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Stereodivergent atom transfer radical cyclization by engineered cytochromes P450

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Abstract

Bringing new catalytic functions to naturally occurring enzymes can dramatically expand the repertoire of enzymology and generate novel biocatalysts. Due to the lack of exploitable stereocontrol elements in synthetic systems, steering the absolute and relative stereochemistry of these free radical processes is notoriously difficult in asymmetric catalysis. Inspired by the innate redox properties of first-row transition-metal cofactor, we repurposed cytochromes P450 to catalyze stereoselective atom transfer radical cyclization. Furthermore, a set of metalloenzymes was engineered to impose excellent stereocontrol over the radical addition step and the halogen rebound step in these unnatural processes, allowing enantio- and diastereodivergent radical catalysis to be easily carried out. This evolvable metalloenzyme platform represents a promising solution to tame fleeting radical intermediates for asymmetric catalysis.

One-Sentence Summary:

P450 enzymes were repurposed and engineered to enable a novel mode of stereoselective new-to-nature metalloredox radical biocatalysis.

As nature's privileged catalysts, enzymes are well-known for their ability to exert exquisite control over the stereochemical outcome of chemical reactions (1). Over the past three decades, the advent of directed evolution (2, 3) has enabled the rapid development of customized enzymes to furnish excellent catalytic activity and stereoselectivity which are

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Author contributions: Y.Y. designed the overall research. Y.Y. and Q.Z. performed initial evaluation of metalloenzymes and reaction conditions. Q.Z., M.C. and Y.Y. performed directed evolution of $P450_{ATRCases}$. Q.Z. and M.C. performed substrate scope study. Y.F. carried out the computational studies with P.L. providing guidance. Y.Y. wrote the manuscript with the input from all other authors.

Competing interests: A provisional patent application has been filed through the University of California Santa Barbara based on the results presented here.

Supplementary Materials
Materials and Methods
Figs. S1 to S6
Tables S1 to S17

often complementary to those of traditional small-molecule catalysts (4). However, until recently, the catalytic repertoire of enzymes has been mostly limited to reactions found in nature, posing constraints on the types of products available from enzyme catalysis. To merge the excellent tunability and stereocontrol of biocatalysts with the synthetic versatility of abiotic systems, the discovery and development of new-to-nature enzymatic activity are widely recognized as preeminent objectives at the interface of modern biocatalysis and organic synthesis (5, 6).

Our group initiated a research program to repurpose naturally occurring metalloenzymes to catalyze unnatural radical reactions in a stereocontrolled fashion. Due to the highly reactive nature of radical intermediates and the lack of synthetically realizable stereoinduction strategies, imposing enantio- and diastereocontrol over free radical-mediated bond forming processes has remained a daunting challenge in asymmetric catalysis (7, 8). On the other hand, natural enzymes (9) such as radical SAM enzymes (10) facilitate radical reactions with excellent chemo-, regio- and stereoselectivity. However, the vast majority of natural radical enzymes display an extremely narrow substrate scope. Alternatively, by repurposing widely used, biotechnologically important enzymes to catalyze new-to-nature radical reactions, novel biocatalytic methods can be developed, thereby allowing for a diverse array of easily available substrates to be converted with excellent operational simplicity. In this respect, recent groundbreaking contribution from the Hyster and Zhao laboratories (11) demonstrated the use of visible light to unveil excited state activities of ketoreductases (12) and ene reductases (13–18), allowing for a range of stereoselective hydrogen transfer transformations to be developed.

