Biocatalytic asymmetric C(sp\textsuperscript{3})–H fluorination enabled by directed evolution of nonheme Fe enzymes

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Due to the scarcity of C–F bond forming enzymatic activities in nature and the contrasting ubiquity of organofluorine moieties in bioactive compounds, developing new biocatalytic fluorination reactions represents a preeminent challenge in enzymology, biocatalysis, and synthetic biology. Additionally, catalytic asymmetric C(sp\textsuperscript{3})–H fluorination remains a challenging problem facing synthetic chemists. Although many nonheme Fe halogenases have been discovered to promote C(sp\textsuperscript{3})–H halogenation reactions, to date, efforts to convert these Fe halogenases to fluorinases have remained unsuccessful. We repurposed a plant-derived natural nonheme enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) to catalyze unnatural enantioselective C(sp\textsuperscript{3})–H fluorination via a radical rebound mechanism. Directed evolution afforded C–H fluorinating enzyme ACCO\textsubscript{CHF} displaying 200-fold higher activity, substantially improved chemoselectivity and excellent enantioselectivity, converting a range of substrates into enantioenriched organofluorine products. Notably, almost all the beneficial mutations were found to be distal to the Fe centre, underscoring the importance of substrate tunnel engineering in nonheme Fe biocatalysis. Computational studies revealed that the radical rebound step with the Fe(III)–F intermediate has an exceedingly low activation barrier of 3.4 kcal/mol, highlighting a new avenue to expand the catalytic repertoire of enzymes to encompass asymmetric C–F bond formation.

Introduction

Over the past decade, groundbreaking studies on unnatural biocatalysis have transformed the biochemical landscape of natural enzymes to encompass biocatalytic reactions that were never previously encountered in nature\textsuperscript{1-5}. Recently, biocatalysis researchers have discovered a range of useful unnatural enzymatic activities, particularly asymmetric free radical transformations with metalloenzymes\textsuperscript{6-10} as well as nicotinamide\textsuperscript{11,12}, flavin\textsuperscript{11,12}, and pyridoxal phosphate (PLP)\textsuperscript{13}-dependent enzymes. Together, these efforts substantially expanded the reaction territory of enzyme
catalysis and furnished powerful tools for asymmetric synthesis. Despite these notable advances, new-to-nature enzymatic C–F bond formation has long eluded the biocatalysis community. The dearth of fluorination enzymatic functions is in stark contrast to the ubiquity of organofluorine compounds in the pharmaceutical and agrochemical industries, as the incorporation of fluorine into organic compounds can often lead to improved bioavailability, enhanced metabolic stability, and desirable protein binding affinity\(^\text{14-17}\). In light of the broad utility of organofluorine compounds, particularly those possessing a fluorinated stereogenic centre\(^\text{18}\), fluorine biocatalysis\(^\text{19}\) and fluorine synthetic biology\(^\text{20-23}\) have long captivated synthetic chemists and synthetic biologists. Among the various fluorination methods\(^\text{24,25}\), biocatalytic enantioselective C(sp\(^3\))–H fluorination would be particularly valuable\(^\text{10}\), as only a handful of chemocatalytic asymmetric C(sp\(^3\))–H fluorination methods are available to non-enolate systems\(^\text{26,27}\), further highlighting the challenge in imposing stereocontrol over fluorination processes. Among these, highly enantioselective C(sp\(^3\))–H fluorination using a free radical mechanism\(^\text{26}\) is particularly challenging.

**Fig. 1. Enzymatic fluorination: an overview.** a, Natural fluorinases that catalyse the conversion of S-adenosylmethionine (SAM) to 5'-fluorodeoxyadenosine (5'-FDA). b, Natural nonheme Fe C(sp\(^3\))–H halogenases. c, Our previously evolved unnatural heme-dependent atom transfer halogenases. d, This work: evolved nonheme Fe enzymes for unnatural enantioselective C(sp\(^3\))–H fluorination. HAT = hydrogen atom transfer, XAT = halogen atom transfer.

