

An enzymatic platform for the asymmetric amination of primary, secondary and tertiary C(sp³)-H bonds

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The ability to selectively functionalize ubiquitous C–H bonds streamlines the construction of complex molecular architectures from easily available precursors. Here we report enzyme catalysts derived from a cytochrome P450 that use a nitrene transfer mechanism for the enantioselective amination of primary, secondary, and tertiary C(sp³)-H bonds. These fully genetically encoded enzymes are produced and function in bacteria, where they can be optimized by directed evolution for a broad spectrum of enantioselective C(sp³)-H amination reactions. These catalysts can aminate a variety of benzylic, allylic, and aliphatic C–H bonds in excellent enantioselectivity with access to either antipode of product. Enantioselective amination of primary C(sp³)-H bonds in substrates bearing geminal dimethyl substituents furnished chiral amines featuring a quaternary stereocentre. Moreover, these enzymes enabled the enantioconvergent transformation of racemic substrates possessing a tertiary C(sp³)-H bond to afford products bearing a tetrasubstituted stereocentre, a process that has eluded small molecule catalysts. Further engineering allowed for the enantioselective construction of ‘methyl-ethyl’ stereocentres, which is notoriously challenging in asymmetric catalysis.

The development of general systems for the highly enantioselective transformation of C(sp³)-H bonds lies at the forefront of current efforts to advance transition-metal-catalysed C–H functionalization.¹⁻⁵ In principle, several classes of enantioselective C(sp³)-H functionalization can be developed, depending on the topicity and degree of substitution (primary, secondary, or tertiary) of the sp³-hybridized carbon atom undergoing functionalization (Fig. 1). As depicted in Fig. 1a, effective enantiodiscrimination of the two prochiral secondary C(sp³)-H bonds at a methylene unit leads to the formation of a trisubstituted stereogenic centre.¹ On the other hand, asymmetric primary C(sp³)-H functionalization by differentiating the two prochiral methyl substituents can serve as a powerful means to access challenging all-carbon quaternary stereocentres (Fig. 1b).^{1,6,7} As a distinct alternative, the enantioselective functionalization of a tertiary C(sp³)-H bond will enable the conversion of readily available precursors into valuable products featuring a tetrasubstituted stereocentre (Fig. 1c). Due to the presence of a pre-existing stereogenic centre at the site of attachment, this process would require the enantioconvergent functionalization⁸ of a tertiary C(sp³)-H bond to convert both enantiomers of the racemic substrate into the same major enantiomer, a daunting challenge that so far remains out of the reach of small-molecule transition-metal catalysts. In this context, identifying a set of structurally related catalysts as a unified platform for the asymmetric functionalization of all three types of aliphatic C–H bonds will accelerate further development and application of C–

H functionalization technologies.

Enabled by numerous potentially cooperative protein-substrate interactions within the elaborate chiral scaffold of the active site, enzymes can exert exquisite control over the stereochemical outcome of various catalytic reactions, including C–H functionalization.⁹ Among naturally occurring enzymatic C–H functionalization processes, cytochrome P450-catalysed C(sp³)–H hydroxylation represents a venerable example of outstanding stereocontrol.^{10,11} Over the past six years, our laboratory^{12,13} and others¹⁴⁻¹⁷ have repurposed these enzymes and other heme proteins to catalyse synthetically useful reactions that are not known to nature. In particular, we have engineered heme proteins for abiological C–H functionalization leading to the formation of C–C and C–N bonds via a carbene¹⁸ or nitrene¹⁹⁻²¹ transfer mechanism. Collectively, these results showcase enzymes' potential for performing abiological asymmetric C–H functionalization reactions, using earth-abundant iron in a fully genetically-encodable protein that can be tuned by evolution. The capabilities of enzymes to solve key outstanding problems in asymmetric catalysis, however, have not yet been tested in these C–H functionalization processes.

Herein, we describe the development of general cytochrome P450-based biocatalysts for the asymmetric amination of primary, secondary and tertiary C(sp³)–H bonds in the synthesis of chiral diamines, a key pharmacophore in numerous antiviral and antibacterial agents (Fig. 1d and Supplementary Information Fig. 1).^{22,23} Inspired by the pioneering work of other groups in the area of transition-metal-catalysed intramolecular C(sp³)–H amination^{4,5,24-30} as well as our own studies¹⁹⁻²¹, we envisioned a unified enzymatic strategy for the catalytic asymmetric assembly of diverse 1,2- and 1,3-diamines from abundant amine precursors. As outlined in Fig. 1d, using a previously established one-step procedure,²³ the aliphatic amine (**I**) can be readily converted to the corresponding sulfamoyl azide (**II**) in excellent yield. Our proposed biocatalytic C(sp³)–H amination would lead to the formation of the enantioenriched cyclic sulfamide (**III**). Subsequent excision of the sulfonyl unit using known procedures²³ would then furnish the desired chiral diamine (**IV**, see the Supplementary Information for details of converting sulfamides to diamines). In addition to its synthetic utility, we postulated that this chiral diamine synthesis could serve as an ideal platform to identify and evolve heme proteins for the asymmetric amination of all three types of C(sp³)–H bonds (Fig. 1a-c), especially those that have not succumbed to small molecule-catalysed asymmetric C–H functionalizations.

