Enantioselective Synthesis of α-Trifluoromethyl Amines via Biocatalytic N−H Bond Insertion with Acceptor-Acceptor Carbene Donors

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ABSTRACT: The biocatalytic toolbox has recently been expanded to include enzyme-catalyzed carbene transfer reactions not occurring in Nature. Herein, we report the development of a biocatalytic strategy for the synthesis of enantioenriched α-trifluoromethyl amines through an asymmetric N−H carbene insertion reaction catalyzed by engineered variants of cytochrome c552 from Hydrogenobacter thermophilus. Using a combination of protein and substrate engineering, this metalloprotein scaffold was redesigned to enable the synthesis of chiral α-trifluoromethyl amino esters with up to >99% yield and 95:5 er using benzyl 2-diazotrifluoropropanoate as the carbene donor. When the diazo reagent was varied, the enantioselectivity of the enzyme could be inverted to produce the opposite enantiomers of these products with up to 99.5:0.5 er. This methodology is applicable to a broad range of aryl amine substrates, and it can be leveraged to obtain chemoenzymatic access to enantioenriched β-trifluoromethyl-β-amino alcohols and halides. Computational analyses provide insights into the interplay of protein- and reagent-mediated control on the enantioselectivity of this reaction. This work introduces the first example of a biocatalytic N−H carbeneoid insertion with an acceptor−acceptor carbene donor, and it offers a biocatalytic solution for the enantioselective synthesis of α-trifluoromethylated amines as valuable synthons for medicinal chemistry and the synthesis of bioactive molecules.

INTRODUCTION

The incorporation of fluorine can favorably alter the physicochemical and biological properties of bioactive molecules.1−14 Fluorine-containing building blocks are increasingly used in medicinal chemistry, as the introduction of fluorine substituents can improve the pharmacokinetic and pharmacological properties of small-molecule drugs, including their potency, cell permeability, and metabolic stability.14,15

One group of organofluorines of great interest in drug discovery and development are chiral α-trifluoromethyl amine derivatives, such as substituted trifluoroethylamines,6,8 and α-trifluoromethyl amino esters.9 These fluorinated building blocks can serve as unnatural amino acids useful for generating proteolytically stable peptides with increased lipophilic properties.8,9 Additionally, chiral α-trifluoromethyl amines and amino esters have been utilized as peptide mimics10 and as PLP-dependent enzyme suicide inhibitors,11,12 respectively, prompting considerable efforts toward the development of methodologies to afford these important fluorinated building blocks.13 Reported methods for the construction of chiral α-trifluoromethyl amines include the asymmetric reduction of N-arylimino trifluoropropanoic acid esters,14 asymmetric organocatalytic Strecker reactions,15 catalytic asymmetric umpolung reactions with trifluoromethylamines,16,17 and palladium-catalyzed vicinal fluoroarylation of gem-difluoro-2-azadienes,18 among others (Scheme 1).19−21 Despite this progress, these methods offer moderate levels of stereoselectivity and require the use of a preinstalled trifluoromethyl group, rare metals, or multiple steps to access the desired α-trifluoromethyl amino core. The transition-metal-catalyzed asymmetric insertion of carbeneoids into N−H bonds represents an attractive strategy for the synthesis of optically active amines.22,23 We further appreciated that, while a carbene N−H insertion reaction involving fluoroalkyl-substituted α-diazo esters could provide a direct route to optically active α-trifluoromethyl amino esters, no methods have so far been reported to realize this transformation.