To date, fluorinases discovered from bacteria and archaea represent the only natural enzyme class capable of incorporating a fluorine atom to organic substrates (**Fig. 1a**).\(^\text{28}\) Using a simple
bimolecular nucleophilic substitution (S$_{2}$2) mechanism, natural fluorinases catalyse the conversion of $\text{S}$-adenosylmethionine (SAM) to 5'-fluoro-5'-deoxyadenosine (5'-FDA), giving rise to fluorometabolites through downstream biosynthetic pathways.$^{20,28}$ On the other hand, an array of $\alpha$-ketoglutarate ($\alpha$KG)-dependent nonheme Fe enzymes has been discovered and engineered to facilitate C–H (pseudo)halogenation reactions$^{29-32}$, including chlorination$^{31,33}$, bromination$^{34}$, azidation$^{35-37}$ and nitration$^{35}$ (Fig. 1b). Mechanistically, these nonheme Fe enzymes generate a carbon-centred radical by hydrogen atom transfer (HAT) via the intermediacy of (X)Fe(IV)=O (X = (pseudo)halide). The incipient carbon-centred radical subsequently undergoes halogen atom transfer with the Fe(III)–X intermediate, leading to C–H halogenated products$^{29-32}$. Despite extensive studies, to date, converting nonheme Fe halogenases to fluorinases has not been met with success. Several mechanistic rationales, including the low binding affinity of Fe enzymes toward the aqueous fluoride ion ($\text{F}^-$) owing to the high hydration energy of $\text{F}^-$, challenging formation of the ferryl intermediate from the putative Fe(II)–F species, and difficulties steering the radical rebound activity toward the Fe(III)–F over Fe(III)–OH moiety, have been postulated to account for this long-standing challenge.

To address these limitations, we capitalised on a new-to-nature enzymatic mechanism to discover and evolve Fe enzymes for asymmetric C–F bond formation. In 2021, our group implemented metallocenodox radical biocatalysis as a general means to develop unnatural biocatalytic radical reactions (Fig. 1c)$^{6-8}$. In our prior study, we engineered P450 enzymes as atom transfer radical cyclases to catalyse stereocontrolled bromine atom transfer$^{6,7}$. Contemporaneous to our studies, elegant work from the Huang laboratory led to highly efficient nonheme Fe azidases using a radical relay strategy$^9$. Parallel to the study from the Huang group, we independently posited that if we could concurrently generate a transient carbon-centred radical and a Fe(III)–F intermediate through a metallocenodox atom transfer mechanism (Figs. 1c and 1d), we would be able to bypass the energetically demanding fluoride binding step as well as the ferryl intermediate formation for radical generation, thus directly interrogating the feasibility of C–F bond formation with Fe-dependent enzymes. Specifically, inspired by non-stereoselective small-molecule Fe catalysis$^{39,40}$, our proposal for the biocatalytic asymmetric fluorination using Fe enzymes is outlined in Fig. 1d. Starting from the N-fluoroamide (I), fluorine atom transfer to the Fe(II) enzyme (II) would lead to a nitrogen-centred radical (i.e., amidyl radical III) and an Fe(III)–F species (IV). Due to the high N–H bond dissociation enthalpy (BDE (N–H) = 103.7 kcal/mol as determined by our DFT calculations (see the Supplementary Information for details), rapid 1,5-hydrogen atom transfer (1,5-HAT) of III would lead to a new carbon-centred radical (V). At this stage, stereocontrolled radical rebound of V with the enzymatic Fe(III)–F intermediate IV would lead to C–F bond formation VI and convert the ferric enzyme to its ferrous state, thereby completing the catalytic cycle. If successfully implemented, this work would lead to the first examples of metallocenzymes capable of forming C–F bonds. Furthermore, it would provide a rare example of highly enantioselective C(sp$^3$)–H fluorination using an open-shell mechanism.