Results and discussion

We commenced our study by evaluating a panel of heme proteins including variants of cytochromes P450, cytochromes P411 (P450 with the iron coordinating cysteine residue replaced by a serine), cytochromes *c* and globins in intact *Escherichia coli* cells for enantioselective diamine synthesis (Fig. 2a). We focused our initial investigation on the asymmetric synthesis of 1,2-diamine derivatives due to the lack of highly enantioselective C–H amination methods for synthesizing these compounds.³¹ Among the heme proteins we tested, a few variants from the cytochrome P450 superfamily showed low levels of C–H amination activity (see Supplementary Information for details). In particular, a truncated P411 variant lacking the FAD domain (P411_{Diane1}, P411 diamine synthase), which we developed during an earlier study on Fe-catalysed carbene insertion into C(sp³)–H bonds,¹⁸ was over 10 times more active than other heme proteins, providing a total turnover number (TTN) of 450 and an enantiomeric excess (ee) of 94% for the 1,2-diamine product (**2a**). This demonstrates that the reductase domain of cytochrome P450 is not needed for C–H amination. Finally, the absolute stereochemistry of **2a** was ascertained by single crystal X-ray diffraction analysis.

We used P411_{Diane1} as the starting template for directed evolution of 1,2-diamine synthase (Fig. 2a). In an effort to further improve the enzyme's activity and enantioselectivity for this C–H amination process, we performed iterative rounds of site-saturation mutagenesis (SSM) and screening, targeting amino acid residues close to the heme cofactor. For each round of engineering, enzyme libraries were expressed and screened in 96-well plates in the form of whole *E. coli* cells. Beneficial mutations I327P, Y263W and Q437F were introduced in three rounds, furnishing a 10-fold improvement in activity as well as further enhancements in enantioselectivity. Notably, undesired nitrenoid reduction was also effectively suppressed. Using final variant P411_{Diane2}, the C(sp³)–H amination product formed in 3490 TTN and 99.9% ee as determined by chiral gas chromatography (GC). Moreover, by further lowering the cell density in whole-cell biotransformations, this enzymatic C–H amination provided the diamine product in 72,000 TTN and 99.9% ee, thereby demonstrating the excellent catalytic efficiency of the engineered P411 enzyme relative to previously developed transition-metal catalysts.

Using *E. coli* whole cells harbouring P411_{Diane2}, we surveyed the substrate scope of this C(sp³)–H amination process for 1,2-diamine synthesis (Fig. 2b). Electron-donating and electron-withdrawing substituents on the aromatic ring were compatible with this process (**2a–2g**), affording 1,2-diamines with uniformly high levels of enantioselectivity. Additionally, substrates bearing a halogen functional group handle for further derivatization were accepted by the enzyme (**2e** and **2f**). Steric hindrance at the *ortho* position was also compatible (**2h**), although lower activity was observed. Structural perturbations such as replacement of the aryl ring by thiophene (**2i**) and variance of the *N*-substituent (**2j**) were well-tolerated by this biocatalytic C(sp³)–H amination. Moreover, we found that the starting variant P411_{Diane1} could be employed for the asymmetric synthesis of 1,3-diamine derivatives using C(sp³)–H amination. In addition to the asymmetric amination of benzylic C(sp³)–H bonds (**2k–2n**), allylic C(sp³)–H bonds (**2o**) were also effectively aminated with excellent enantioselectivity. In contrast to Rh(OAc)₂-based systems,²⁴ competing aziridination product was not observed, highlighting the chemoselectivity of these iron-based biocatalysts.

Furthermore, directed evolution of P411_{Diane1} furnished a complementary set of enzymatic catalysts allowing for the enantiodivergent amination of unactivated secondary aliphatic C(sp³)–H bonds (Fig. 3). Amino acid residue 87 located in a loop in the active site and known for its importance in substrate recognition in P450-catalysed oxidation and carbene transfer reactions^{32,33} was found to play a dominant role in determining the sense of absolute stereochemistry of the C–H amination product. Specifically, a single A87I mutation inverted the absolute configuration of the newly formed stereocentre. With this initial result, additional rounds of SSM and screening led to P411_{Diane1} L82M A87I Y263W I327S, delivering the C(sp³)–H amination product in 96% ee (*R* enantiomer). On the other hand, leaving A87 unchanged, a single mutation I327T resulted in a significant enhancement in enantioselectivity (32% ee to 71% ee). This is a rare example of enantiodivergent C(sp³)–H amination reactions catalysed by engineered heme proteins.

