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An enzymatic platform for the asymmetric amination of primary, secondary and tertiary C(sp^3)–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(sp^3)–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(sp^3)–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(sp^3)–H bonds in substrates that bear geminal dimethyl substituents furnished chiral amines that feature a quaternary stereocentre. Moreover, these enzymes enabled the enantioconvergent transformation of racemic substrates that possess a tertiary C(sp^3)–H bond to afford products that bear 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(sp^3)–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(sp^3)–H functionalization can be developed, depending on the topicity and degree of substitution (primary, secondary or tertiary) of the sp^3-hybridized carbon atom undergoing functionalization (Fig. 1). As depicted in Fig. 1a, effective enantiodiscrimination of the two prochiral secondary C(sp^3)–H bonds at a methylene unit leads to the formation of a trisubstituted stereogenic centre[1]. On the other hand, performing asymmetric primary C(sp^3)–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(sp^3)–H bond will enable the conversion of readily available precursors into valuable products that feature 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 functionalization[8] of a tertiary C(sp^3)–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 the application of C–H functionalization technologies.

Enabled by numerous potentially cooperative protein–substrate interactions in the elaborate chiral scaffold of the active site, enzymes can exert 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(sp^3)–H hydroxylation represents a venerable example of outstanding stereocontrol[10,11]. Over the past six years, our laboratory[12,13] and others[14–17] have repurposed these enzymes and other haem proteins to catalyse synthetically useful reactions that are not known to nature. In particular, we have engineered haem proteins for abiological C–H functionalization leading to the formation of C–C and C–N bonds through a carbene[18] or nitrene[19–21] transfer mechanism. These results collectively 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(sp^3)–H bonds in the synthesis of chiral diamines, a key pharmacophore in numerous antiviral and antibacterial agents (Fig. 1d and Supplementary Fig. 1)[22,23]. Inspired by the pioneering work of other groups in the area of transition metal-catalysed intramolecular C(sp^3)–H amination[4,5,24–30], as well as our own studies[19–21], 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(sp^3)–H amination would lead to the formation of the enantioenriched cyclic sulfamide (III). Subsequent excision of the sulfonyl unit using known procedures[23] would then furnish the desired chiral diamine (IV, see the Supplementary Information for details on 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 haem proteins for the asymmetric amination of all

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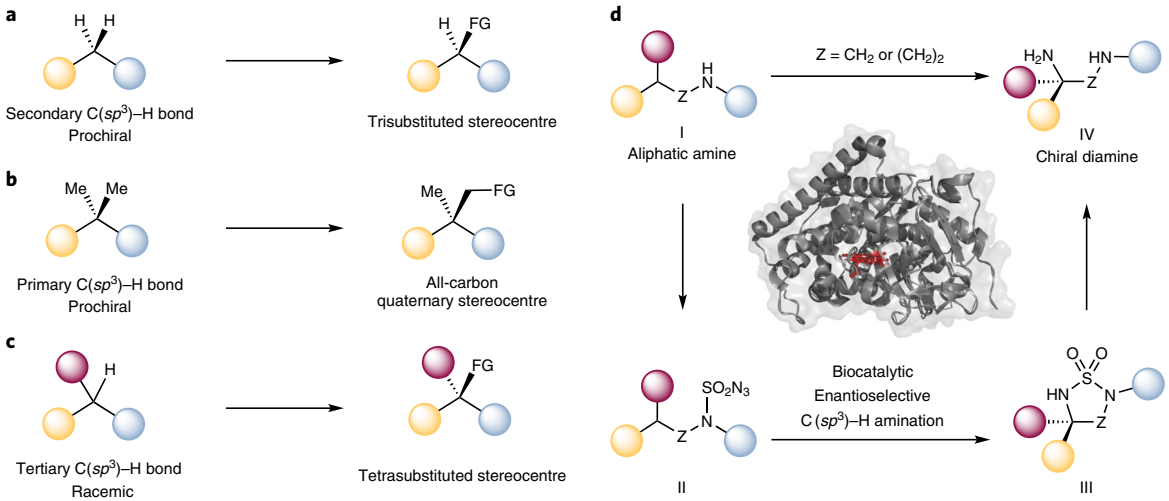