Results and discussion

Discovery of C–H fluorinating enzyme activities

We commenced our investigation by evaluating an in-house collection of ca. 200 metallocproteins and their variants, including diverse heme and nonheme Fe proteins, by high throughput experimentation using 24- or 96-well plates. All the identified active biocatalysts were then validated, and representative results are summarized in Fig. 2a. Among all the heme proteins we evaluated, only reduced amide 3a derived from the N-fluoroamide substrate 1a was observed
in varying yields (Table S1). For example, our previously evolved P450 atom transfer radical cyclases P450\textsubscript{ATRCase1} possessing a Fe-binding serine residue (Table S1, entry 1) and P450\textsubscript{ATRCase2} lacking an Fe-binding residue (Table S1, entry 2) provided 3a in 14\% and 17\% yield, respectively, with no measurable 2a formation. In contrast to heme proteins, a set of fluorination biocatalysts emerged from the nonheme Fe enzyme superfamily. Among these, isopenicillin N synthase (IPNS) from \textit{Emericella nidulans} with a two-histidine-one-carboxylate facial triad\textsuperscript{41} provided the desired C–H fluorination product 2a in 0.2\% yield and 10:90 enantiomeric ratio (e.r.) (Fig. 2a, see Tables S1 and S2 for further details). Anthocyanidin synthase from \textit{Arabidopsis thaliana}\textsuperscript{12} furnished 2a in 0.4\% yield and 37:63 e.r.\end{table}

Additionally, quercetin 2,3-dioxygenase from \textit{Bacillus subtilis} (BsuQuestD) with a three-histidine-one-carboxylate coordination sphere\textsuperscript{43} also afforded 1a in 0.2\% yield and 29:71 e.r. Furthermore, 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) from \textit{Petunia hybrida}\textsuperscript{34,45}, a plant-derived nonheme Fe enzyme with a two-histidine-one carboxylate facial triad whose native function is to produce ethylene from 1-aminocyclopropane-1-carboxylic acid\textsuperscript{44}, furnished fluorinated 2a in 0.9\% yield and 10:90 e.r. Finally, natural Fe- and \textalpha{}KG-dependent C–H halogenases, including SyrB2 (Table S1, entry 7),\textsuperscript{46,47} WelO5 (Table S1, entry 8),\textsuperscript{33,48-53} and BesD (Table S1, entry 9),\textsuperscript{54-56} were found to be ineffective in facilitating this unnatural C(sp\textsuperscript{3})–H fluorination.

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|}
\hline
entry & ACCO variant & yield (e.r.) of 2a & TTN & yield of 3a & 2a:3a \\
\hline
1 & wt ACCO & (0.9 ± 0.1)\% (10:90) & 3 & (5.9 ± 0.1)\% & 17.83 \\
2 & ACCO I184A & (1.4 ± 0.1)\% (12:88) & 5 & (5.6 ± 0.5)\% & 23.77 \\
3 & ACCO I184AK158N & (2.3 ± 0.1)\% (19:81) & 8 & (6.0 ± 0.1)\% & 29.71 \\
4 & ACCO I184AK158I & (4.5 ± 0.2)\% (74:26) & 21 ± 1 & (5.7 ± 0.3)\% & 44.56 \\
5 & ACCO I184AK158IF91L & (29.5 ± 0.5)\% (84:16) & 73 ± 3 & (6.6 ± 0.2)\% & 82:18 \\
6 & ACCO I184AK158IF91LK172Y & (37.3 ± 0.8)\% (88:12) & 140 ± 3 & (6.4 ± 0.4)\% & 85.15 \\
7 & ACCO I184AK158IF91LK172YK93Q & (76 ± 2)\% (92:8) & 266 ± 7 & (2.8 ± 0.4)\% & 96.4 \\
8 & ACCO I184AK158IF91LK172YK93Q & (97 ± 2)\% (95:5) & 211 ± 5 & (1.4 ± 0.1)\% & 98:2 \\
9 & ACCO\textsubscript{CHF}(OD\textsubscript{500} = 5) & (92 ± 2)\% (95:5) & 601 ± 5 & (1.7 ± 0.1)\% & 98:2 \\
10 & ACCO\textsubscript{CHF}(OD\textsubscript{500} = 5) & (63 ± 1)\% (95:5) & 820 ± 10 & (1.1 ± 0.1)\% & 98:2 \\
\hline
\end{tabular}
\caption{Development of chemo- and enantioselective nonheme Fe enzyme ACCO\textsubscript{CHF} for new-to-nature C(sp\textsuperscript{3})–H fluorination: enzyme mining and engineering. a, Discovery of fluorine}
atom transfer activity among Fe-dependent enzymes. **b**, Directed evolution of ACCO\textsubscript{CHF} as a highly chemo- and enantioselective C–H fluorinating enzyme. Active-site illustration of ACCO was made from PDB ID: 1W9Y.