Because small-molecule complexes of first-row transition metals were reported to catalyze unselective radical reactions via ground-state single electron transfer between the metal center and the substrate, we envisioned a powerful strategy to control the stereoselectivity of these radical reactions by engaging enzymes bearing a redox-active metallocofactor in these unnatural processes. By fine tuning the intimate interaction between the reactive radical intermediate and the metalloprotein scaffold using directed evolution, these metalloenzyme catalysts could be engineered to achieve levels of enantio- and diastereocontrol that are unmatched by small-molecule catalysts. With this strategy, a diverse array of unnatural radical metalloenzymes could be potentially developed, as metalloredox couples spanning a wide window of potentials, including Fe(II)/Fe(III) (19, 20), Co(I)/Co(II) (21), and Cu(I)/Cu(II) (22), are readily available in a myriad of native metalloenzymes. In particular, inspired by polymer chemists' research employing heme based systems to generate radical species for polymerization (23, 24), we saw numerous opportunities in leveraging heme proteins as a general platform for asymmetric radical transformations. Previous elegant work from Arnold, Fasan and others demonstrated that heme proteins can be repurposed to catalyze unnatural carbene and nitrene transfers analogous to the natural oxene transfer observed in heme oxygenases (25–30). Thus, the highly promiscuous and evolvable nature of heme proteins make them particularly promising for the development of new-to-nature stereocontrolled metalloredox radical chemistry.

We envisioned a new family of unnatural biocatalytic reaction, namely stereoselective atom transfer radical cyclization (ATRC) (31, 32), to be advanced with metalloenzymes. In

our proposed atom transfer radical cyclization catalyzed by Fe-dependent enzymes (Fig. 1(A)), the metalloprotein catalyst in its ferrous state undergoes single electron transfer with an organic halide, producing a transient radical intermediate in the active site. Rapid addition of the nascent radical species to an unsaturated system would afford a new radical, which subsequently react with the ferric halide to produce the product and regenerate the metalloenzyme catalyst. The envisioned atom transfer radical cyclization simultaneously installs an alkyl group and a halogen atom across the C=C double bond of an olefin substrate, thereby generating valuable products with added stereochemical complexity (31). Despite the extensive study and widespread utility of atom transfer radical cyclization reactions, the development of general methods for stereocontrolled ATRC presents significant hurdles for small-molecule catalysts (7, 33, 34). Due to the difficulty in maintaining tight association between the chiral catalyst and the radical intermediate, general catalytic strategies to exert enantiocontrol over the C−C bond forming radical addition step remains unavailable. Imposing catalyst-controlled diastereoselectivity in the subsequent halogen-rebound step is similarly challenging. Herein, we describe the development of metalloprotein catalysts that provide excellent enantio- and diastereocontrol for this daunting problem in asymmetric catalysis. Notably, our protein engineering efforts allow for a toolbox of stereocomplementary catalysts to be developed, granting enantio- and diastereodivergent access to ATRC products.

In the present study, we evaluated both heme (P450s, globins and cytochromes *c*) and non-heme Fe-dependent proteins for the targeted transformation (Table S1). Atom transfer radical cyclization of substrate **1a** was selected as the model reaction, as $FeCl_2$ was known to facilitate these processes (35) and the corresponding enantioenriched lactams derived from these cyclization processes are ubiquitous in medicinally relevant scaffold (36). The utility of Fe enzymes in our library was evaluated in the form of purified proteins, cell-free lysates, as well as whole-cell catalysts. Although several metalloproteins were found to promote the desired radical cyclization (Table S1), among all the metalloprotein catalysts we examined, a serine-ligated variant of *Bacillus megaterium* P450 (CYP102A1) (37), “P” (38), furnished a measurable enantiomeric excess (60:40 e.r., e.r. = enantiomeric ratio) and the highest activity. Notably, intact *Escherichia coli* cells harboring P exhibited an order of magnitude higher activity than that of the purified protein catalyst with identical enantioselectivity (Table S4 and S5). The ability to use whole bacterial cells as catalysts without further manipulation dramatically accelerated this protein engineering campaign.

To further improve the enantioselectivity of this radical cyclization process, we next performed directed evolution through iterative cycles of site-saturation mutagenesis (SSM) and screening (Fig. 1(B) and (C)). In each round of engineering, 90 clones were screened. By targeting active site residues proximal to the heme cofactor, including residues 327, 263, 181, 82, and 266, through five rounds of protein engineering, the enantiomeric ratio of the desired product (**2a**) could be rapidly enhanced. With our final metalloenzyme variant, $P450_{ATRCase1}$ (P T327I I263Q L181F A82T H266T), the desired product formed with excellent yield and enantioselectivity (89% yield, 8,110 TTN, and 97:3 e.r.). By further decreasing the catalyst loading, a total turnover number of 20,000 was achieved without affecting the enantioselectivity.

