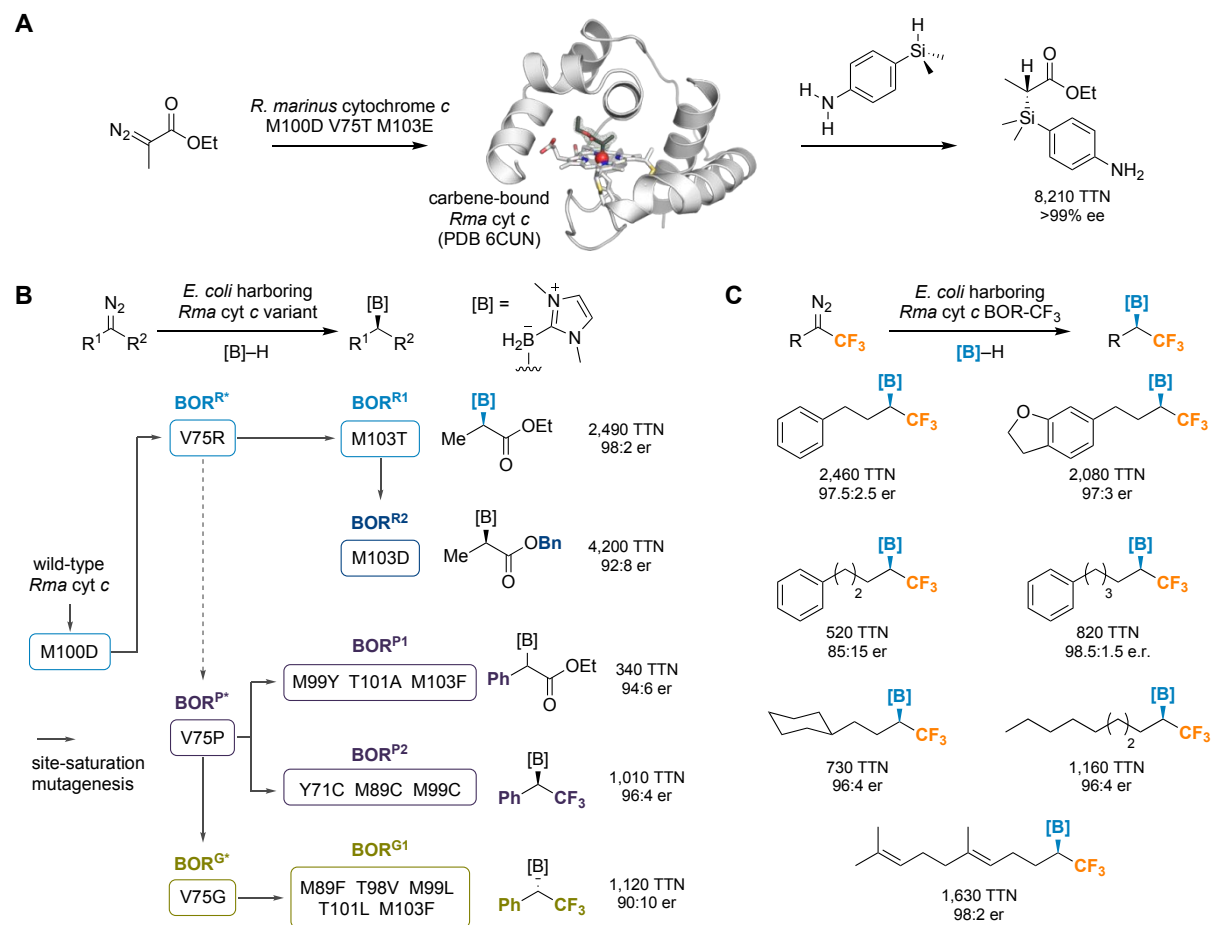
Scheme 3. Cytochrome P411_{BM3}-catalyzed bicyclobutanation and cyclopropenation.¹²³

New chemical bonds

Carbon–silicon and carbon–boron bonds are absent in biology but commonly found in man-made molecules and materials in our modern world. By engineering cytochrome *c* protein from the thermohalophilic bacterium *Rhodothermus marinus* (*Rma cyt c*), the Arnold group described carbene insertion into Si–H and B–H bonds as a means to coax bacteria to forge carbon–silicon and carbon–boron bonds in living systems for the first time.^{124, 125} With only three mutations, directed evolution turned wild-type *Rma cyt c* into a silylation enzyme that exhibits up to 8,200 turnovers (**Scheme 4A**), capable of making 20 different organosilicon products with 95–99% ee.¹²² The mechanism of biological carbon–silicon bond formation was elucidated by the Arnold and Houk groups by capturing the catalytic iron–carbene intermediate in the active site of *Rma cyt c* crystallographically (**Scheme 4A**) and by probing experimentally and computationally how the iron–carbene electronic state and protein conformational dynamics affect the reaction.¹²⁶ These efforts are complemented by the mechanistic work of other groups,