**Directed evolution of chemo- and enantioselective C–H fluorinating enzyme ACCO\textsubscript{CHF}**

Due to the high levels of enantioselectivity and slightly better initial activity observed with wild-type (wt) *P. hybrida* ACCO, we selected this nonheme Fe enzyme as the template for the further development of an efficient C(sp\(^3\))–H fluorinating biocatalyst (**Fig. 2b**). Guided by the crystal structure of ACCO and our molecular docking studies, we first performed site-saturation mutagenesis (SSM) and screening by targeting amino acid residues in proximity to the nonheme Fe centre. Surprisingly, only a single beneficial mutation I184A with modest activity improvement was found among residues in the closest sphere of Fe (**Fig. 2b**, entry 2 and **Table S3**). In light of the relatively small size of ACCO’s native substrate 1-aminocyclopropane-1-carboxylic acid (see the SI for further details), we postulated that substrate tunnel engineering might be required for this nonheme enzyme to better accommodate bulky non-native substrates. We thus turned our attention to amino acid residues in the β-sheets of the substrate tunnel in protein engineering. In each round of engineering, we selected four tunnel residues for SSM. The best performing variant from the four SSM libraries was then selected as the basis for further optimisation. Through iterative saturation mutagenesis (ISM) and screening, five beneficial mutations distal to the Fe centre were identified in the tunnel-defining β-sheets. Among these, residue 158 was found to play a critical role in modulating the fluorination activity and enantioselectivity. While the incorporation of K158N into ACCO I184A resulted in 1.4-fold higher yield and similar enantioselectivity (**Fig. 2b**, entry 3, (2.3 ± 0.1)% yield, 19:81 e.r.), the K158I variant of ACCO I184A furnished 2a with 3.2-fold higher yield and inverted enantiopreference (entry 4, (4.5 ± 0.2)% yield, 74:26 e.r.). The inverted stereochemistry observed with ACCO I184A K158I relative to that with ACCO I184A is surprising, as the α-carbon of K158 is 10.8 Å away from the Fe centre. These results demonstrated that remote residues such as 158 could influence not only the activity but also the enantioselectivity of ACCO in the current unnatural C–H fluorination.