During the course of this study, we also found that another serine-ligated P450 variant ($P411_{Diane2}$) we previously engineered for enantioselective C−H amidation (39) also exhibited promiscuous activity for this atom transfer radical cyclization with inverted enantiopreference (Fig. 1(B)). $P411_{Diane2}$ was further evolved via recursive site saturation mutagenesis (SSM) and screening (Fig. 1(D)). Axial ligand effect was investigated in this unnatural metalloredox radical chemistry. Strikingly, the “S400A” variant lacking a coordinating axial ligand (“S400A” = $P411_{Diane2}$ P327C S400A) facilitated the unnatural radical reaction with a 1.4-fold improvement in TTN relative to the S400 variant without affecting the enantioselectivity (1160 TTN, 32:68 e.r. to 1590 TTN, 32:68 e.r., Table S7). By targeting other active site residues including 327, 181, 438 and 436, we arrived at $P450_{ATRCase2}$ (= $P411_{Diane2}$ P327C **S400A** L181V T438Q L436T) with substantially enhanced activity and enantioselectivity (3,350 TTN, 91:9 e.r.).

We next surveyed the substrate scope of this new-to-nature atom transfer radical cyclization process (Fig. 2). A range of functional groups on the nitrogen substituent was readily tolerated, providing the corresponding γ-lactam in excellent TTN and enantioselectivity (**2a**−**2i**). Chloro- (**2g**) and bromo (**2h**) substituents were also compatible with this enantioselective biocatalytic ATRC, providing a valuable functional group handle for further derivatization. Substrates bearing a heterocycle such as thiophene (**2i**) were also transformed with excellent enantiocontrol. Together, these results demonstrated that evolved $P450_{ATRCase}$ variants are highly active and stereoselective for this type of transformation. Additionally, substituted olefins (**1j** and **1k**) as well as α,α-difluorinated (**1l**) substrates could also be converted into the desired γ-lactam products, including **2j** bearing contiguous quaternary-quaternary stereocenters. The absolute configuration of **2b** was ascertained by X-ray crystallography. Furthermore, using these engineered metalloenzyme catalysts, β- and δ-lactams (**2m** and **2n**) readily formed in an enantioselective fashion, indicating the potential of this biocatalytic platform to access a diverse array of enantioenriched lactam products. Products **2k** and **2m** were derived from dehydrohalogenation of the ATRC product.

Given the success of engineered metalloenzymes in exerting excellent stereocontrol over the C−C bond forming step, we questioned whether we could further leverage this metalloredox biocatalytic platform to control the relative stereochemistry in the C−Br bond forming halogen rebound event (Fig. 3(A)). To this end, we re-examined all the P450 variants we accumulated in this protein engineering effort. Two hits were identified, including $P450_{ATRCase}$ gen-4 (P T327I I263Q L181F A82T) and P’ (P L181V L437F S438Q), giving rise to promising levels of opposite diastereopreferences, respectively. Through iterative rounds of SSM and screening, we engineered an orthogonal set of highly selective biocatalysts including $P450_{ATRCase3}$ and $P450_{ATRCase4}$, allowing for the diastereodivergent synthesis of either **2o** or **2o-*dia*** (Fig. 3(B)). For comparison, the use of traditional tris(2-pyridylmethyl)amine copper(I) catalyst for ATRC (40) furnished **2o** and **2o-*dia*** with low diastereoselectivity (1:1.6 and 2.1:1 d.r. from (*E*)- and (*Z*)-**1o**, respectively, see the SM for details). Moreover, with $P450_{ATRCase3}$ and $P450_{ATRCase4}$, (*E*)- and (*Z*)-**1o** provided the same major diastereomer (see the SM). Thus, starting from easily accessible mixtures of (*E*)- and (*Z*)-**1o**, the diastereoconvergent biocatalytic transformation with $P450_{ATRCase3}$ or $P450_{ATRCase4}$ delivered the corresponding γ-lactam product in good diastereoselectivity.