Fig. 1 | The three major types of asymmetric C(sp^3)–H functionalization and our envisioned biocatalytic C(sp^3)–H amination. a, Enantioselective functionalization of secondary C(sp^3)–H bonds. **b**, Enantioselective functionalization of primary C(sp^3)–H bonds (that is, the desymmetrization of geminal dimethyl substituents). **c**, Enantioconvergent functionalization of tertiary C(sp^3)–H bonds. **d**, Enzymatic synthesis of chiral diamines using C(sp^3)–H amination. Red, yellow and blue spheres are generic substituents of the molecule. FG, functional group.

three types of C(sp^3)–H bonds (Fig. 1a–c), especially those that have not succumbed to small molecule-catalysed asymmetric C–H functionalization.

Results and discussion

We commenced our study by evaluating a panel of haem proteins—including variants of cytochromes P450, cytochromes P411 (P450 but 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 haem proteins we tested, a few variants from the cytochrome P450 superfamily showed low levels of C–H amination activity (see the Supplementary Information for details). In particular, a truncated P411 variant lacking the FAD domain (P411 diamine synthase, also known as $P411_{Diane1}$) that we developed during an earlier study on iron-catalysed carbene insertion into C(sp^3)–H bonds[18] was over ten times more active than other haem proteins, providing a total turnover number (TTN) of 450 and an enantiomeric excess (e.e.) 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 haem 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 tenfold improvement in activity as well as further enhancements in enantioselectivity; undesired nitrenoid reduction was also effectively suppressed. Using final-variant $P411_{Diane2}$, the C(sp^3)–H amination product formed in 3,490 TTN and 99.9% e.e., as determined by chiral gas chromatography. 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% e.e., thereby demonstrating the excellent catalytic efficiency of the engineered P411 enzyme relative to previously developed transition metal catalysts.

Using *E. coli* whole cells that harbour $P411_{Diane2}$, we surveyed the substrate scope of this C(sp^3)–H amination process for 1,2-diamine synthesis (Fig. 2b). Electron-donating and -withdrawing substituents on the aromatic ring were compatible with this process (**2a–2g**), affording 1,2-diamines with uniformly high levels of enantioselectivity. Furthermore, 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(sp^3)–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(sp^3)–H amination. In addition to the asymmetric amination of benzylic C(sp^3)–H bonds (**2k–2n**), allylic C(sp^3)–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.

Directed evolution of $P411_{Diane1}$ furnished a complementary set of enzymatic catalysts that allow for the enantiodivergent amination of unactivated secondary aliphatic C(sp^3)–H bonds (Fig. 3). Amino acid residue 87, which is 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, further rounds of SSM and screening led to $P411_{Diane1}$ L82M A87I Y263W I327S, delivering the C(sp^3)–H amination product in 96% e.e. (as the *R* enantiomer). On the other hand, when leaving A87 unchanged, a single mutation I327T resulted in a considerable enhancement in enantioselectivity (32% e.e. to 71% e.e.). This is a rare example of enantiodivergent C(sp^3)–H amination reactions that are catalysed by engineered haem proteins.

