Biocatalytic Generation of Trifluoromethyl Radicals by Nonheme Iron Enzymes for Enantioselective Alkene Difunctionalization

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ABSTRACT: The trifluoromethyl ($-CF_3$) group represents a highly prevalent functionality in pharmaceuticals. Over the past few decades, significant advances have been made in the development of synthetic methods for trifluoromethylation. In contrast, there are currently no metalloenzymes known to catalyze the formation of $C(sp^3)$-CF_3 bonds. In this work, we demonstrate that a nonheme iron enzyme, hydroxymandelate synthase from *Amycolatopsis orientalis* (*Ao*HMS), is capable of generating CF_3 radicals from hypervalent iodine(III) reagents and directing them for enantioselective alkene trifluoromethyl azidation. A high-throughput screening (HTS) platform based on Staudinger ligation was established, enabling the rapid evaluation of *Ao*HMS variants for this abiological transformation. The final optimized variant accepts a range of alkene substrates, producing the trifluoromethyl azidation products in up to 73% yield and 96:4 enantiomeric ratio (e.r.). The biocatalytic platform can be further extended to alkene pentafluoroethyl azidation and diazidation by altering the iodine(III) reagent. In addition, anion competition experiments provide insights into the radical rebound process for this abiological transformation. This study not only expands the catalytic repertoire of metalloenzymes for radical transformations but also creates a new enzymatic space for organofluorine synthesis.

The trifluoromethyl group ($-CF_3$) is among the most crucial functional groups in medicinal chemistry.[1] Strategic introduction of trifluoromethyl groups can significantly enhance the medicinal properties of organic compounds by increasing their metabolic stability, lipophilicity, and bioavailability.[2] In drug development, the trifluoromethyl group and trifluoromethyl-containing moieties are widely employed as bioisosteres to modulate the potency and metabolic profile of lead compounds.[3] Given the prevalence of trifluoromethyl groups in pharmaceuticals, a variety of trifluoromethylation reactions have been established over the past decades, providing important synthetic tools to access diverse trifluoromethyl-containing compounds.[4-6]

In contrast to the success of synthetic trifluoromethylation chemistry, biocatalytic systems for trifluoromethylation remain underdeveloped, despite the recent rapid progress in enzymatic systems for synthetically useful transformations for organofluorine synthesis.[7-9] A handful of biocatalytic strategies have recently emerged for fluoroalkyl group incorporation (**Figure 1a**). Natural methyl transferases have been repurposed to facilitate fluoroalkylation reactions through the use of chemically or enzymatically produced fluoroalkyl-substituted *S*-adenosyl-L-methionine (SAM) analogs.[10-14] Heme proteins have been engineered to catalyze fluoroalkyl-substituted carbene-transfer reactions such as cyclopropanation,[15,16] C–H fluoroalkylation,[17] and B–H and N–H bond insertions.[18,19] More recently, ene reductases have been employed for alkene hydrofluoroalkylation via enzymatic photoredox catalysis.[20] Although these methods are able to install various fluoroalkyl groups, the direct incorporation of trifluoromethyl groups in enzymatic systems remains a significant challenge. In this regard, laccase was employed for the trifluoromethylation of phenols using *tert*-butyl hydroperoxide (^{t}BuOOH) and triflinate salts like CF_3SO_2Na and $Zn(SO_2CF_3)_2$ (**Figure 1b**).[21,22] While this method directly constructs C–CF_3 bonds, the role of laccase is to generate phenol cation radicals that react with CF_3 radicals produced from ^{t}BuOOH and triflinate salts. As a result, the formation of C–CF_3 bonds occurs in solution and is not directed by the enzyme active site. Another promising system for the introduction of trifluoromethyl groups is through the use of pyridoxal. In a recent report, the quinonoid form of the cofactor was shown to induce CF_3 radical generation from a trifluoromethyl iodine(III) reagent.[23] However, the realization of this process with a pyridoxal phosphate (PLP)-dependent enzyme has not yet been achieved.