Having engineered a set of enzymes for the asymmetric amination of secondary C(sp³)–H bonds, we questioned whether this enantioselective amination could be extended to the conversion of unactivated primary aliphatic C(sp³)–H bonds, a thermodynamically more challenging process.²⁶ Examination of our P411_{Diane} collection revealed that the P411_{Diane1} I327P variant already displayed excellent activity and enantioselectivity for the aminative desymmetrization of geminal dimethyl substituents, providing the desired 1,3-diamine possessing an all-carbon quaternary stereocentre at the β position in 99% ee (Fig. 4a). In the realm of asymmetric catalysis, the desymmetrization of geminal dimethyl groups has been recognized as a promising solution to bypass the long-standing problem of enantioselective

methylation.³⁴ In this context, our biocatalytic desymmetrization represents a valuable example of C–N bond formation for the construction of methyl-substituted stereocentres. Subsequent examination of substrate scope revealed that this desymmetrization is also applicable to other substrates bearing various aryl groups (**4a–4d**). The absolute configuration of **4a** was determined by X-ray diffraction analysis.

The enantioselective functionalization of tertiary C(sp³)–H bonds found in racemic substrates represents an unsolved problem for small molecule catalysts. In particular, enantioconvergent protocols to transform both antipodes of the racemic C–H substrate into the same major enantiomer of the product remain elusive, although conceptually similar enantioconvergent transformations of tertiary alkyl halides have recently received considerable attention.^{35–37} Previous studies indicated that iron nitrene-mediated C–H amination may involve a radical mechanism,^{38,39} thus suggesting the possibility of achieving stereochemical convergence in the enzymatic tertiary C(sp³)–H functionalization. Nonetheless, such enzymatic enantioconvergent transformations would require the same heme protein to accommodate both antipodes of the racemic substrate, a process rarely found in nature’s biochemical repertoire. To our delight, we found that P411_{Diane1} could effect the enantioconvergent C(sp³)–H amination to provide diamine product **5a** featuring a tetrasubstituted stereocentre (99% ee). Iterative SSM and screening generated improved variant P411_{Diane3}. Bearing beneficial mutations L78A, A87G, Q437G and I327V, P411_{Diane3} provided the diamine product in 76% yield, 1910 TTN and 99% ee (Fig. 4b). More importantly, P411_{Diane3} was found to be effective in the enantioconvergent amination of other substrates (**5a–5f**). Electron-donating (**5b**), electron-withdrawing (**5c**) and *ortho*-substituents (**5d**) were effectively tolerated under these conditions. Substrates bearing heterocycles (**5e**) and other branching alkyl groups (**5f**) could also be transformed with excellent enantioselectivity.

The effective discrimination between two minimally differentiated methyl- (Me-) and ethyl- (Et-) groups to construct ‘methyl-ethyl’ stereocentres is a notoriously difficult problem in asymmetric catalysis.^{40,41} We envisioned that engineered heme proteins could provide a powerful platform to address this challenge (Fig. 4c). Indeed, directed evolution of P411_{Diane1} led to P411_{Diane4} bearing five additional mutations (L82C, L181V, I327T, A330M and Q437G), culminating in the enantioconvergent formation of ‘methyl-ethyl’ stereocentre (**5g**) in 82% yield, 2120 TTN and 87% ee. This result represents a rare example of solving the ‘methyl-ethyl’ problem using a C(sp³)–H functionalization strategy.

Mechanistic and computational investigations provided further insight into this enantioconvergent C(sp³)–H amination process. First, we prepared sulfamoyl azide substrates bearing a stereochemically well-defined olefin moiety ((*Z*)-**1d** and (*E*)-**1d**) and subjected them to the enzymatic reactions (Fig. 5a). Partial scrambling of the olefin geometry was observed in both the (*E*)- and (*Z*)-substrates, with the (*Z*)-substrate providing a substantial amount of the scrambled product bearing a thermodynamically more stable (*E*)-olefin. The erosion of C=C double bond stereochemistry is consistent with the formation of a carbon-centred radical at the allylic position and does not agree with a concerted C–H insertion mechanism. Consistent with literature reports on related iron-based catalyst systems,^{38,39,42} these findings support a radical mechanism for this cytochrome P450-catalysed C(sp³)–H amination process.