Having engineered a set of enzymes for the asymmetric amination of secondary C(sp^3)–H bonds, we questioned whether this enantioselective amination could be extended to the conversion

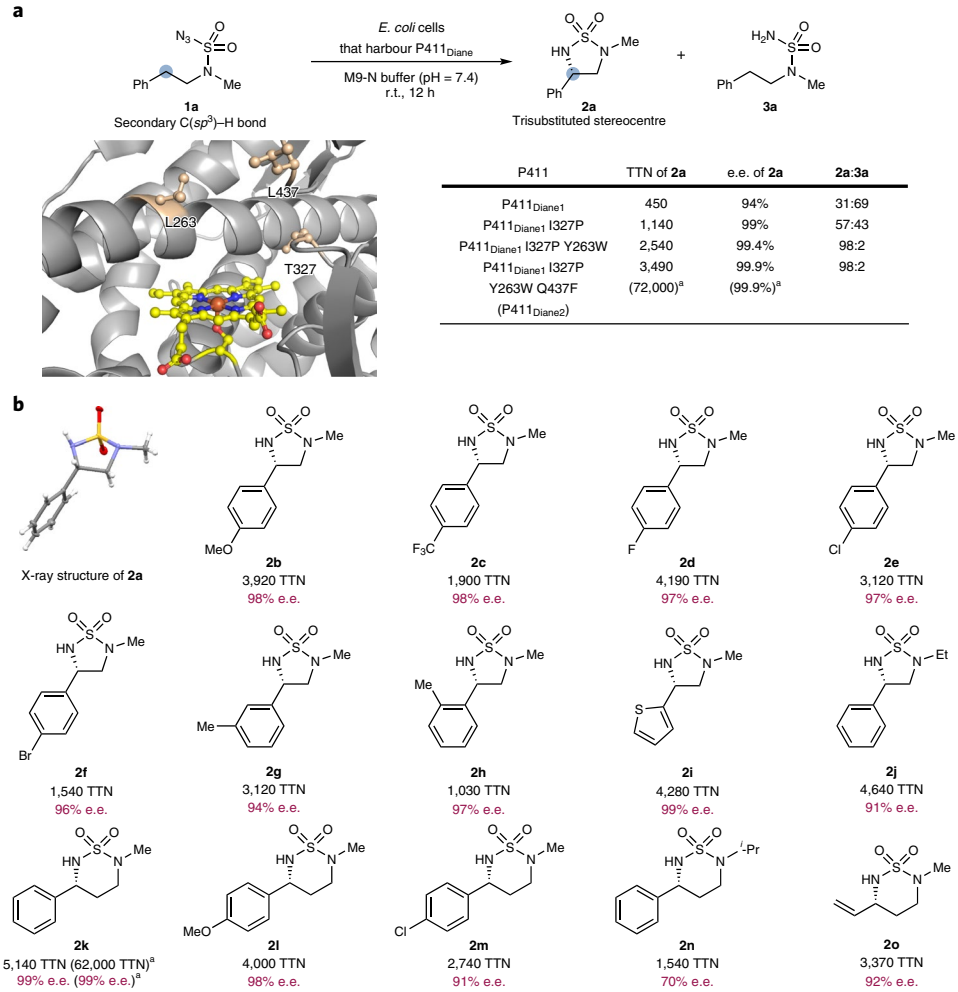

Fig. 2 | Enantioselective amination of secondary C(sp^3)–H bonds. a, The directed evolution of P411ΔFAD for the enantioselective synthesis of 1,2-diamines. The crystal structure of a variant closely related to $P411_{Diane1}$ is shown (PDB ID: 5UCW). **b**, The substrate scope of 1,2-diamine and 1,3-diamine synthesis. The experiments were performed using *E. coli* that expresses cytochrome $P411_{Diane}$ (OD_{600} = 30) with 10 mM substrate at room temperature (r.t.) under anaerobic conditions for 12–24 h. See the Supplementeary Information for details. [a]Performed using *E. coli* expressing cytochrome $P411_{Diane2}$ or $P411_{Diane1}$ (OD_{600} = 1.9) with 20 mM substrate at room temperature under anaerobic conditions for 24 h.

of unactivated primary aliphatic C(sp^3)–H bonds, which is 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 that possesses an all-carbon quaternary stereocentre at the β position in 99% e.e. (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 the substrate scope revealed that this desymmetrization is also applicable to other substrates that bear various aryl groups (**4a**–**4d**). The absolute configuration of **4a** was determined by X-ray diffraction analysis.