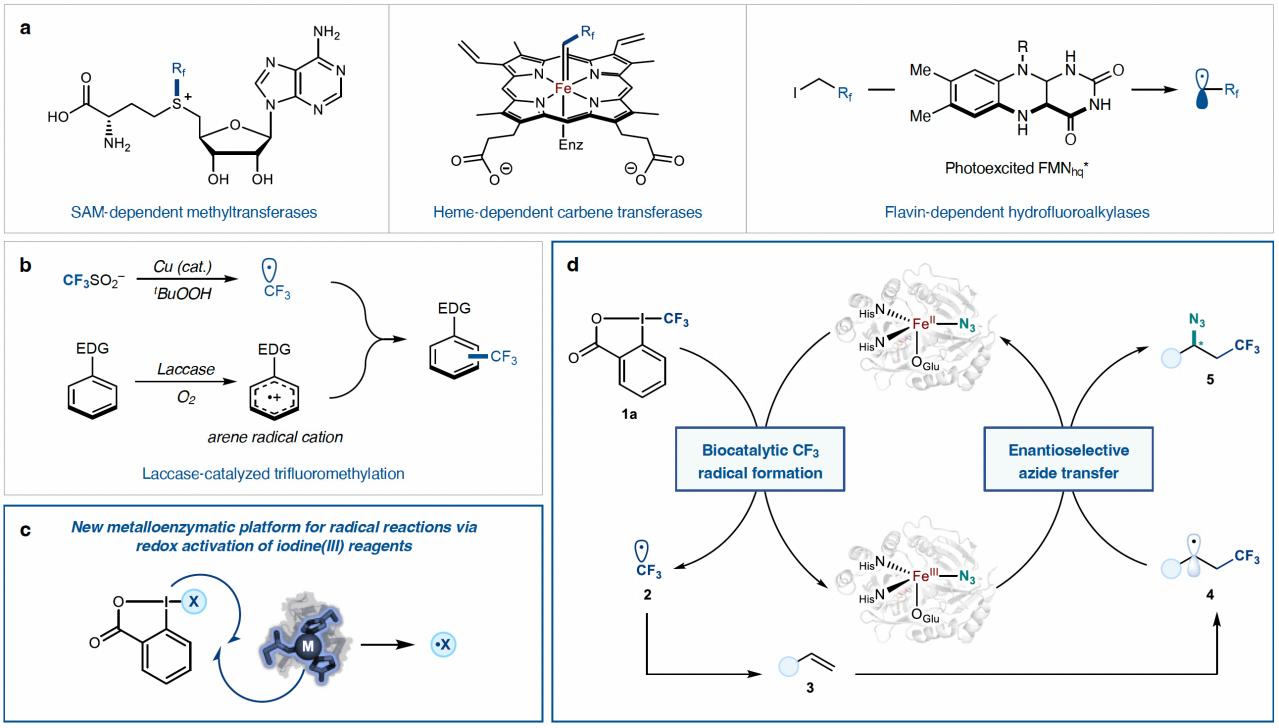


Figure 1. Biocatalytic fluoroalkylation and trifluoromethylation. (a) Current development of biocatalytic strategies for the introduction of fluoroalkyl groups. **(b)** C(sp^2)–H trifluoromethylation of electron-rich arenes catalyzed by fungal laccase. **(c)** New biocatalytic radical transformations through redox activation of iodine(III) reagents. **(d)** Nonheme iron enzyme-catalyzed trifluoromethyl radical generation for enantioselective alkene carboazidation.