Thus, we postulate that this enantioconvergent amination comprises a stereoablative hydrogen atom transfer (HAT) and an enantioselective C–N bond formation. As described in Fig. 5b, reaction of the ferrous heme cofactor with the racemic azide substrate leads to an open-shell Fe-nitrenoid intermediate. Subsequent stereoablative HAT results in the formation

of a carbon-centred radical, which then undergoes the final radical rebound step with high levels of enantioselectivity. This mechanistic proposal is further corroborated by our DFT calculations (Fig. 6). Computational studies on an iron porphyrin model system showed that the triplet state of the key Fe-nitrenoid intermediate **7** is 3.0 kcal/mol more stable than the open-shell singlet state and 11.6 kcal/mol more stable than the quintet state (see the Supplementary Information for the comparison between open-shell singlet, triplet, and quintet free energy profiles). The calculated Mulliken spin density of **7** revealed a spin density of 0.84 on the N atom and 1.16 on the Fe atom, demonstrating significant radical characters on both the nitrogen and the iron centre. In addition, our computations suggest that the hydrogen atom transfer is irreversible and occurs through triplet transition state **TS1** with an energy barrier of 15.7 kcal/mol. Furthermore, the subsequent radical rebound step proceeding through quintet transition state **TS2** requires an activation energy of 18.4 kcal/mol, suggesting sufficient lifetime of the carbon-centred radical to allow for stereoablation and the subsequent enantioselective C–N bond formation observed in the enzymatic reaction.

In conclusion, we have developed a biocatalytic platform for the asymmetric amination of a variety of C(sp³)–H bonds, permitting a diverse range of synthetically useful chiral diamines to be prepared with excellent enantioselectivity. These biocatalysts are fully genetically encoded, and thus can be easily tuned and reconfigured through DNA manipulation. Empowered by directed evolution, this genetically encoded platform allowed for the rapid development of highly active biocatalysts for the enantioselective amination of primary, secondary, and tertiary aliphatic C–H bonds. Notably, some of these processes, such as the enantioconvergent tertiary C(sp³)–H amination, have not been successfully implemented with small molecule catalysts. We anticipate that this biocatalytic platform can be further leveraged to tackle other challenges in enantioselective C–H functionalization and asymmetric catalysis in general.

Methods

Expression of P411 variants. *E. coli* (*E. coli* BL21(DE3)) cells carrying plasmid encoding the appropriate P411 variant were grown overnight in 4 mL LB_{amp}. Preculture (3 mL) was used to inoculate 27 mL of HB_{amp} in a 125 mL Erlenmeyer flask. This culture was incubated at 37 °C, 230 rpm for 2.5 h. The culture was then cooled on ice for 20 min and induced with 0.5 mM IPTG and 1.0 mM 5-aminolevulinic acid (final concentrations). Expression was conducted at 20 °C, 130 rpm, for 16–18 h. *E. coli* cells were then pelleted by centrifugation (4500 g, 3 min, 4 °C). Media was removed and the resulting cell pellet was resuspended in M9-N buffer to OD₆₀₀ = 30–40. An aliquot of this cell suspension (2 mL) was taken to determine P411 concentration using the hemochrome assay after lysis by sonication. When applicable, the remaining cell suspension was further diluted with M9-N buffer to the OD₆₀₀ used for the biotransformation and the concentration of P411 protein in the biotransformation was calculated accordingly.

C–H amination reactions using whole *E. coli* cells harbouring P411. Suspensions of *E. coli* cells expressing the appropriate heme protein variant in M9-N buffer (typically OD₆₀₀ = 30) were kept on ice. In another centrifuge tube, a solution of D-glucose (250 mM in M9-N) was prepared. All solutions were then transferred into an anaerobic chamber for reaction set up. To a 2 mL vial were added a GOx oxygen depletion solution (20 μL of stock solution containing 14,000 U/mL catalase and 1,000 U/mL glucose oxidase in M9-N buffer), D-glucose (40 μL of 250 mM stock solution in M9-N buffer), the suspension of *E. coli* expressing P411 (320 μL), and the sulfamoyl azide substrate (20 μL, typically 400 mM stock solution in EtOH) in succession. The vials were sealed and shaken at room temperature and

500 rpm for 12-20 h and then analyzed by GC-MS or LC-MS.

Competing Interest: A provisional patent application (inventors: Yang Yang; Inha Cho) has been filed through the California Institute of Technology. The provisional patent covers the development and application of engineered cytochromes P450 for the synthesis of chiral diamine derivatives by C-H amination.

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Data and materials availability: All data are available in the main text and the Supplementary Materials. Solid-state structures of **2a**, **4a**, **5a** and **5f** are available free of charge from the Cambridge Crystallographic Data Centre under reference numbers CCDC 1905551, 1905553, 1905552 and 1905554. Plasmids encoding the enzymes reported in this study are available for research purposes from F.H.A. under a material transfer agreement with the California Institute of Technology.

Author contributions

Y.Y. designed the overall research with F.H.A. providing guidance. Y.Y. and I.C. designed and performed the initial screening of heme proteins and directed evolution experiments. Y.Y. designed and performed the substrate scope study and mechanistic study. X.Q. carried out the computational studies with P.L. providing guidance. Y.Y. and F.H.A. wrote the manuscript with the input of all other authors.