Although conceptually similar enantioconvergent transformations of tertiary alkyl halides have recently received considerable attention[35–37], enantioconvergent protocols to transform both antipodes of a racemic C–H substrate into the same major enantiomer of the product remain elusive. Previous studies indicate that iron nitrene-mediated C–H amination may involve a radical mechanism[38,39], thus suggesting the possibility of achieving

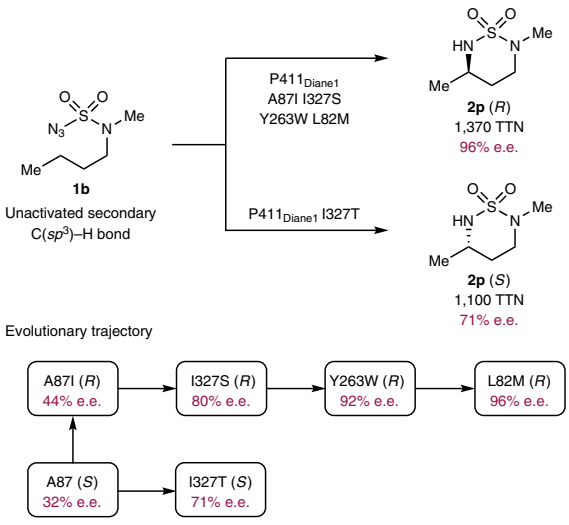

Fig. 3 | Amination of unactivated secondary C(sp^3)–H bonds. The enantiodivergent amination of unactivated secondary C(sp^3)–H bonds to access either the (*R*)- or the (*S*)-product using engineered $P411_{Diane}$ variants.