In this study, our objective was to establish a new enzymatic platform for trifluoromethylation reactions, which we sought to achieve by using hypervalent iodine(III) reagents (**Figure 1c**). 1-Trifluoromethyl-1,2-benziodoxol-3-(1H)-one (**1a**), commonly known as Togni reagent II, has been utilized extensively as a source of trifluoromethyl radicals for diverse trifluoromethylation applications, including aromatic trifluoromethylation and trifluoromethylative difunctionalization of alkenes.[4,24] Despite their wide synthetic utility, the use of iodine(III) reagents for stereoselective radical transformations remains underdeveloped in biocatalysis. This is in stark contrast to the rapid advancements in radical biocatalysis over the past decade, which have incorporated a variety of non-natural reaction mechanisms into natural enzymes to facilitate diverse radical transformations.[25-32] We hypothesized that nonheme iron enzymes could be used to bridge this gap between chemical and biocatalysis, considering the wide use of synthetic iron catalysts for radical trifluoromethylation[33] and the recent demonstrations of nonheme iron enzymes for abiotic radical transformations.[32,34-38] To establish the feasibility of this concept, we chose alkene trifluoromethyl azidation as the target reaction[39]. The chiral azide products that are formed are synthetically valuable, as they can be readily derivatized to various functionalized trifluoromethyl-containing compounds. However, achieving enantioselective carboazidation has long been recognized as a synthetic challenge, primarily due to the complexity associated with controlling the stereochemistry of the rapid radical azide transfer step.[40] Concerning alkene trifluoromethyl azidation, high enantioselectivity has only been realized with α,β-unsaturated carbonyl substrates, where the carbonyl group can interact with the metal center to facilitate enantioselective azide transfer.[41] In cases lacking such an interaction with the metal center, there is only a single reported example, wherein 3-chlorostyrene undergoes iron-catalyzed trifluoromethyl azidation utilizing a tridentate chiral NON-pincer ligand to form the corresponding product with moderate enantioselectivity.[40]

Our biocatalytic system would provide a platform for achieving this challenging transformation by harnessing the genetic tunability and the chiral active-site environment of enzymes for reaction control. The demonstrated capability of nonheme iron enzymes for enantioselective radical azide transfer lends additional support for the feasibility of this transformation.[32,36,37] A proposed mechanism for the envisioned alkene trifluoromethyl azidation is outlined in **Figure 1d**. Activation of Togni reagent II (**1a**) would oxidize the Fe(II) center of the enzyme to Fe(III) and generate CF_3 radical **2**. Subsequent radical addition of **2** to the alkene substrate **3** would form substrate radical **4**, which would under radical rebound with the Fe(III)–N_3 intermediate to afford the chiral trifluoromethyl-azide product **5**.

Following the mechanistic analysis above, a panel of wild-type nonheme iron enzymes was evaluated using styrene (**3a**) as the substrate, sodium azide (NaN_3) as the azide source, and Togni reagent II (**1a**) as the trifluoromethylation reagent. A hydroxymandelate synthase from *Amycolatopsis orientalis* (*Ao*HMS) showed the highest activity, forming trifluoromethylazide product **5a** in 4% yield (**Figure 2a, Table S1**). No product was formed when the enzyme catalyst was omitted from the reaction or when using a double alanine mutant where the two iron-coordinating histidine residues were replaced with alanine (**Table S2**), indicating that the reaction was facilitated by the Fe(II) center of the enzyme. We then tested a variety of substituted styrenes and selected 4-

methoxystyrene (**3b**) as the new model substrate, as it exhibited comparable activity to **3a** (**Table S3**). We expected the more electron-rich substrate **3b** would facilitate further reaction development due to its faster rate of CF_3 radical addition.[42] With **3b** as the substrate, we evaluated a number of engineered *Ao*HMS variants from our lab and discovered a sextuple mutant, *Ao*HMS-QGHLYV (F188Q/T214G/Q305H/F307L /F330Y/I335V), that exhibited improved activity and enantioselectivity compared to wild-type *Ao*HMS, forming product **5b** in 14% yield and 69:31 e.r. Using *Ao*HMS-QGHLYV, we further optimized reaction conditions and were able to improve the yield of **5b** to 31% running the reactions under cell lysate conditions with final concentrations of 1 mM Fe(II), 50 mM NaN_3, 5 mM substrate, and 10 mM Togni reagent II for a reaction duration of 3 hours (**Table S5-S7**).