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Previous efforts by our group and others have led to the development of hemoprotein-based biocatalysts for a growing number of abiological carbene transfer reactions, including carbene insertions into heteroatom–hydrogen bonds (N–H, S–H, Si–H, and B–H). In particular, engineered myoglobins, P450 enzymes, and artificial metalloenzymes have been reported for (nonasymmetric) N–H insertion reactions involving acceptor-only diazo esters. More recently, the first example of a biocatalytic asymmetric N–H insertion with donor–acceptor α-alkyl-substituted diazo esters was accomplished. On the other hand, laboratory-evolved variants of cytochrome from Rhodothermus marinus have proven useful for stereoselective Si–H insertion reactions in the presence of donor–acceptor diazo compounds. Despite this progress, bio- and chemocatalytic methods for asymmetric carbene N–H insertions remain scarce, and none have yet been made available for the synthesis of α-trifluoromethyl amino esters. Using engineered variants of cytochrome from Hydrogenobacter thermophilus, we report herein the first biocatalytic strategy for the enantioselective carbene N–H insertion of acceptor–acceptor alkyl 2-diazo-3,3,3-trifluoropropanoates (DTPs) (Scheme 1). Furthermore, the combination of protein engineering with substrate engineering achieved by tuning of the diazo compound is shown to provide an effective approach to achieve high enantioselectivity as well as enantiodivergence in this reaction. This biocatalytic strategy represents a sustainable and efficient approach to afford enantioenriched α-trifluoromethylamino alkyl–amino alcohols and halides.

RESULTS AND DISCUSSION

Biocatalyst Screening for N–H Insertion with Ethyl α-Diazotrifluoropropanoate. In initial studies, we tested the activity of wild-type sperm whale Mb and variants thereof toward catalyzing the conversion of p-anisidine into α-trifluoromethyl amino ester in the presence of ethyl 2-diazo-3,3,3-trifluoropropanoate (EtDTP, 2a), under anaerobic and reducing conditions using sodium dithionite as a reductant (Table 1, entries 2 and 4). Unfortunately, the formation of the desired product 3a was not detected. In the presence of Mb(H64V,V68A), a highly active catalyst for N–H insertion with acceptor-only α-diazo esters, only trace amounts of the desired N–H insertion product were detected and no enantioselectivity was observed (Table 1, entry 3). On the basis of these results, we turned our attention to cytochrome from Hydrogenobacter thermophilus, herein referred to as Hi-Cc552, a highly thermostable electron transfer protein (Tm > 110 °C) whose structure both in solution and in crystal form are known. Since the “distal” axial position of the heme c cofactor in this protein is occupied via coordination by a methionine residue (Met59), an M59G variant was initially designed to enhance its reactivity in the desired reaction. A similar strategy has previously proven useful for improving the carbene transfer activity of cytochrome c from Rhodothermus marinus.

Table 1. Catalytic Activity and Enantioselectivity of Mb, Hi-Cc552 Variants, and Hemoproteins for N–H Carbene Insertion of p-Anisidine with EtDTP (2a)

| entry | catalyst | conditions | yield (%) | TON | er |
|-------|----------|------------|-----------|-----|----|
| 1     | hemin`   | std        | 0         | nd  | nd |
| 2     | wt Mb    | std        | 0         | nd  | nd |
| 3     | Mb(H64V,V68A) | std | 2         | 2   | 50:50 |
| 4     | Hi-Cc552 | std        | 0         | nd  | nd |
| 5     | Hi-Cc552(M59G) | std | 33      | 27  | 64:36 |
| 6     | Hi-Cc552(M59G) | no reductant | 0 | nd  | nd |
| 7     | Hi-Cc552(M59G) | aerobic   | 1.4      | 1   | nd |