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3 further our understanding of how iron porphyrins and heme proteins catalyze carbene transfer to olefins,
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5 N–H, and C–H bonds.¹²⁷⁻¹³³
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7 Genetically programmable chiral organoborane synthesis was also realized by the laboratory
8 evolution of *Rma cyt c*.¹²³ This platform successfully yields structurally distinct organoboranes through
9
10 divergent directed evolution (**Scheme 4B**), providing bacterial borylation catalysts that are suitable for
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12 gram-scale biosynthesis, and offering up to 15,300 turnovers and excellent selectivity (99:1 e.r., 100%
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14 chemoselectivity). Moreover, these enzymes are readily tunable to accommodate the synthesis of lactone-
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16 based organoboranes¹³⁴ and a broad range of chiral α -trifluoromethylated organoboranes (**Scheme 4C**).¹³⁵
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18 The latter demonstration is particularly appealing as it represents a means to introduce fluorine-containing
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20 motifs to small molecules,⁹⁵ orthogonal to existing strategies for organofluorine biosynthesis using
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22 engineered fluorinase enzymes or precursor-directed biosynthesis of fluorine-containing polyketides,
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24 peptides, and alkaloids.^{136, 137}
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Scheme 4. Engineered *R. marinus* cytochrome *c*-catalyzed carbon–silicon¹²⁴ and carbon–boron bond formation.^{125, 135}

C–H functionalization

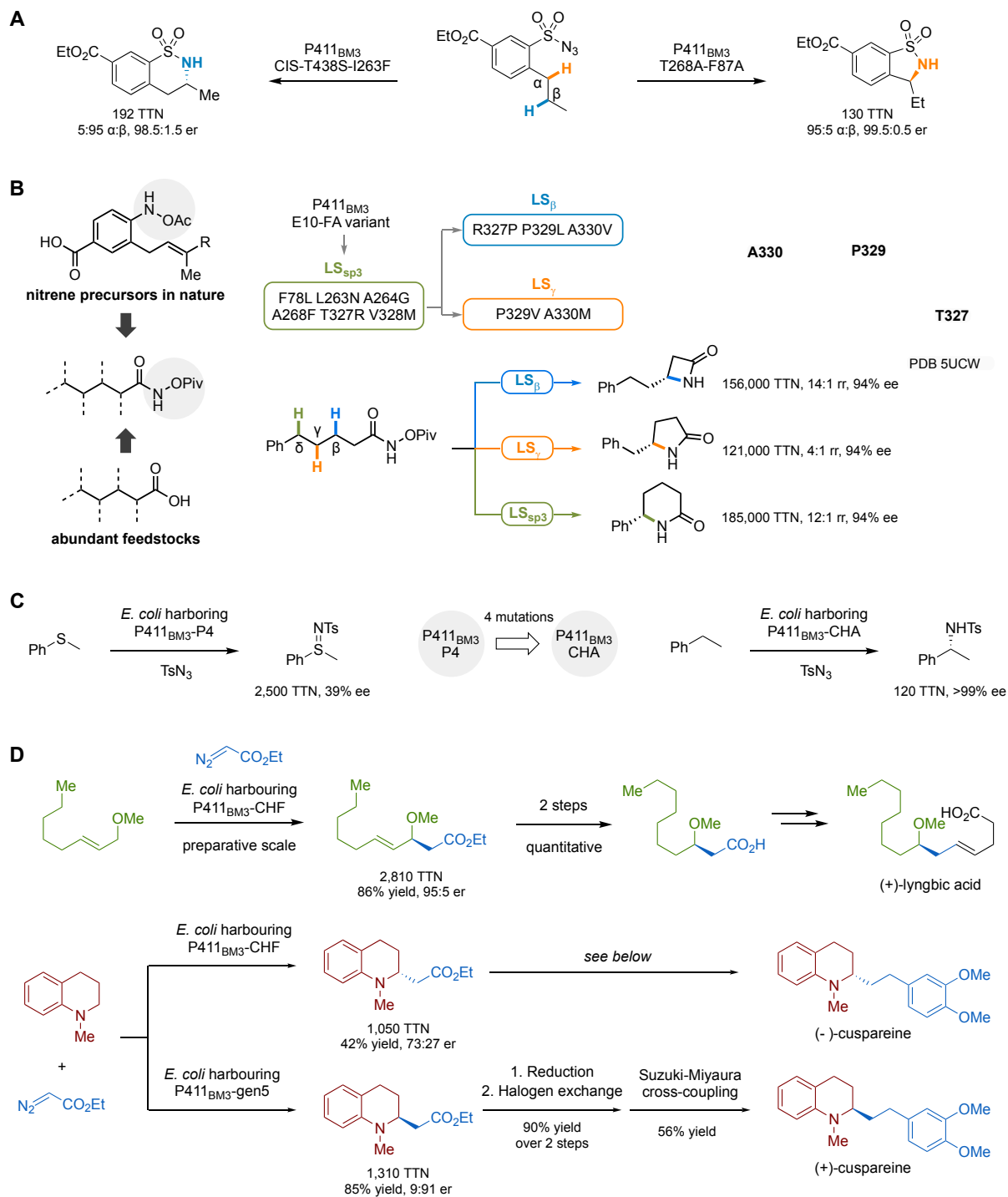
Evolving heme proteins to functionalize C–H bonds in new ways is of interest to the Arnold group and others in the biocatalytic community.¹³⁸ While native P450 enzymes are well versed in oxidizing even unactivated C–H bonds through a reactive, high-valent iron-oxene intermediate (Compound I),¹³⁹ other than oxygen atoms, examples of heme proteins inserting other groups or functionalities into C–H bonds are sporadic in the natural and unnatural worlds. Inspired by a 1985 report describing a C–H amination reaction catalyzed by rabbit liver cytochrome P450,¹⁴⁰ the Arnold and Fasan group independently discovered that engineered P411 (P411_{BM3}-T268A and P411_{BM3}-CIS)¹⁴¹ and P450_{BM3} variants,⁸⁹ as well as other