We then focused on improving the catalytic performance of *Ao*HMS-QGHLYV through directed evolution. To accelerate the engineering effort, we developed a high-throughput screening (HTS) platform by leveraging the differing reactivity of inorganic azide and organic azide in fluorogenic click chemistry.[43] We previously used this approach to optimize a radical-relay azidation reaction via fluorogenic copper-catalyzed azide alkyne cycloaddition (CuAAC).[32] However, this CuAAC-based screening method proved to be unsuitable for trifluoromethyl azidation due to the high reactivity of Cu(I) with Togni reagent **1a**. To address this issue, we designed a new HTS platform based on Staudinger ligation (**Figure 2b**).[44] In this system, the fluorescein-based Staudinger

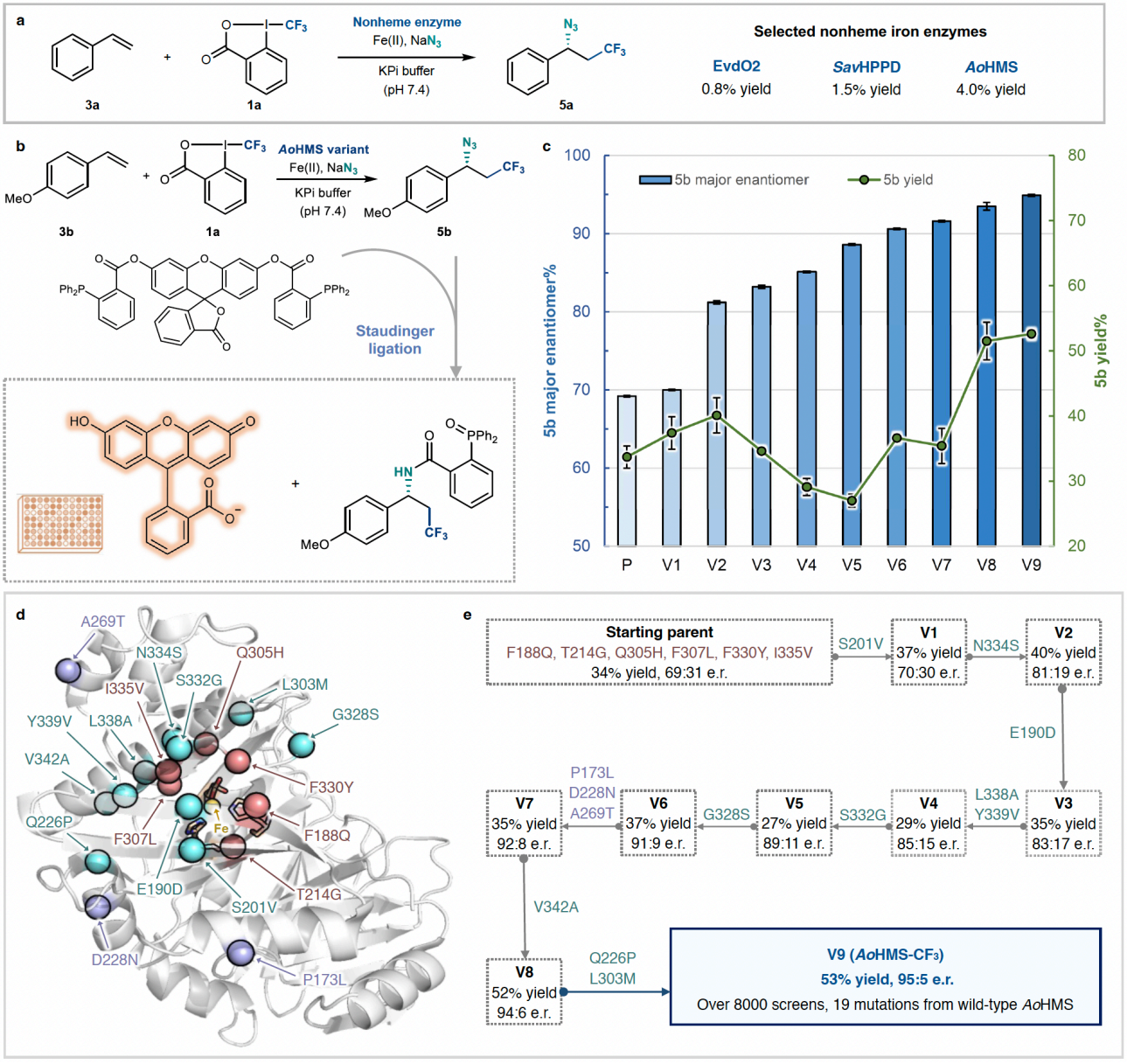


Figure 2. Directed evolution of *Ao*HMS for alkene trifluoromethyl azidation. **(a)** Initial activity discovery. **(b)** Development of Staudinger ligation-based high-throughput screening platform. **(c)** Representative variants discovered during directed evolution of *Ao*HMS. Standard conditions: Lysate (OD = 40); Fe(II) (1 mM), NaN_3 (50 mM), **3b** (5 mM), **1a** (10 mM); 3 hours. **(d)** Crystal structure of WT *Ao*HMS (PDB: 2R5V). Spheres shown in salmon represent mutations of the initial parent (*Ao*HMS-QGHLYV). Spheres shown in aquamarine represent mutations accumulated during directed evolution. Spheres shown in slate represent mutations introduced as a result of error-prone PCR. **(e)** Yields, enantiomeric ratios, and accumulated mutations of variants in the *Ao*HMS evolutionary lineage.

probe selectively reacts with organic azide, even in the presence of high concentrations of NaN_3, to release a fluorescein reporter for product quantification. Since Staudinger ligation does not require a copper catalyst, the conditions not only avoided undesired side reactions with Togni reagent **1a** but also greatly improved the operational simplicity of the screening process. Quantitative evaluation of the fluorescence assay showed that organic azide can be detected in cell lysate with a detection limit of 4 μM and a coefficient of variation of 7.6% when reactions were run under standard conditions with *Ao*HMS-QGHLYV expressed in 96-well plate format (**Figure S1-S3**).

We utilized the newly developed HTS platform to conduct extensive rounds of single and double site-saturation mutagenesis (SSM) and screening with *Ao*HMS-QGHLYV (denoted as "P") as the initial parent (**Figure 2c-2e**). The targeted residues for SSM were located primarily in the three C-terminal α-helix and β-strand region containing the residues that form the nonheme catalytic triad. These regions are critical for accommodating the native 4-hydroxyphenylpyruvate substrate and enabling catalytic acvivity.[45,46] Since the HTS method we used is activity-based and might overlook mutants with improved enantioselectivity, a combined screening strategy was implemented, wherein the fifteen