`Standard reaction conditions: 5 mM p-anisidine (1a), 10 mM EtDTP (2a), 60 μM (1.2 mol %) catalyst in KPi buffer (50 mM, pH 7.0), 10 mM Na2S2O4, rt, 16 h, under an argon atmosphere. nd = not determined. Yield, TON, and enantiomeric ratios based on chiral supercritical fluid chromatography (SFC) analysis using calibration curves generated from racemic analytical standards. `15% DMF.
Gratifyingly, while Hi-Cc552 exhibited no detectable activity in the N−H insertion reaction between p-anisidine and EtDTP, the Hi-Cc552(M59G) variant produced 3a with significantly higher efficiency (33% yield), albeit with modest enantioselectivity (64:36 er) (Table 1, entry 5). Control experiments showed that the product formation was abolished when the reaction was carried out in the absence of the reductant (Na2S2O4) or under aerobic conditions (Table 1, entries 6 and 7), indicating that ferrous Hi-Cc552 is the catalytically active species and that molecular oxygen inhibits the reaction, likely preventing or interfering with the formation of the iron porphyrin carbene intermediate. Importantly, the heme cofactor alone does not catalyze the N−H carbene insertion reaction (Table 1, entry 1), highlighting the critical role of the protein scaffold in promoting catalysis. Furthermore, the superior catalytic activity of Hi-Cc552(M59G) vs Mb(H64V,V68A) in the reaction suggested a beneficial effect of the heme c cofactor in Hi-Cc552 in comparison to the heme b cofactor in myoglobin toward activation of the acceptor−acceptor diazo reagent EtDTP. Indeed, Mb(H64V,V68A) was previously found to catalyze the N−H functionalization of aniline with ethyl 2-diazopropanoate, which is sterically similar to but electronically different from EtDTP. This difference in reactivity with EtDTP may be ascribed, at least in part, to the more positive redox potential of the Hi-Cc552 scaffold in comparison to the myoglobin scaffold (i.e., ∼+250 mV42 and +60 mV,43,44 respectively, vs SHE). Indeed, our group recently demonstrated that myoglobin-based carbene transferases featuring increased redox potentials as a result of structural alterations to the heme cofactor and the first metal-coordination sphere enhanced reactivity toward the cyclopropanation of electron-deficient substrates. Consistent with this hypothesis, we determined experimentally that wild-type Hi-Cc552 features an Fe3+/Fe2+ reduction potential (E°(Fe3+/Fe2+)) of +245(±2) mV (Figure S8)—in excellent agreement with a prior literature value45—whereas the Hi-Cc552(M59G) variant features an even higher E°(Fe3+/Fe2+) value that exceeds +300 mV (Figure S8), as estimated on the basis of the upper limit for measurable E°(Fe3+/Fe2+) values using the spectrophotometric method applied for these analyses.45 Using the same method, the reduction potential (E°(Fe3+/Fe2+)) of Mb(H64V,V68A) was previously determined to be +54 mV.46 Thus, in addition to facilitating access of the diazo reagent to the active iron center of the heme c cofactor, the beneficial M59G mutation could favor the reaction by shifting the redox potential of the metalloprotein toward a more positive value.

**Protein Engineering of Hi-Cc552 Variants for Enantioselective N−H Insertion.** On the basis of these initial results, Hi-Cc552(M59G) was selected as a promising starting point for further development of a biocatalyst for the reaction via protein engineering. To this end, we created and screened an “active-site mutational landscape” library that sampled all 19 possible amino acid substitutions at positions Pro60, Pro61, and Gln62, which reside within a loop region above the heme pocket (Figure 1). From these libraries, a Q62R mutation (i.e., Hi-Cc552(M59G,Q62R)) was found to be particularly beneficial to improve both the efficiency (33% → 81% yield) and enantioselectivity (64:36 vs 76:24 er) of the metalloprotein over the parental sequence (Figure 2 and Figures S1−S3 in the Supporting Information). Using Hi-Cc552(M59G,Q62R) as the parent, the introduction of the P60E mutation induced a further improvement in enantioselectivity (78:22 er; Figure 2 and Figures S1−S3 in the Supporting Information), without affecting the catalytic activity, resulting in the identification of Hi-Cc552(M59G,P60E,Q62R) as a significantly improved biocatalyst for the N−H insertion reaction over Hi-Cc552(M59G). Next, the amino acid positions lle46, Gly49, and Gly50, which reside within the inner side of the heme pocket (Figure 1), were targeted for site-saturation mutagenesis, due to their close proximity to the heme c cofactor. From these libraries, the improved variant Hi-Cc552(G50T,M59G,P60E,Q62R) was identified that is able to produce the desired α-trifluoromethyl amino ester 3a in nearly quantitative yield (93%) and with further improved enantioselectivity (81:19 er) (Figure 2 and Figure S4 in the Supporting Information).