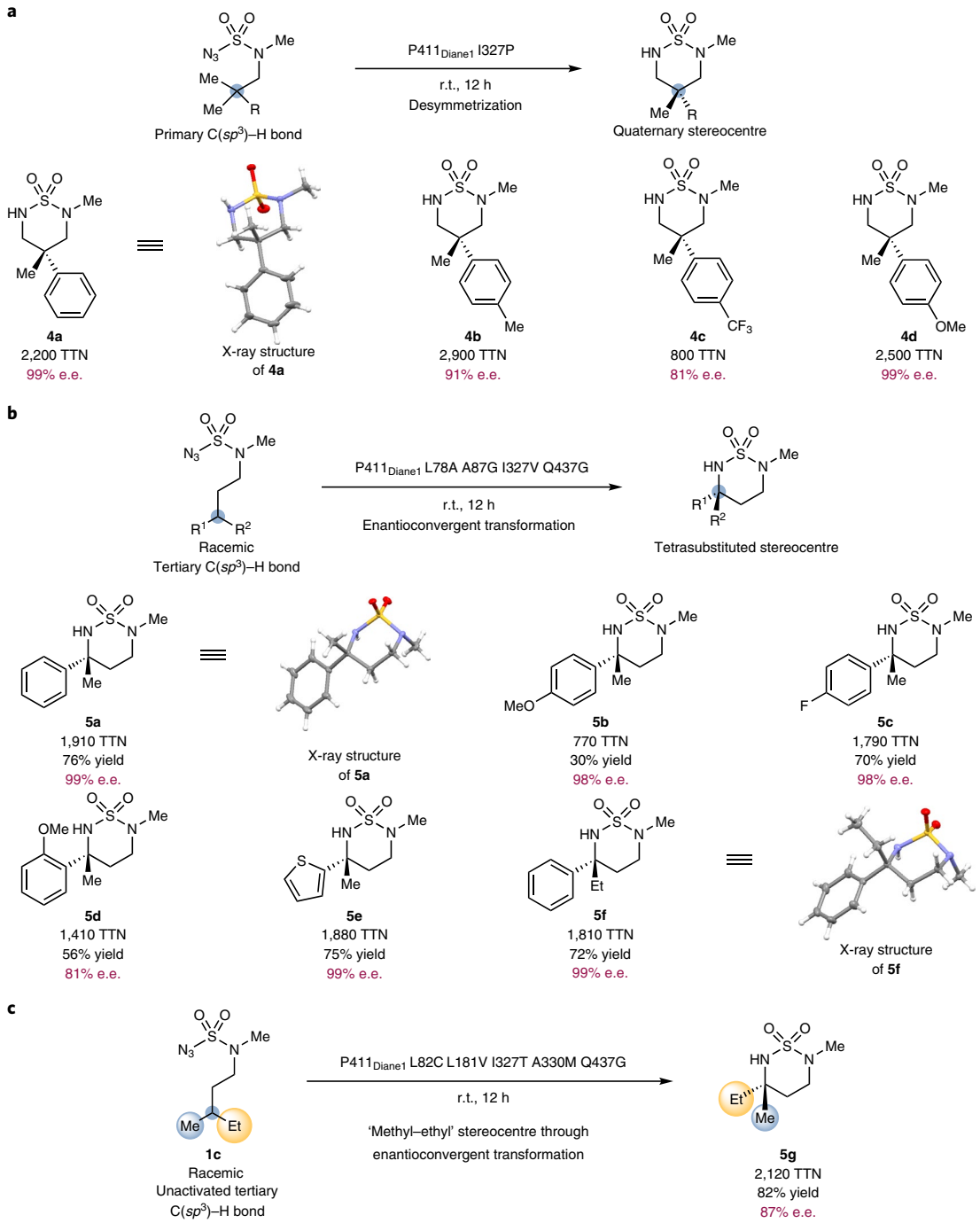

Fig. 4 | Engineered P411$_{Diane}$ variants for the enantioselective amination of primary and tertiary C(sp^3)–H bonds. a, Asymmetric amination of primary C(sp^3)–H bonds (that is, desymmetrization of geminal dimethyl substituents). **b**, Enantioconvergent amination of tertiary C(sp^3)–H bonds. **c**, The enantioconvergent construction of a methyl–ethyl stereocentre using tertiary C(sp^3)–H amination. Experiments were performed using *E. coli* that expresses cytochrome P411$_{Diane1}$ I327P, P411$_{Diane1}$ L78A A87G I327V Q437G and/or P411$_{Diane1}$ L82C L181V I327T A330M Q437G (OD_{600} = 30–40) with 10 mM substrate at room temperature under anaerobic conditions for 12–24 h.

stereochemical convergence in the enzymatic tertiary C(sp^3)–H functionalization. Nonetheless, such enzymatic enantioconvergent transformations would require the same haem protein to accommodate both antipodes of the racemic substrate, a process rarely found in nature's biochemical repertoire. We found that P411$_{Diane1}$ could effect the enantioconvergent C(sp^3)–H amination to provide diamine product **5a**, which features a tetrasubstituted stereocentre (99% e.e.). Iterative SSM and screening generated an improved variant, P411$_{Diane3}$, which bears the beneficial mutations L78A, A87G, Q437G and I327V and provides the diamine product in 76% yield, 1,910 TTN and 99% e.e. (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 that bear heterocycles (**5e**) and other branching alkyl groups (**5f**) could also be transformed with excellent enantioselectivity.