In further directed evolution, F91L was found to be another key mutation providing 6.6-times higher yield and improved enantioselectivity of 2a (entry 5, (29.5 ± 0.5)% yield and 84:16 e.r.). Notably, ACCO I184A K158I F91L was the first variant in this evolutionary lineage favouring the formation of C–H fluorination over the undesired reduction (2a:3a = 82:18), giving rise to substantially enhanced chemoselectivity. Ultimately, after an additional three rounds of SSM and screening (entries 6–8), directed evolution led to ACCO I184A K158I F91L K172Y K93Q T89A (ACCO\textsubscript{CHF}, CHF = C–H fluorination), furnishing the enantioenriched fluorination product 2a in (97 ± 2)% yield, 95:5 e.r., 98:2 chemoselectivity and (211 ± 5) total turnover number (TTN, entry 8). By further lowering the biocatalyst loading via decreasing the optical density of *E. coli* cells (OD\(_{600} = 10\)) overexpressing ACCO\textsubscript{CHF}, a TTN of (601 ± 5) could be achieved without lowering the yield of 2a (entry 9). Further decreasing OD\(_{600}\) to 5 led to a TTN of (820 ± 10). Importantly, among all the nonheme Fe enzymes we investigated, ACCO\textsubscript{CHF} represents the only biocatalyst capable of delivering enantioenriched fluorinated products in excellent yields without forming other side products, such as the reduction products and C–H hydroxylation products\(^{57}\). Furthermore, 550 mg of evolved ACCO\textsubscript{CHF} could be obtained from 1 L *E. coli* culture in TB, which represented a 1.7-fold improvement in protein expression level relative to wt ACCO and underscored its utility in preparative biotransformations (**Table S15** and **Fig. S12**).
We further characterized the Michaelis–Menten kinetics of two intermediate variants ACCO I184A K158I F91L K172Y, ACCO I184A K158I F91L K172Y K93Q and the final variant ACCO(CHF) using purified enzymes in the presence of stoichiometric quantities of dithionite as the reductant (Figs. S6–S8). Michaelis–Menten kinetics showed that while the $k_{\text{cat}}$ value of these ACCO variants remained approximately unchanged (2.1 min$^{-1}$, 2.5 min$^{-1}$ and 2.3 min$^{-1}$, respectively), the $K_M$ value of this evolutionary series decreased from 520 µM to 420 µM to 210 µM. Together, the $k_{\text{cat}}/K_M$ value of these variants increased from 4.0 mM$^{-1}$min$^{-1}$ to 6.0 mM$^{-1}$min$^{-1}$ to 11 mM$^{-1}$min$^{-1}$. Thus, these results indicated that the improved enzymatic activity towards C–H fluorination originated from enhanced substrate binding, which may arise from substrate tunnel engineering.

![Diagram](https://doi.org/10.26434/chemrxiv-2024-pt58m)

**Fig. 3.** Substrate scope of ACCO(CHF)-catalysed asymmetric C(sp$^3$)–H fluorination. Reaction conditions: 6.7 mM 1, 0.67 mM (NH$_4$)$_2$Fe(SO$_4$)$_2$, see the Supplementary Information for details. $^{a}$1.5-gram scale synthesis was carried out using whole E. coli cells derived from 1 L of TB expression culture. See the Supplementary Information for details.

**Substrate scope of biocatalytic asymmetric C(sp$^3$)–H fluorination**

With the evolved variant ACCO(CHF) in hand, we next examined the substrate scope of this biocatalytic enantioselective C(sp$^3$)–H fluorination. Substituents at the 3, 4, and 5 positions of the aromatic ring were compatible, providing the corresponding fluorinated products in good to
excellent enantioselectivity (2b, 2c, and 2d). Electron-withdrawing fluorine (2e) and chlorine (2f) substituents as well as electron-donating methoxy (2g) were compatible with this transformation. Tert-Amyl amides (2h) could also be transformed with good enantioselectivity. Furthermore, extended aliphatic substituents, including a propyl (2i), a homoallyl (2j) and a methoxyethyl (2k) group, could be efficiently fluorinated with excellent enantiocontrol. A tetrahydropyranthalene core (2l) also underwent biotransformation to furnish the corresponding enantioenriched fluorinated product. Additionally, tertiary C(sp3)–H bonds could also be efficiently and chemoselectively fluorinated to provide the desired fluorination product (2i) in excellent yields. A thiophene containing substrate 1n could also undergo C–H fluorination with excellent enantioselectivity and chemoselectivity (90:10 e.r., 2n:3n = 88:12, see the SI for details). Additional examples of unsuccessful substrates are provided in the SI. For example, primary C(sp3)–H bonds (1o) could not be efficiently fluorinated with evolved ACCOCHF. N-fluoroacrylamide 1p was transformed with low activity but excellent enantio- and chemoselectivity.

Importantly, the excellent catalytic activity, enantioselectivity, and chemoselectivity of ACCOCHF allowed this biocatalytic C–H fluorination to be easily carried out on a gram-scale with a relatively small amount of whole-cell biocatalysts, demonstrating the practicality of this biotransformation. The absolute stereochemistry of 2a was ascertained by single crystal X-ray diffraction analysis (see the SI for details).