**Tuning of Hi-Cc552 Variant Enantioselectivity via Diazoreagent Engineering.** In previous studies, we found that re-engineering of the diazo reagent can furnish a valuable and complementary strategy (to protein engineering) for fine-tuning the enantioselectivity of carbene transfer biocatalysts. Armed with this knowledge, we investigated the possibility of increasing the enantioselectivity of the Hi-Cc552(G50T,M59G,P60E,Q62R)-catalyzed N−H insertion reaction by tuning the diazo reagent to a more electron-rich analog (e.g., benzyl diazirine). In these experiments, using benzyl diazirine, we observed only the formation of 3a at low yields (20−30% er) and with moderate enantioselectivity (48:52 er). This suggests that the diazo reagent is a key determinant of enantioselectivity for the Hi-Cc552 variant.

**Figure 1.** X-ray crystal structure of Hi-Cc552 (PDB IYNR) shown as a ribbon model. Amino acid residues targeted for site-saturation mutagenesis are highlighted in green, and the heme c cofactor is shown in pink.

**Figure 2.** Directed evolution of Hi-Cc552 for the enantioselective N−H insertion of p-anisidine with EtDTP. Reactions were carried out using 60 µM (1.2 mol %) purified Hi-Cc552 variants, 5 mM 1a, and 10 mM 2a, in KPi buffer (50 mM, pH 7.0) at room temperature under anaerobic conditions (=standard reaction conditions or s.r.c.).
Scheme 2. Tuning of Ht-Cc552(G50T,M59G,P60E,Q62R) Enantioselectivity via Diazo Reagent Engineering

\[
\begin{align*}
\text{PH}_{3} & + \text{F}_2\text{C} & & \text{N}_{2} \\
\text{1a} & & & \text{2a-h} \\
\text{Na}_2\text{S}_2\text{O}_4 & & \text{KPi buffer (pH 7.0)} \\
& & \{\text{s.r.c.}\} \\
\rightarrow & & \\
\text{3a-h} & & \% \text{yield} \\
& & \text{e.r.} \ (R:S)
\end{align*}
\]

\[\text{s.r.c. = Standard reaction conditions as in Figure 2. The product conversion and stereoselectivity were determined by chiral SFC using a calibration curve with authentic standards.}\]

Scheme 3. Substrate Scope of Ht-Cc552-Catalyzed R-Enantioselective N–H Insertion Reaction in the Presence of BnDTP (2c)

\[
\begin{align*}
\text{ArNH}_2 & + \text{F}_2\text{C} & & \text{O} \\
1a-n & & \text{2c} \\
& & \text{Ht-Cc552} \\
& & \{\text{1.2 mol}\%\} \\
& & \{\text{10 mM}\} \\
& & \text{KPi (50 mM, pH 7.0)} \\
& & \text{anaerobic, rt, 16 h} \\
\rightarrow & & \\
\text{4a-n} & & \% \text{yield} \\
& & \text{e.r.} \ (R:S)
\end{align*}
\]

\[\text{s.r.c. = standard reaction conditions as in Figure 2. Asterisks denote 5% DMF. The product conversion and stereoselectivity were determined by chiral SFC and GC using a calibration curve with authentic standards.}\]
reaction by varying the alkyl ester group in the diazo reagent, with the goal of exploiting beneficial steric interactions between this group (e.g., in the heme-bound carbene intermediate) and the surrounding protein residues. To this end, we developed an efficient and versatile synthetic route to afford 2-diazo-3,3,3-trifluoropropionate esters from inexpensive trifluoroacetic acid and p-anisidine (Scheme S1) and applied this protocol to produce a diverse set of DTP carbene donors (compounds 2b−h, Scheme 2) bearing ester groups of varying size (e.g., 2d vs 2c), bulk (e.g., 2b vs 2c), or substitution patterns on the benzyl ring (e.g., 2e vs 2g vs 2h).