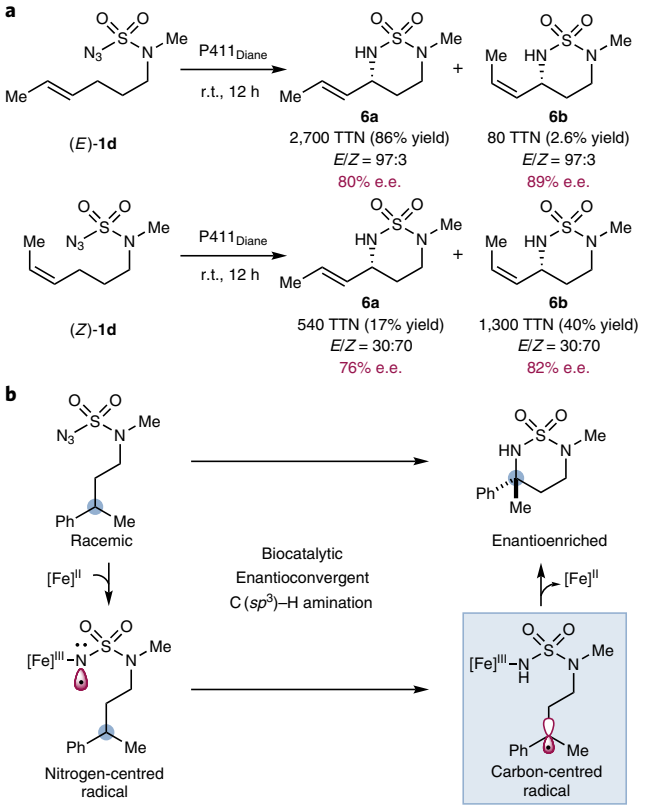

Fig. 5 | Mechanistic insight. a, Scrambling of olefin stereochemistry during the allylic C(sp^3)–H amination of (*E*)- and (*Z*)-**1d** using P411 biocatalyst ($P411_{Diane1}$ I327P). **b**, The proposed mechanism for the enantioconvergent amination of tertiary C(sp^3)–H bonds.

The effective discrimination between two minimally differentiated methyl and ethyl groups to construct methyl–ethyl stereocentres is a notoriously difficult problem in asymmetric catalysis[40,41]. We envisioned that engineered haem 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 a methyl–ethyl stereocentre (**5g**) in 82% yield, 2,120 TTN and 87% e.e. This result represents a rare example of solving the methyl–ethyl problem using a C(sp^3)–H functionalization strategy.

Mechanistic and computational investigations provided further insight into this enantioconvergent C(sp^3)–H amination process. First, we prepared sulfamoyl azide substrates that bear a stereochemically well-defined olefin moiety ((*Z*)-**1d** and (*E*)-**1d**) and subjected them to 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 that bears a thermodynamically more stable (*E*)-olefin. The erosion of C=C 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(sp^3)–H amination process.

We thus postulate that this enantioconvergent amination comprises a stereoablative hydrogen atom transfer and an enantioselective C–N bond formation. As described in Fig. 5b, reaction of the ferrous haem cofactor with the racemic azide substrate leads to an open-shell iron nitrenoid intermediate. Subsequent stereoablative hydrogen atom transfer 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 density functional theory calculations (Fig. 6). Computational studies on an iron porphyrin model system showed that the triplet state of the key iron nitrenoid intermediate **7** is 3.0 kcal mol^{-1} more stable than the open-shell singlet state and 11.6 kcal mol^{-1} more stable than the quintet state (see the Supplementary Information for comparisons between open-shell singlet, triplet and quintet free energy profiles). The calculated Mulliken spin densities of **7** revealed a value of 0.84 on the nitrogen atom and 1.16 on the iron atom, demonstrating substantial radical characters on both the nitrogen and iron centres. Furthermore, 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^{-1}; the subsequent radical rebound step that proceeds through quintet transition state **TS2** requires an activation energy of 18.4 kcal mol^{-1}, 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(sp^3)–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(sp^3)–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. cloni* BL21(DE3)) cells carrying plasmid encoding the appropriate P411 variant were grown overnight in 4 ml of lysogeny broth with ampicillin. A preculture (3 ml) was used to inoculate 27 ml of hyper broth with ampicillin in a 125 ml Erlenmeyer flask. This culture was incubated at 37 °C and 230 r.p.m. for 2.5 h. The culture was then cooled on ice for 20 min and induced with 0.5 mM isopropyl-β-D-thiogalactoside and 1.0 mM 5-aminolevulinic acid (final concentrations). The expression was conducted at 20 °C and 130 r.p.m. for 16–18 h. *E. coli* cells were then pelleted by centrifugation (4,500 g, 3 min, 4 °C). The media was removed and the resulting cell pellet was resuspended in M9-N buffer to OD_{600} = 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 OD_{600} 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 that harbour P411. The suspensions of *E. coli* cells that express the appropriate haem protein variant in M9-N buffer (typically OD_{600} = 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 setup. A GOx oxygen depletion solution (20 µl of stock solution) containing 14,000 U ml^{-1} catalase and 1,000 U ml^{-1} 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) were added in succession to a 2 ml vial. The vials were sealed and shaken at room temperature and 500 r.p.m. for 12–20 h and then analysed by gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry.