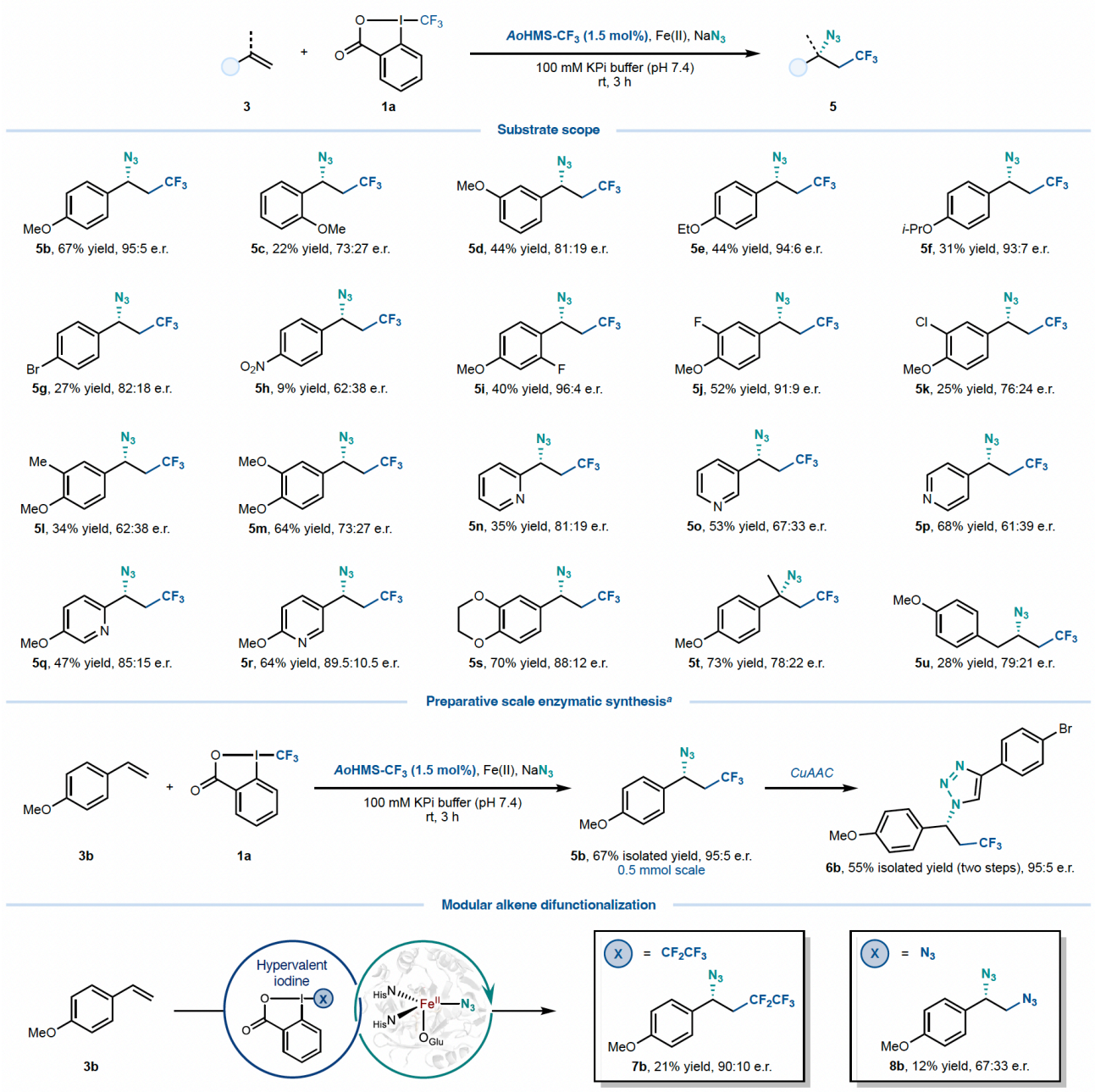


Figure 3. Substrate scope and additional applications for alkene trifluoromethyl azidation catalyzed by *Ao*HMS-CF_3. Standard conditions: Lysate (OD = 40, 1.5 mol% enzyme); Fe(II) (1 mM), NaN_3 (50 mM), alkene (2.5 mM), **1a** (5 mM); 3 hours. Values shown for the substrate scope and additional difunctionalization reactions represent the average of biological triplicates. [a]5 mM **3b** and 10 mM **1a** were used for preparative enzymatic synthesis (Section XIV in Supporting Information).

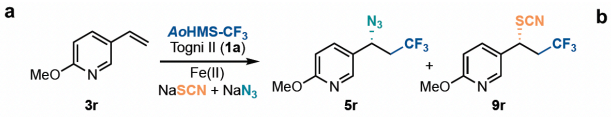
most active hits in each mutagenesis library identified through the Staudinger assay were further assessed based on their enantioselectivity. This approach ensured high screening efficiency while still considering mutants that could enhance enantioselectivity but might slightly decrease activity. This engineering campaign resulted in the discovery of seven mutations, S201V, N334S, E190D, L338A, Y339V, S332G, and G328S, (*Ao*HMS-V6), that led to an improvement in enantioselectivity from 69:31 e.r. to 91:9 e.r. and a slight improvement in activity. Up to this point, all residues for mutagenesis were selected by examining the crystal structure of wild-type *Ao*HMS, and beneficial mutations were identified through exhaustive screening of over 5000 clones. To further enhance the catalytic performance of the enzyme, we turned to error-prone PCR (epPCR) random mutagenesis to explore a broader sequence space. This screening effort led to the discovery of three mutations (P173L, D228N, and A269T) that further improved the enantioselectivity to 92:8 e.r. (*Ao*HMS-V7). Notably, all three mutations are located at sites that are solvent-exposed and distant from the active site. With *Ao*HMS-V7 as the parent, we carried out another set of SSM and screening and introduced three more mutations, V342A, Q226P, and L303M, to give the final variant, *Ao*HMS-CF_3. The final variant contained nineteen mutations relative to the wild-type *Ao*HMS and formed product **5b** in 53% yield and 95:5 e.r.