To our delight, a notable improvement in the enantioselectivity of the reaction with *Ht-Cc552* (G50T,M59G,P60E,Q62R) was obtained upon substitution of the ethyl group in the EtDTP reagent with a larger group (i.e., a cyclohexyl (2b), benzyl (2c), or naphthyl group (2d)), resulting in the formation of the desired N−H insertion product in up to 97:3 enantiomeric ratio (3b−d vs 3a, Scheme 2). Further analysis of 3b−d via chiral chromatography and other control experiments (see the Supporting Information for details) showed that they share the R configuration of 3a. Thus, across this compound series, the increase in R enantioselectivity was found to correlate largely with the increasing size of the ester group in the carbene donor reagent (3d ≈ 3c > 3b > 3a). In consideration of the higher enantioselectivity but comparably high reactivity vs EtDTP (83% vs 93% yield), the benzyl ester derivative 3c was chosen as the optimal reagent for the formation of the R enantiomer of the N−H insertion product. A variation of the reaction conditions did not lead to a further improvement in yield and/or enantioselectivity (Table S1). Additional experiments demonstrated that *Ht-Cc552* (G50T,M59G,P60E,Q62R) supported up to 752 total turnovers under catalyst-limited conditions (entry 11, Table S1) and it catalyzes the reaction with an initial product formation rate of 8 and 1.5 TON min⁻¹ in the presence of EtDTP and BnDTP, respectively (Figure S5). Under the optimized reaction conditions, no significant loss in the Soret band of the protein (<10%) or protein precipitation was noted during the reaction (Figure S6), indicating that destruction of the heme cofactor does not play a major role in limiting the catalyst performance and that the biocatalyst may be recyclable, an aspect that will be investigated as part of future studies.

Intriguingly, substitution of the benzyl group in the diazo reagent with one or two methyl groups (2e−h) led to a complete switch of the biocatalyst’s enantioselectivity to give the corresponding S-configured N−H insertion products 3e−h in up to 99% enantiomeric excess (3g, Scheme 2). On the basis of its superior performance in terms of both enantioinduction and yield, the 2,5-dimethylbenzyl-containing diazo compound 2g was selected as the optimal carbene donor reagent for favoring S enantioselectivity in the *Ht-Cc552* (G50T,M59G,P60E,Q62R)-catalyzed N−H insertion reaction. Altogether, these studies highlighted the value of combining protein engineering of the metalloprotein scaffold with diazo substrate engineering for both tuning and inverting the enantioselectivity of a carbene transferase enzyme. Indeed, while it has been previously possible to obtain enantiocomplementary carbene transfer biocatalysts by re-engineering of the enzyme,⁴¹,⁴⁷,⁴⁸ to our knowledge this is the first example in which enantiomivergence has been achieved within a single enzyme through engineering of the diazo reagent.

**Substrate Scope of R and S Enantioselective N−H Insertion Reactions with *Ht-Cc552***

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*Scheme 4. Substrate Scope of *Ht-Cc552*-Catalyzed S-Enantioselective N−H Insertion Reaction in the Presence of (2,5-Dimethyl)benzyl 2-Diazo trifluoropropionate (2g)"*

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"s.r.c. = standard reaction conditions as in Figure 2. The product conversion and stereoselectivity were determined by chiral SFC and GC using a calibration curve with authentic standards.
α-functionalized anilines could be readily converted to the corresponding N−H insertion product. Notably, variously substituted anilines, including para- (1a,b,e,f–i,j,k,m), meta- (1c,n), and ortho-substituted (1d) anilines were readily accepted by the Ht-Cc552 variant to produce the desired α-trifluoromethyl amino esters 3c, 4b–f in good to quantitative yields (41–99%) and with high enantioselectivity (90:10 to 95:5 er) (Scheme 3). A doubly substituted aniline substrate such as 3-chloro-4-fluoropropanoate (1g) was also converted to the corresponding N−H insertion product 4g with good enantioselectivity (88:12 er), albeit in more modest yield (32%). The reactions with aniline derivatives carrying electron-withdrawing groups or large substituents in the para position generally, albeit not always (e.g., 3c, 4b–f, 4i) resulted in a broad substrate scope of the diastereomeric N−H insertion reactions with good enantioselectivity (88:12 er), albeit in more modest yield (32%). The reactions with aniline derivatives carrying electron-withdrawing groups or large substituents in the para position generally, albeit not always (e.g., 4f,m), displayed higher levels of enantioselectivity as indicated by the results with 4e,f (94:6 er), 4h (93:7 er), and 4i (95:5 er) in comparison to 3c and 4b. An opposite trend was observed for meta-substituted anilines (4n vs 4c). Notably, keto- and nitrile-functionalized anilines could be readily converted to the desired α-trifluoromethyl amine derivatives (4l,m), despite the potential reactivity of the keto group with the amine substrate and the tendency of nitrile compounds to bind metal centers, respectively. Substrates 1j,k were also tested to explore the scope of the reaction across other aromatic amines. These naphthyl- and benzoxyl-substituted amines were both accepted to give the desired N−H insertion products 4j,k, respectively, in high yields (68–99%) and in high enantiomeric ratios (91:9 to 92:8 er), further demonstrating the broad substrate profile of the engineered Ht-Cc552-based biocatalyst.