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3 hemoproteins¹⁴², are able to catalyze and intramolecular nitrene insertion into benzylic C–H bonds on aryl
4 sulfonyl azides. By further evolving these P411 variants through active site site-saturation mutagenesis and
5 recombination of beneficial mutations, the regioselectivity of the cyclization process could be altered to
6 yield either five-membered (benzylic C–H insertion) or six-membered ring (homobenzylic C–H insertion)
7 products, representing a powerful example of how directed evolution can tune an enzyme active site to
8 favor functionalization of higher-energy C–H bonds (**Scheme 5A**).¹⁴³
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16 Recently, this concept was further expanded and showcased in the divergent synthesis of β -, γ -, and
17 δ -lactams by P411_{BM3}-catalyzed intramolecular C–H amidation.¹⁴⁴ Using acyl-protected hydroxamates as
18 carbonyl nitrene precursors^{90, 145}, a collection of P411_{BM3}-derived lactam synthases with complementary
19 cyclization modes was obtained (**Scheme 5B**). Remarkably, these biocatalysts can override intrinsic
20 substrate bias from sterics, electronic preference, and ring strain to deliver lactams of different ring sizes
21 with excellent selectivities and up to a million total turnovers.
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29 Intermolecular C–H amination is a significantly challenging transformation and few catalytic
30 systems are known to be both efficient and selective across diverse substrates. To overcome this, many
31 heme proteins were tested in the Arnold group for intermolecular nitrene C–H insertion activity: none was
32 found to be active until they tested a cytochrome P411_{BM3} variant, called P4, engineered for the nitrene
33 transfer to sulfides and allylic sulfides.^{146, 147} The promiscuous activity observed may arise from transition
34 state similarities between sulfimidation and nitrene C–H insertion. Introducing four mutations to P4 (A82L,
35 A78V, F263L, and E267D) led to P411_{CHA}, a highly enantioselective benzylic C–H amination biocatalyst
36 capable of catalyzing a reaction which the iron cofactor alone cannot perform (**Scheme 5C**). Interestingly,
37 P4 A82L also exhibits intermolecular C–H alkylation activity, unlocking a whole new world of
38 opportunities for developing enzymatic C–H functionalization methods by carbene transfer.¹⁴⁸ After 13
39 rounds of mutagenesis and screening and removal of the enzyme FAD domain, P411-CHF was created
40 from P4 A82L, which demonstrates 140-fold turnover increase relative to the parent protein in carbene
41 transfer C–H insertion reaction of *p*-methoxybenzyl methyl ether. P411-CHF is broad in scope: it
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functionalizes benzylic, allylic, and α -amino C–H bonds with up to 3750 turnovers and >99:1 er, and is applicable to streamlining the synthesis of natural products (**Scheme 5D**). Because this biocatalyst is fully genetically encoded, produced in *E. coli*, and functions at room temperature, it offers a desirable alternative to noble-metal catalysts for intermolecular C–H functionalization chemistry.



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3 **Scheme 5.** C–H functionalization enabled by enzymatic nitrene and carbene transfer C–H insertion: **(a)**
4 regioselective cyclization via intramolecular nitrene insertion into benzylic C–H bonds;¹⁴³ **(b)** divergent
5 synthesis of β -, γ -, and δ -lactams by P411_{BM3}-catalyzed intramolecular C–H amidation;¹⁴⁴ **(c)** P411_{BM3}-
6 catalyzed intermolecular C–H amination;¹⁴⁷ **(d)** P411_{BM3}-catalyzed intermolecular C–H alkylation.¹⁴⁸
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13 **Summary**

14
15 Frances Arnold pioneered the development of directed evolution for enzyme engineering. Her career has
16 not only had a profound impact in the field of enzyme engineering and biocatalysis but also deeply
17 influenced the lives of many of her students and postdocs, including the three of us. This account articles
18 revisit some of her inspiring accomplishments over the past three decades and we are sure that more
19 groundbreaking contributions will be forthcoming in the coming years, as a direct result of her fearless and
20 tireless pursuit of excellence in research.
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31
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43 **Notes**

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45 The authors declare no competing financial interest.
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