The relative stereochemistry of **2o** was determined by X-ray diffraction analysis (see the SM for details).

To further demonstrate the synthetic utility of engineered radical metalloenzymes, we performed this whole-cell biotransformation on a gram scale (Fig. 3(C)). Excellent yield and enantioselectivity were observed, thus showcasing the practicality of this new-to-nature biocatalytic reaction. Furthermore, the presence of a bromine functional group in enantioenriched ATRC products allowed for a range of diversification reactions to be conveniently carried out. S_N2-type nucleophilic substitution with a range of nucleophiles furnished synthetically versatile azide (**3a**), cyanide (**3b**) and xanthate (**3c**) products in good yields while maintaining the stereochemical purity. Combined with the enantioselective biocatalytic atom transfer radical cyclization, these derivatization reactions allowed a range of formal enantioselective carbofunctionalization reactions, including carboazidation (**3a**), carbocyanation (**3b**), and carboxanthation (**3c**), to be achieved.

Consistent with initial hypothesis, our radical clock experiments using hemin as the catalyst led to ring opening products (see the SM for details), indicating radical intermediates are involved in this process. To gain further insights into the reaction mechanism and the origin of the biocatalyst's activity, we performed density functional theory (DFT) calculations using a model system (39) for the axial serine-ligated P450 catalyst (Fig. 6, see the Supplementary Material for details). DFT calculations showed that the Fe porphyrin catalyst remains high-spin throughout the catalytic cycle in this biocatalytic ATRC process. This contrasts with the previously studied native oxene transfer (41) and analogous nitrene transfer (39) chemistry of P450 enzymes, wherein spin crossover is involved. With the model system, the radical initiation step (**TS1**) has a relatively low activation barrier ($\Delta G^{\ddagger}$) of 17.7 kcal/mol. Considering that the enzyme environment may further facilitate this process by promoting substrate binding to form complex **5**, this Fe-catalyzed radical initiation is expected to be kinetically facile. The electron-rich nature of the Fe center allows for the facile single electron reduction of the substrate, as evidenced by the substantial electron transfer (0.44 e^-) from **4** to **1a** in **TS1**. After the selective 5-*exo*-trig cyclization (**TS2-*exo***) to form the primary carbon radical **8**, the bromine rebound step (**TS3**) is highly exergonic with a low activation barrier of 13.1 kcal/mol. The fast trapping of this newly formed carbon radical via bromine atom transfer renders the C−C bond formation step irreversible and enables kinetic control of reaction stereochemistry. The bromine rebound reactivity is promoted by the conversion of a weaker Fe–Br bond in the ferric bromide species (6) to a stronger primary C(sp^3)–Br bond in **2a**. Therefore, the unique ability of the Fe porphyrin system to promote both radical initiation and bromine rebound steps makes it an excellent ATRC biocatalyst. The results described herein represent our first foray into the development of a new mode of unnatural metalloredox biocatalysis for stereoselective radical chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data are available in the main text or the supplementary materials. Solid-state structure of **2b** and **2o** is available free of charge from the Cambridge Crystallographic Data Centre under reference number CCDC 2087196 and 2087196, respectively.