With the final variant in hand, we performed an additional round of reaction optimization and found that reducing the substrate concentration to 2.5 mM further increased the reaction yield to 67% without compromising the enantioselectivity. Using these optimized conditions, we surveyed the substrate scope of *Ao*HMS-CF_3 for the alkene trifluoromethyl azidation reaction (**Figure 3**). In general, electron-rich alkenes exhibited higher activity compared to their electron-deficient counterparts. Common functionalities such as alkoxyl, nitro, and halogen groups were tolerated (**5b** to **5h**). The reaction showed compatibility with various disubstituted styrenes (**5i** to **5m**), forming the corresponding products with yields ranging from 25% to 64% and enantioselectivities ranging from 62:38 e.r. to 96:4 e.r. Heteroaromatics such as vinylpyridines were effective substrates (**5n** to **5r**), and the corresponding trifluoromethyl azidation products were formed with 35% - 80% yield and 61:39 to 85:15 e.r. When employing a 1,1-disubstituted styrene substrate, the corresponding tertiary azide product **5t** was formed in 73% yield and 78:22 e.r. Notably, the reaction could be extended to unactivated alkenes such as 4-allylanisole, affording product **5u** in 28% yield and 79:21 e.r. The biotransformation could also be performed on a preparative scale, as demonstrated by the enzymatic synthesis of **5b** on a 0.5 mmol scale in 67% isolated yield and 95:5 e.r. The incorporation of an azide group provides an efficient handle for derivatization into various nitrogen-containing molecules using well-established synthetic methods. In this regard, we subjected compound **5b** to CuAAC, forming triazole product **6b** in 55% isolated yield and 95:5 e.r.

A noteworthy feature of this biocatalytic platform is its modularity, as the same iodine(III) activation mechanism should be applicable for the generation of radicals other than CF_3 radicals. We found that the reaction demonstrated broad applicability with additional iodine(III) reagents (**Table S11**). Replacing Togni reagent **1a** with the analogous pentafluoroethyl iodine(III) reagent yielded the corresponding pentafluoroethyl azidation product **7b** in 21% yield and 90:10 e.r. Utilizing azidobenziodoxolone (ABX), also known as the Zhdankin reagent,[47] resulted in the formation of diazidation product **8b** in 12% yield and 67:33 e.r. While these reactions exhibited lower yields and enantioselectivities compared to trifluoromethyl azidation, they underscore the versatility of this biocatalytic platform in facilitating new enzymatic alkene difunctionalization reactions.

We next tested other transferrable ligands such as halides and pseudohalides for alkene difunctionalization with *Ao*HMS-CF_3 (**Figure S12**). Thiocyanate (SCN^-) was the only anion that exhibited radical transfer activity, affording thiocyanation product **9r** in 33% yield and 80.5:19.5 e.r. Notably, we confirmed that SCN transfer in our reaction leads to the formation of a C–SCN bond rather than a C–NCS bond. This finding is intriguing, as Fe-NCS bonding mode has predominantly been observed in both nonheme iron enzymes[48-50] and model compounds,[51-53] suggesting that the radical recombines with the terminal sulfur of the Fe(III)-NCS intermediate. However, we cannot completely rule out the possibility that the enzyme adopts an Fe-SCN bonding mode and that radical recombination occurs at the iron-bound sulfur.