**Mechanistic insights from computational and experimental investigations**

Our radical clock experiment with a cyclopropyl substrate indicated the formation of cyclopropane ring-opening product, consistent with the proposed radical mechanism (see the SI for details). To gain further insights into the mechanism and the roles of active site residues of this new-to-nature biocatalytic C(sp3)–H fluorination, we performed computational studies using classical molecular dynamics (MD) simulations and density functional theory (DFT) calculations (see the SI for computational details). First, classical MD simulations of the complexes of N-fluoroamide 1a with both wt ACCO and ACCOCHF variants were carried out to study the preferred substrate binding modes and to identify key active site residues involved in substrate binding (Fig. 4). In order to model the substrate near-attack-conformations that promote the fluorine atom transfer step, the Fe–F distance was restrained to be within 3.0–3.2 Å using a harmonic potential of 100 kcal·mol<sup>−1</sup>·Å<sup>−2</sup>. The restrained MD simulations showed that in order to minimise unfavourable steric repulsions with the protein scaffold, substrate 1a approaches the Fe centre from the coordination site trans- to H234. Furthermore, these MD studies indicated that mutations K158I, F91L, and T89A widen the substrate entrance tunnel, providing more space to facilitate the transport of the bulky N-fluoroamide substrate 1a into the active site. In particular, mutations K158I and F91L greatly reduce the bulkiness of these tunnel bottleneck residues, increasing the tunnel bottleneck radius from 3.12 Å in wt ACCO to 4.17 Å in ACCOCHF (see Fig. S15). In addition, the I184A mutation opens up more space to accommodate 1a, allowing the fluorine atom in 1a to approach the Fe centre while circumventing unfavourable steric clashes between the adjacent N-tBu group of 1a and active site residues.
Fig. 4. The most populated structures from restrained MD simulations of the enzyme–substrate complexes of 1a with the (a) wt ACCO and (b) ACCOCHF variants. Substrate entrance tunnels are shown in orange.

Next, to elucidate the reactivities of the unnatural fluorine atom transfer and fluorine rebound steps, we performed DFT calculations of the reaction energy profile of this biocatalytic C–H fluorination. We constructed a truncated model based on the active site geometry of the ACCO enzymes, in which imidazole (Im) and acetate (AcO) groups are used to model aspartate and histidine residues, respectively. Our DFT calculations indicate that all the intermediates and transition states in the catalytic cycle feature a high-spin quintet Fe(II) and sextet Fe(III) centres (see Fig. S18 for computed energy profile at the less favorable intermediate-spin state). Previous studies indicated that fluorine atom transfer to a transition metal centre is kinetically more challenging than the analogous chlorine and bromine atom transfer, due to the higher bond dissociation energy (BDE) of the fluorine atom donor. In addition, fluorine atom rebound from a transition metal fluoride is also slower than other halogens because of the strong metal fluoride bond. In contrast, the computed reaction energy profile (Fig. 5) reveals a relatively low barrier of 11.8 kcal/mol for the initial fluorine atom transfer (TS-1). Here, the high exothermicity (−14.3 kcal/mol) in converting an activated N–F bond in 4 to a stronger Fe(III)–F bond provides the thermodynamic driving force for substrate activation. In addition, the Fe(II) centre in 4 has a relatively high HOMO energy of −7.03 eV, promoting the charge transfer (0.31 e) from the Fe centre to the N-fluoroamide substrate 1a at the transition state (TS-1).