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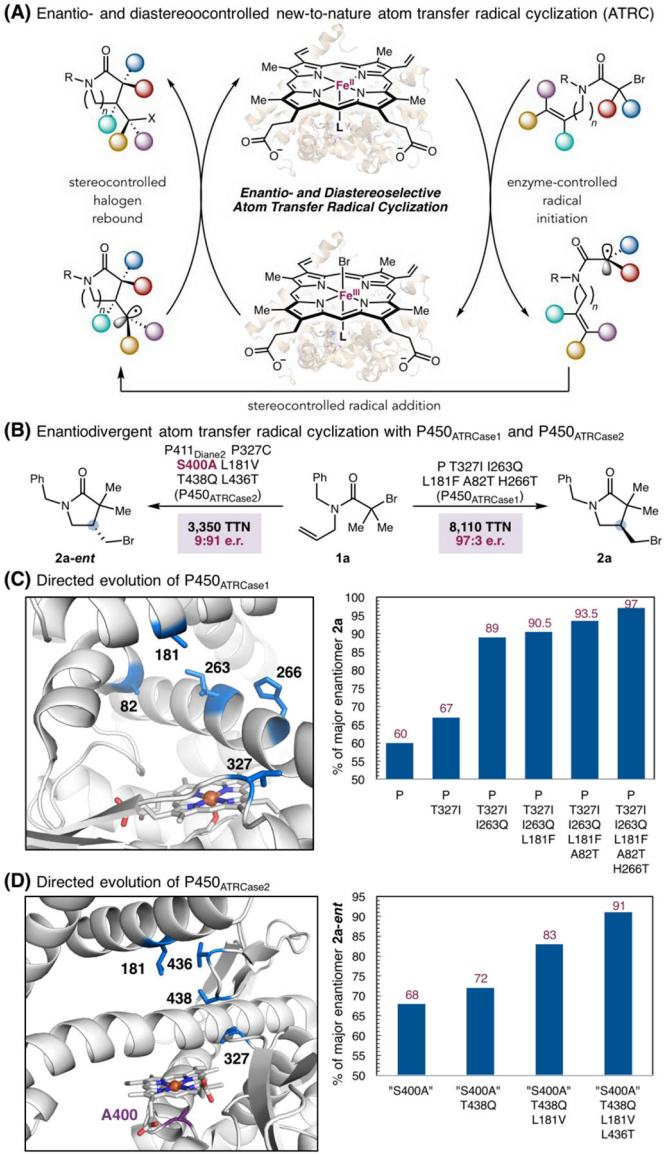

Fig. 1.

(A) Enantio- and diastereocontrolled atom transfer radical cyclization (ATRC). L = Fe-coordinating amino acid residue. **(B) Enantiodivergent atom transfer radical cyclization using P450$_{ATRCase1}$ and P450$_{ATRCase2}$. (C) Directed evolution of P450$_{ATRCase1}$**. Left illustration is made from the crystal structure of a closely related P450 variant (PDB ID: 4H23). "P" = P411-CIS T438S. **(D) Directed evolution of P450$_{ATRCase2}$**. Left illustration is made from the crystal structure of a closely related P450 variant (PDB ID: 5UCW). "S400A" = P411$_{Diane2}$ P327C S400A.