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Fig. 1. Three major types of asymmetric C(sp³)-H functionalization and our envisioned biocatalytic C(sp³)-H amination. a, Enantioselective functionalization of secondary C(sp³)-H bonds. **b**, Enantioselective functionalization of primary C(sp³)-H bonds (i.e., desymmetrization of gem-dimethyl substituents). **c**, Enantioconvergent functionalization of

tertiary C(sp³)-H bonds. **d**, Enzymatic synthesis of chiral diamines using C(sp³)-H amination. FG = functional group.

Fig. 2. Enantioselective amination of secondary C(sp³)-H bonds. **a**, Directed evolution of P411ΔFAD for the enantioselective synthesis of 1,2-diamines. Crystal structure of a variant closely related to P411_{Diane1} is shown (Protein Data Bank ID: 5UCW); **b**, Substrate scope of 1,2-diamine and 1,3-diamine synthesis. Experiments were performed using *E. coli* expressing cytochrome P411_{Diane2} or P411_{Diane1} (OD₆₀₀ = 30) with 10 mM substrate at room temperature under anaerobic conditions for 12–24 h. †Performed using *E. coli* expressing cytochrome P411_{Diane2} or P411_{Diane1} (OD₆₀₀ = 1.9) with 20 mM substrate at room temperature under anaerobic conditions for 24 h.

Fig. 3. Enantiodivergent amination of unactivated secondary C(sp³)-H bonds to access either the (R)- or the (S)-product using engineered P411_{Diane} variants.

Fig. 4. Engineered P411_{Diane} variants for the enantioselective amination of primary and tertiary C(sp³)-H bonds. **a**, asymmetric amination of primary C(sp³)-H bonds (i.e., desymmetrization of geminal dimethyl substituents); **b**, Enantioconvergent amination of tertiary C(sp³)-H bonds; **c**, Enantioconvergent construction of ‘methyl-ethyl’ stereocentre using tertiary C(sp³)-H amination. Experiments were performed using *E. coli* expressing cytochrome P411_{Diane1} I327P, P411_{Diane1} L78A A87G I327V Q437G, and or P411_{Diane1} L82C L181V I327T A330M Q437G (OD₆₀₀ = 30–40) with 10 mM substrate at room temperature under anaerobic conditions for 12–24 h.

Fig. 5. Mechanistic insight. **a**, Scrambling of olefin stereochemistry during the allylic C(sp³)-H amination of (*E*)- and (*Z*)-1d using P411 biocatalyst (P411_{Diane1} I327P). **b**, Proposed mechanism for the enantioconvergent amination of tertiary C(sp³)-H bonds.

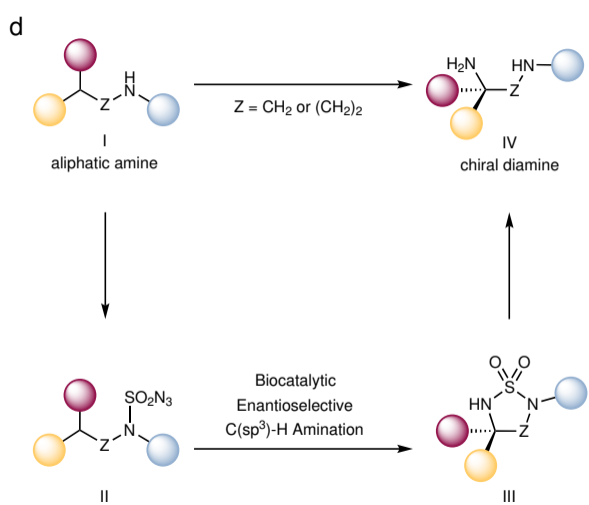
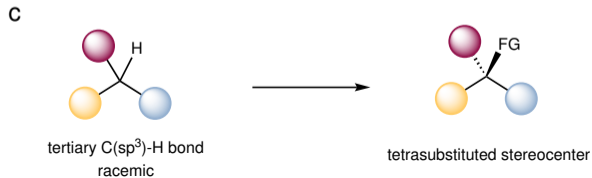
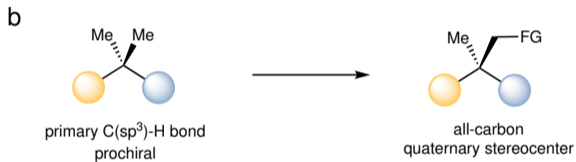
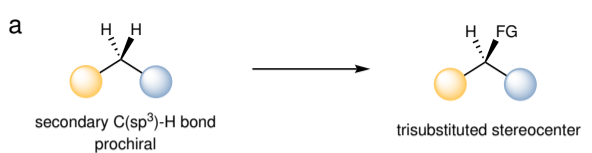
Fig. 6. Free energy profile of the iron porphyrin-catalysed C(sp³)-H amination. Density functional theory (DFT) calculations were performed at the B3LYP-D3(BJ)/6-311+G(d,p)-LANL2TZ(f)/SMD(chlorobenzene)//B3LYP-D3(BJ)/6-31+G(d)-LANL2DZ level of theory. Triplet structures are shown in black and quintet structures are shown in blue. Mulliken spin densities of Fe and N of the key iron nitrenoid **7** are shown in italic (blue).