After the fluorine atom transfer, the nitrogen-centred radical in complex 5 undergoes rapid 1,5-hydrogen atom transfer via TS-2 to provide the benzyl radical intermediate 6. Finally, radical rebound with the Fe(III)–F species leading to C–F bond formation via TS-3 is found to be highly kinetically facile, displaying an activation barrier of 3.4 kcal/mol with respect to 6. Unlike other transition metal fluorides featuring a strong M–F bond with BDEs higher than 100 kcal/mol, the Fe(III)–F bond in 6 has a weaker BDE of 81.4 kcal/mol, allowing thermodynamically downhill fluorine rebound to form the benzylic C–F bond with a BDE of 98.1 kcal/mol. In addition, the heterolytic dissociation of F– from Fe(III)–F in 5 and 6 was found to be thermodynamically unfavourable (Fig. S19), allowing for productive C–F bond formation via the radical rebound pathway. We note that the low
activation energy required for C–F bond formation involving an enzymatic Fe(III)–F intermediate has broad implications in repurposing natural nonheme Fe halogenases as fluorinases.

**Fig. 5.** Computational studies using a truncated active site model. a, DFT-computed reaction energy profile of C–H fluorination of N-fluoroamide 1 using a truncated model based on the active site of ACCO enzyme at the (U)B3LYP-D3(BJ)/def2-TZVP/SMD//(U)B3LYP-D3(BJ)/6-31G(d)–SDD(Fe) level of theory. Energies are with respect to 4. Imidazole (Im) and acetate (AcO) groups are models for aspartate and histidine residues, respectively. b, Optimized geometries of the fluorine atom transfer (TS-1) and fluorine radical rebound (TS-3) transition states.

**Conclusion**

In summary, we repurposed and evolved a nonheme Fe enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) to catalyse C(sp³)–H fluorination reactions with excellent levels of chemo- and enantiocontrol. Our evolved Fe-dependent enzyme ACCO<sub>CHF</sub> constitutes the first new-to-nature C–F bond forming biocatalyst, which has long been sought after in biocatalysis, synthetic biology, and bioinorganic chemistry. By exploiting an underutilised radical mechanism for enzymatic C–F bond formation, the current new-to-nature fluorinating enzymes complement the only naturally occurring fluorinase relying on a closed-shell SN2 mechanism for fluorination. Furthermore, this biocatalytic fluorination represents a rare method for catalytic asymmetric C(sp³)–H fluorination and the only radical C–H fluorination with excellent enantiocontrol. The utility of our newly evolved nonheme enzymes was further demonstrated in the gram-scale preparation of enantioenriched organofluorides. Together, the evolution of efficient and selective Fe-dependent fluorinating enzymes affords a valuable tool for the biological synthesis of chiral organofluorine compounds via a C–H functionalization logic, thereby setting the stage for the further advancement of fluorine biocatalysis and synthetic biology.

**Methods**

**Expression of ACCO<sub>CHF</sub> variants.**

*E. coli* BL21(DE3) cells harbouring recombinant plasmid encoding the appropriate ACCO variant were grown overnight at 37 °C and 230 rpm in 4 mL LB media supplemented with 0.05 mg/mL kanamycin (LB<sub>kan</sub>). Preculture (1.5 mL, 5% v/v) was used to inoculate 28.5 mL TB media supplemented with 0.05 mg/mL kanamycin (TB<sub>kan</sub>) in a 125 mL Erlenmeyer flask. The culture was incubated at 37 °C and 230 rpm for 2 h to reach an OD<sub>600</sub> of approximately 1.5. The culture was
then cooled on ice for 20 min and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentrations). Protein expression was performed at 20 °C and 200 rpm for 20 h. The cells were then transferred to a conical tube (50 mL) and harvested by centrifugation (4000 rpm, 4 min, 4 °C) using an Eppendorf 5910R tabletop centrifuge.

**Analytical scale enantioselective biocatalytic C–H fluorination.** The suspension of *E. coli* cells expressing ACCO in M9-N buffer (typically OD$_{600}$ = 10–30, 540 μL) was added to a vial (2 mL) and kept on ice. This vial was then transferred into an anaerobic chamber, where the solution of Mohr’s salt (30 μL, 13.3 mM stock solution in degassed H$_2$O) and the N-fluoroamide substrate (30 μL, 133 mM stock solution in MeCN) was added. The final reaction volume was 600 μL; the final concentration of the N-fluoroamide substrate was 6.67 mM. (Note: the reaction performed with *E. coli* cells resuspended to OD$_{600}$ = 30 indicates that 540 μL cell suspension with an OD$_{600}$ of 30 were added.) The vials were sealed and shaken on a Corning digital microplate shaker at room temperature and 680 rpm for 24 h. The reaction mixture was then analyzed by chiral HPLC.