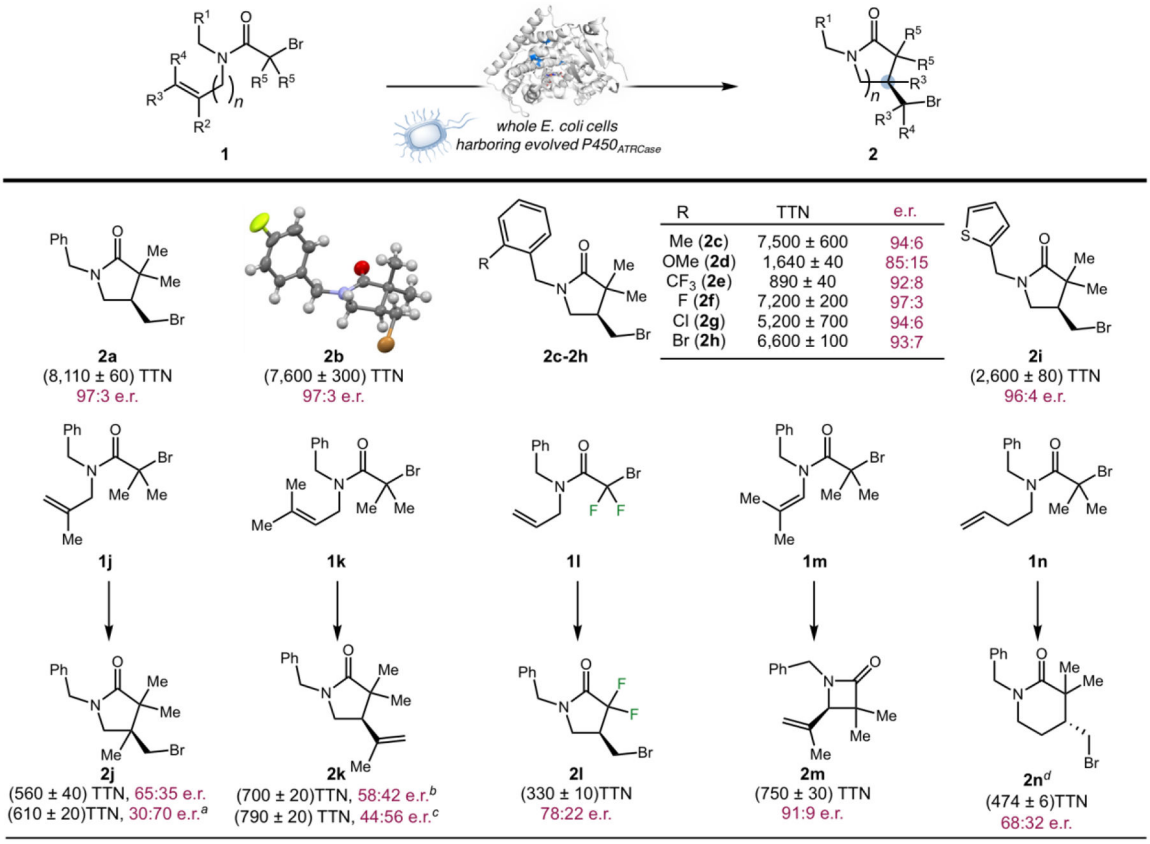

Fig. 2. Substrate scope of new-to-nature enantioselective atom transfer radical cyclization

[a]Variant P' was used. [b]P T327I I263Q was used. [c]$P450_{ATRCase1}$ (P T327I I263Q L181F A82T H266T) was used. [d]$P450_{ATRCase2}$ A330K was used. Whole-cell reactions were carried out at OD_{600} = 5−30. The variation of e.r. values is ≤ 1%. See the SM for details.