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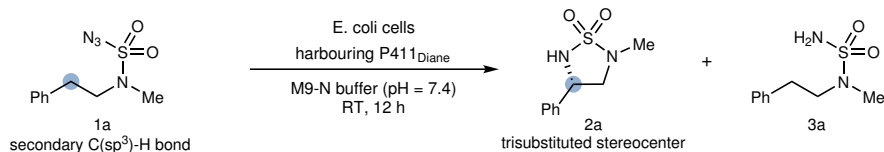
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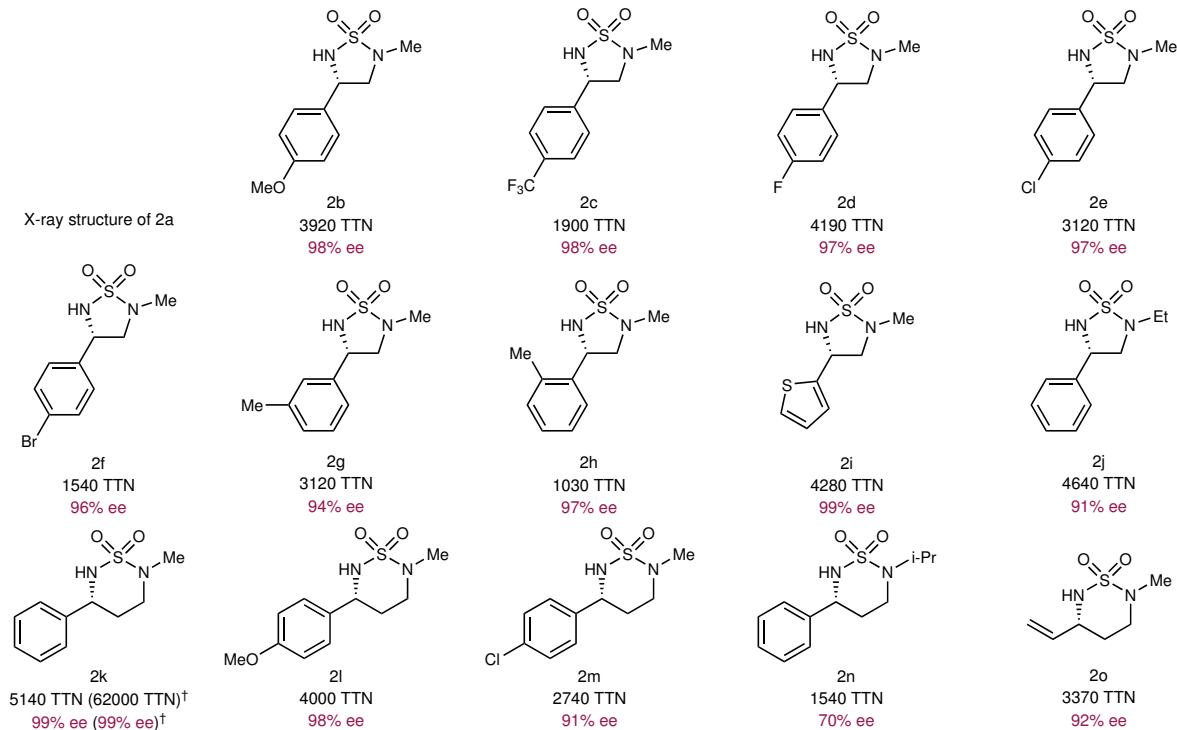


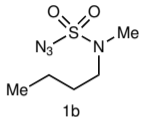
a



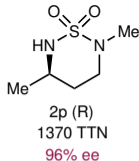
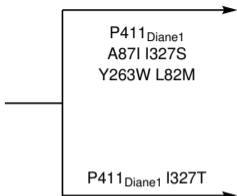
P411	TTN of 2a	ee of 2a	ratio of 2a/3a
P411 _{Diane1}	450	94% ee	31:69
P411 _{Diane1} I327P	1140	99% ee	57:43
P411 _{Diane1} I327P Y263W	2540	99.4% ee	98:2
P411 _{Diane1} I327P	3490	99.9% ee	98:2
Y263W Q437F	(72000) [†]	(99.9% ee) [†]	
(P411 _{Diane2})			