We also investigated the competition between N_3 and SCN for radical capture by conducting the reaction with NaN_3 and NaSCN



Entry	[NaN_3]	[NaSCN]	**5r** yield	**5r** e.r.	**9r** yield	**9r** e.r.
1	40 mM	10 mM	48%	76:24	0.6%	–
2	30 mM	20 mM	44%	69.5:30.5	1.4%	–
3	20 mM	30 mM	40%	65.5:34.5	2.6%	–
4	10 mM	40 mM	35%	61:39	5.8%	80.5:19.5
5	5 mM	45 mM	18%	60:40	6.4%	80.5:19.5
6	2.5 mM	47.5 mM	11%	59:41	11%	80.5:19.5
7	1 mM	49 mM	5.8%	56.5:43.5	18%	80.5:19.5
8	0.5 mM	49.5 mM	2.9%	–	22%	80:20
9	0.25 mM	49.75 mM	1.6%	–	25%	80:20

Figure 4. Mechanistic Studies. (a) N_3 and SCN competition experiment. **(b)** Experimental studies of the CF_3 radical generation and azide transfer steps.

at varying ratios (**Figure 4a, Table S13**). These competition experiments yielded two main findings. First, azidation reaction outcompetes thiocyanation. For example, azidation product **5r** and thiocyanation product **9r** are formed in roughly equal amounts (11% yield) even with a 19-fold excess of NaSCN relative to NaN_3 (**Figure 4a, Entry 6**). These results align with previous studies on nonheme iron model compounds that showed a preference for N_3 transfer over SCN transfer,[54] although differences in ligand binding between N_3 and SCN at the iron center cannot be entirely ruled out. Second, increasing the concentration of SCN leads to substantial decreases in the both the yield and enantioselectivity of the azidation product, with **5r** being formed in only 5.8% yield and 56.5:43.5 e.r. when 49 mM of NaSCN was used (**Figure 4a, Entry 7**). Intriguingly, this perturbation of the enantioselectivity of the azidation product was not observed with the other anions tested (**Table S14**). Control reactions with empty vector and buffer ruled out the possibility of free SCN-bound iron facilitating a background reaction to form racemic **5r** (**Table S15**). While we are still investigating the cause of the diminishing enantioselectivity, one possibility is that SCN binding to the iron alters the primary coordination environment of the radical-intercepting Fe(III)-N_3 intermediates and subsequently impacts the enantiodetermining radical transfer step.

We further carried out a series of experiments to study the mechanistic aspects of this reaction (**Figure 4b**). Substitution of Togni reagent II (**1a**) with the alcohol-based Togni reagent I (**1b**) lowered the reaction yield but maintained the high enantioselectivity (**Figure 4b, Table S11**). This observation implies that the enantiodetermining radical recombination step is not sensitive to the structure of the iodine(III) reagents. The decrease in yield is also consistent with the lower reduction potential of **1b**, which could lower its propensity for trifluoromethyl radical generation compared to **1a**.[4] Next, a TEMPO radical trap was employed in an attempt to intercept any potential radical intermediates. Upon addition of TEMPO, formation of product **5r** was completely inhibited, and unreacted starting material **3r** was observed, suggesting that the reaction proceeds through a radical mechanism. In addition, TEMPO-CF_3 adduct **10** was detected by both GCMS and ^{19}F NMR, supporting the involvement of trifluoromethyl radical in the reaction (**Figure S9 and S10**). To further investigate the radical nature of the transformation, we employed radical clock substrate **3v**, which has a slow rate of ring opening ($k = 1.4 \times 10^5\ s^{-1}$).[55] Under standard reactions conditions, the cyclopropyl ring was preserved and only non-ring opening product **5v** was formed, suggesting a rapid azide-transfer step, consistent with the lower energy barriers calculated for enzymatic radical N_3 transfer in a previous work.[32]

In conclusion, we developed a novel biocatalytic system for the generation of trifluoromethyl radicals to enable a new-to-nature alkene trifluoromethyl azidation reaction catalyzed by nonheme iron enzymes. This work introduces a new activation mode for enzymatic alkene difunctionalization. By varying the hypervalent iodine(III) reagent, the reaction can be expanded to the pentafluoroethyl azidation and diazidation of alkenes, demonstrating the versatility of this platform. We anticipate that this study will stimulate further development in biocatalytic methods for trifluoromethylation and broaden the horizons of enzymatic organofluorine chemistry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interest.

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