**Gram scale enantioselective biocatalytic C–H fluorination.** *E. coli* BL21(DE3) cells harbouring the recombinant plasmid encoding ACCO$_{CHF}$ were grown overnight at 37 °C and 230 rpm in 25 mL LB media supplemented with 0.05 mg/mL kanamycin (LB$_{kan}$). Preculture (20 mL, 2% v/v) was used to inoculate 1 L TB media supplemented with 0.05 mg/mL kanamycin (TB$_{kan}$) in a 4 L Erlenmeyer flask. The culture was incubated at 37 °C and 230 rpm for 2.5 h to reach an OD$_{600}$ of approximately 1.5. The culture was then cooled on ice for 30 min and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentrations). Protein expression was performed at 20 °C and 200 rpm for 20 h.

The cells were harvested by centrifugation (4000 rpm, 5 min, 4 °C) using a Thermo Scientific Sorvall Lynx 6000 superspeed centrifuge and resuspended in M9-N buffer (OD$_{600}$ = 30). An aliquot of *E. coli* cell suspension (2 mL) was taken to determine protein concentration using the SDS-PAGE assay. Cell suspensions in M9-N buffer were kept on ice until use. (Note: leaving the cell suspension at room temperature for an extended period of time will lead to significantly reduced enzyme activity.) To a 1 L Erlenmeyer flask equipped with a screw cap was added a suspension of *E. coli* cells in M9-N buffer expressing the desired enzyme variant. The flask was transferred to an anaerobic chamber, where a solution of Mohr’s salt (5 mL, 150 mM stock solution in degassed H$_2$O) and the N-fluoroamide substrate (1.67 g, 7.5 mmol, 500 mM in MeCN, 15 mL for substrate 1a) were added. The flask was capped, sealed with parafilm, taken out of the anaerobic chamber and allowed to shake in an Eppendorf Innova 44R shaker at room temperature and 230 rpm for 24 h.

Upon the completion of this biotransformation, the reaction mixture was extracted with 300 mL EtOAc. The mixture was transferred to a 1 L ultracentrifugation bucket and spun down (6000 rpm, 10 min) using a Thermo Scientific Sorvall Lynx 6000 superspeed centrifuge to separate the organic layer from the aqueous layer. The aqueous layer was extracted with EtOAc for an additional five times. Combined organic layers were dried over MgSO$_4$ and an aliquot of the organic layer (400 μL) was used for product enantioselectivity via chiral HPLC. Combined organic layers were concentrated in vacuo with the aid of a rotary evaporator and purified by column chromatography with the aid of a Biotage Isolera to afford the desired organofluoride product 2a in 88% yield and 95:5 e.r..

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Data availability: All data are available in the main text and the Supplementary Information or available from the authors upon reasonable request. Plasmids encoding evolved RAPs reported in this study are available for research purposes from Y.Y. under a material transfer agreement with the University of California Santa Barbara. Solid-state structure of 2a is available free of charge from the Cambridge Crystallographic Data Centre under reference number CCDC 2294341.

Author contributions

Y.Y. conceived and directed the project. L.Z. performed all the enzyme engineering, Michaelis–Menten kinetics, substrate synthesis, and substrate scope studies. Y.Y., L.C. and Y.Z. performed enzyme mining. B.K.M. carried out the computational studies with P.L. providing guidance. Y.Y., L.Z., P.L. and B.K.M. wrote the manuscript with the input of all other authors.

Competing interests

The authors declare no competing interests.

References

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In our unpublished work, it was found that other evolved nonheme enzymes provided undesired reduction product and/or C-H hydroxylation product resulting from radical rebound with the Fe(III)-OH intermediate.