b

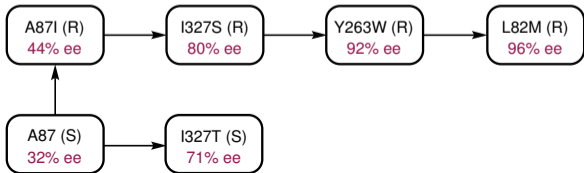


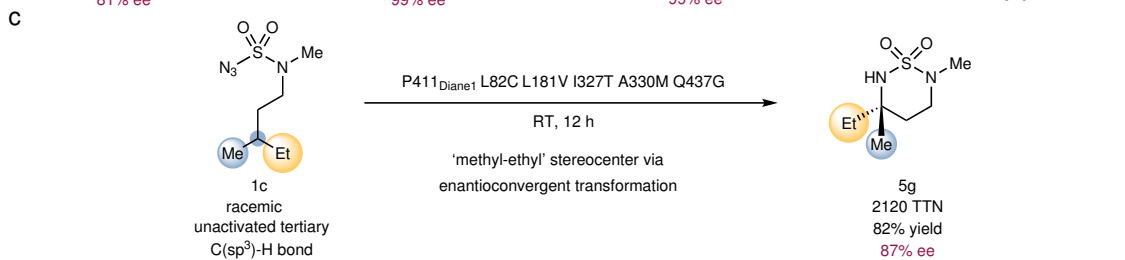
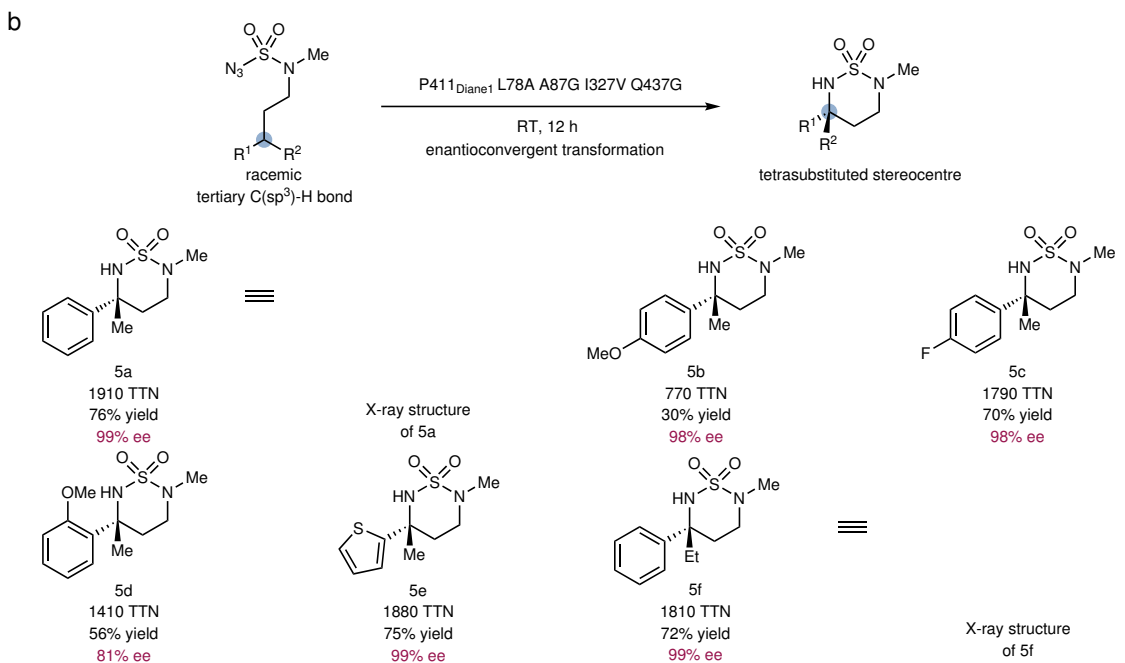
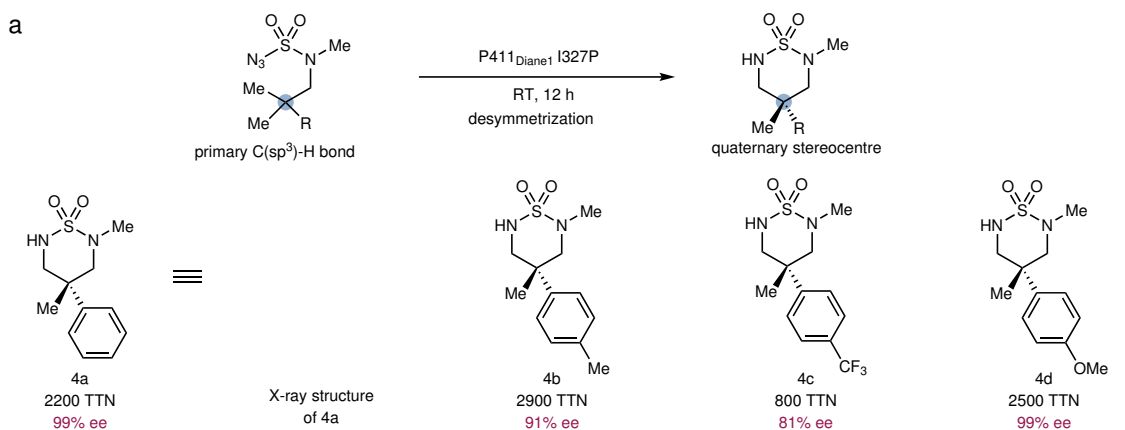