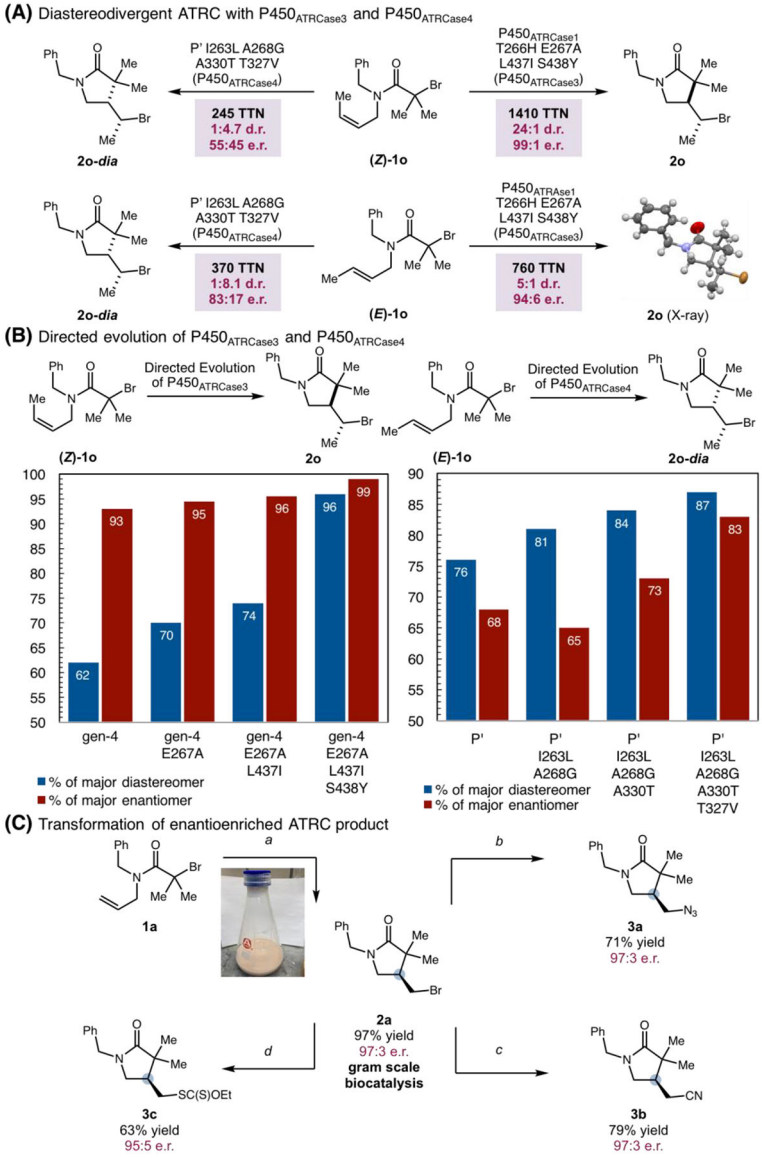

Fig. 3. (A) Diastereodivergent new-to-nature ATRC with P450$_{ATRCase3}$ and P450$_{ATRCase4}$. (B) Directed evolution of P450$_{ATRCase3}$ and P450$_{ATRCase4}$. (C) Transformation of enantioenriched ATRC product.

Conditions: *a.* Whole-cell biotransformation using *E. coli* cells resuspended in M9-N buffer (pH = 7.4) at room temperature for 12 h, 97% yield, 97:3 e.r., *b.* NaN_3, NaI, DMF/H_2O, 60 °C, 16 h, 79% yield, 97:3 e.r., *c.* NaCN, NaI, DMF/H_2O, 60 °C, 16 h, 71% yield, 97:3 e.r., *d.* KSC(S)OEt, acetone, RT, 12 h, 63% yield, 95:5 e.r..

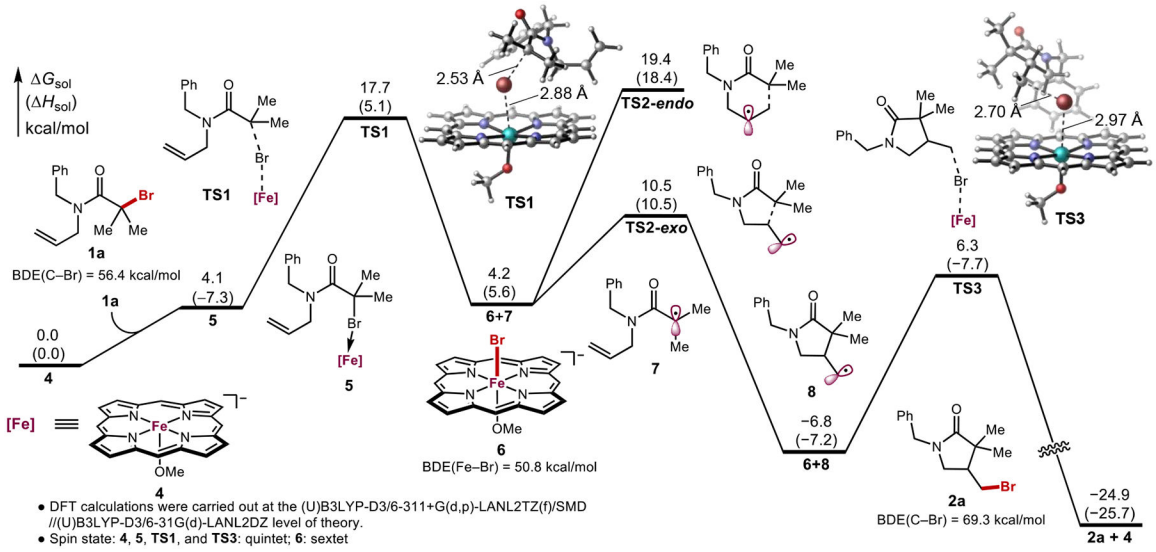

Fig. 4. Reaction energy profile of the current biocatalytic atom transfer radical addition using a model system for the axial serine-ligated Fe-porphyrin catalyst.

A methoxy-bound Fe porphyrin was used as the model system for serine-ligated P450 (39).