Data availability

All data necessary to support the paper's conclusions are available in the main text and the Supplementary Information. Solid-state structures of **2a**, **4a**, **5a** and **5f** are available free of charge from the Cambridge Crystallographic Data Centre under reference nos 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.

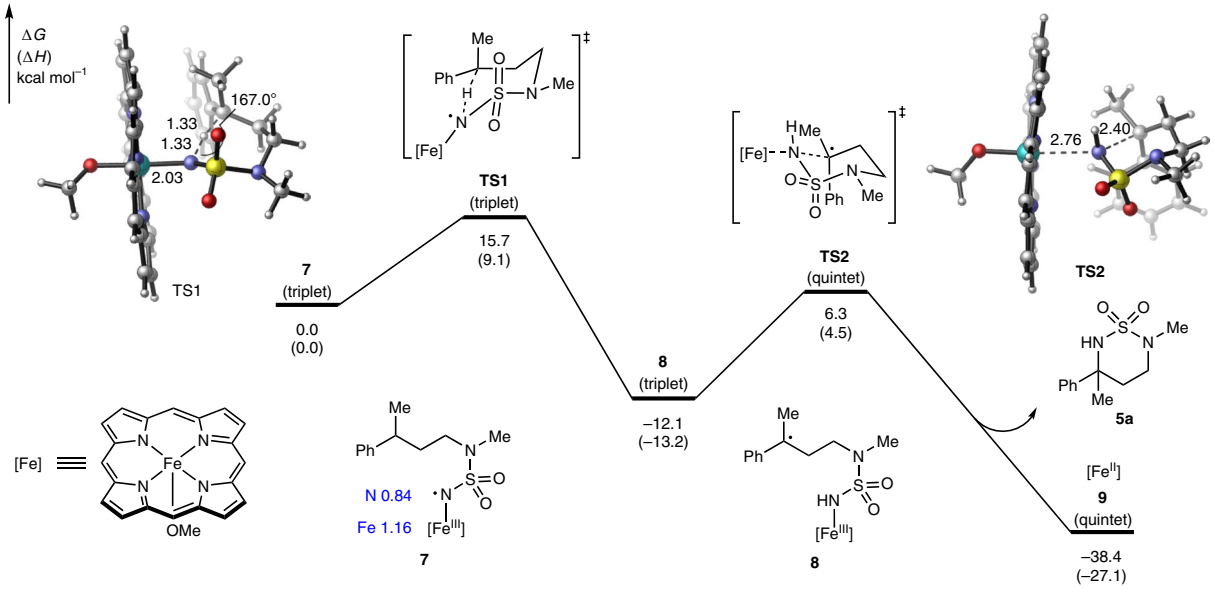

Fig. 6 | Free energy profile of the iron porphyrin-catalysed C(sp^3)–H amination. Density functional theory calculations were performed at the B3LYP-D3(BJ)/6-311+G(*d*,*p*)–LANL2TZ(f)/SMD(chlorobenzene)//B3LYP-D3(BJ)/6-31+G(*d*)–LANL2DZ level of theory. The Mulliken spin densities of iron and nitrogen for the key iron nitrenoid **7** are shown in blue.

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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 haem 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.

Competing interests

A provisional patent application (inventors Y.Y. and I.C.) 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.

Additional information

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