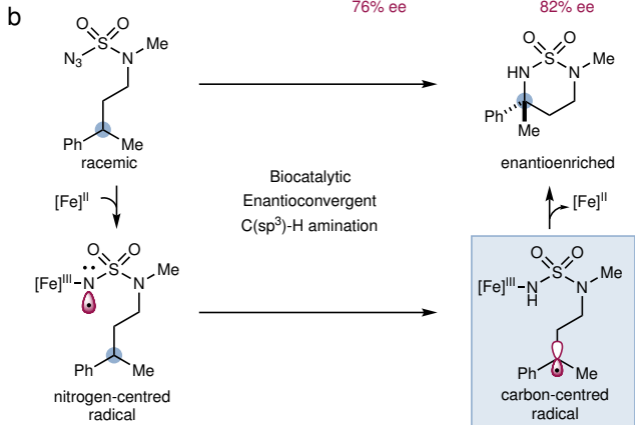
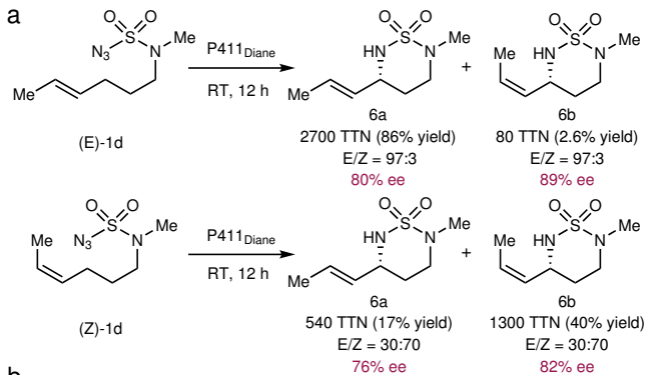
unactivated secondary
C(sp³)-H bond

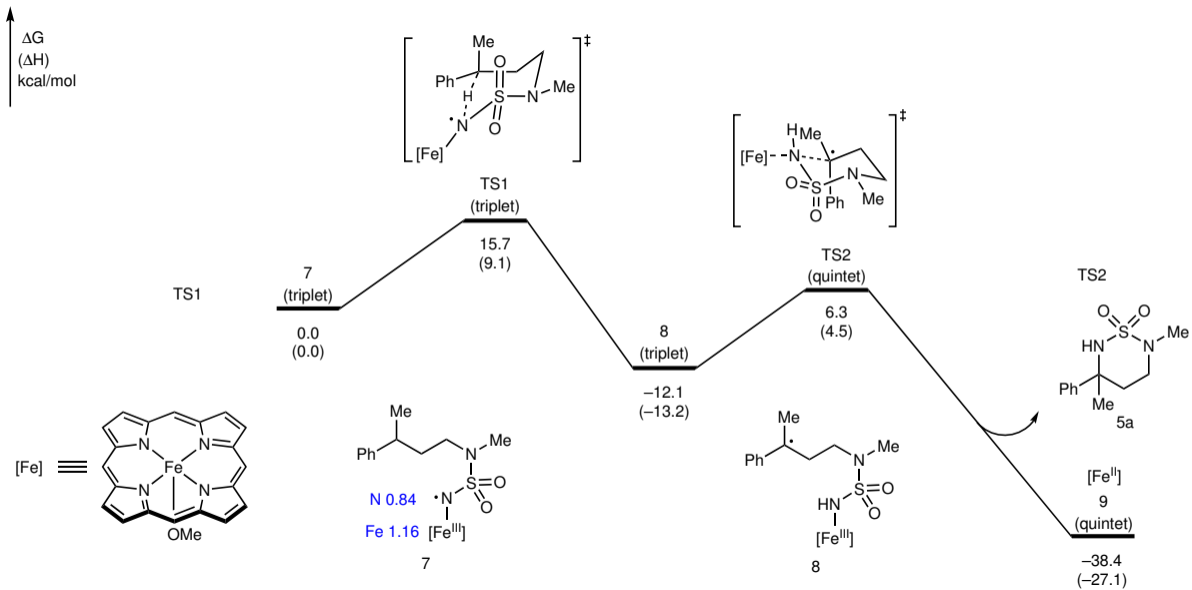


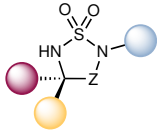
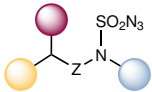
Evolutionary trajectory











primary, secondary,
and tertiary $\text{C}(\text{sp}^3)\text{-H}$ bonds

tri- and tetrasubstituted
stereocentre