Importantly, in all of these reactions the N−H insertion products were obtained with R enantioselectivity, highlighting the conserved and predictable enantiopreference of the biocatalyst under the applied conditions. It is also worth noting that no formation of the dimerization byproducts of BnDTP, dibenzyl 2,3-bis(trifluoromethyl)malate, or double-insertion products were detected in these reactions, further indicating that these biocatalytic transformations proceed with high chemoselectivity. Furthermore, a large-scale reaction using 4-bromoaniline (1f) and 2c was carried out to obtain 60 mg of the α-trifluoromethyl-amino ester 4f in 75% isolated yield, supporting the scalability of the methodology. Product 4f was crystallized and determined to have an R absolute configuration by X-ray diffraction analysis (Scheme 3), serving as a reference for the stereochemical assignment of the other products and their corresponding enantiomers.

S-Enantioselective N−H Insertion Reactions via a Diazo Reagent Switch. To probe the substrate scope of the Ht-Cc552 biocatalyst in the S-selective mode, representative samples of the aniline derivatives described in Scheme 3 were then tested by applying the same enzyme variant and identical reaction conditions but in the presence of 2,5-dimethylbenzyl 2-diazotrifluoropropanoate (2g) as the carbene donor reagent, instead of BnDTP (Scheme 4). Gratifyingly, the desired S-configured N−H insertion products 5a–k were obtained in all cases, showing that the diazo-substrate-induced inversion of enantiopreference is broadly maintained across the different aniline substrates. Although the yields of these reactions were generally lower than observed for the R-selective counterparts (34% vs 68% average yield), they all proceeded with good to excellent enantioselectivity, resulting in the formation of the S-configured products in enantiomeric ratios ranging from 17:83 to 0.5:99.5 (Scheme 4). In addition to the inverted enantioselectivity, distinct structure activity trends were also noted for the two enantiodivergent transformations. For example, whereas aniline derivatives with bulky groups at the para position were well tolerated in the R-selective mode with BnDTP, these represented more challenging substrates for the S-enantioselective variant of the reaction in the presence of 2g, in particular in comparison to other para-substituted analogues (e.g., 12−34% yield for 5h–i vs 38−62% for 3g, 5f,5j). This difference clearly suggested distinct steric requirements with respect to the amine substrate during the Ht-Cc552-catalyzed reaction in the two enantiodivergent fashions. Overall, the results summarized in Schemes 3 and 4 demonstrate the utility

Figure 3. (A) Stereochemical model for enantioselective N−H insertion reaction catalyzed by Ht-Cc552 variants. (B) DFT-calculated model of the proS(−) heme-bound ylide reaction intermediate.

Cc552(G50T,M59G,P60E,Q62R). To explore the substrate scope of the Ht-Cc552(G50T,M59G,P60E,Q62R) biocatalyst in the R-enantioselective mode, the enzyme was challenged with a panel of aniline derivatives and other aryl amines in the presence of benzyl 2-diazotrifluoropropanoate 2c (Scheme 3). Notably, variously substituted anilines, including para- (1a,b,e–i,j,k,m), meta- (1c,n), and ortho-substituted (1d) anilines were readily accepted by the Ht-Cc552 variant to produce the desired α-trifluoromethyl amino esters 3c, 4b–f in good to quantitative yields (41–99%) and with high enantioselectivity (90:10 to 95:5 er) (Scheme 3). A doubly substituted aniline substrate such as 3-chloro-4-fluoropropanoate (1g) was also converted to the corresponding N−H insertion product 4g with good enantioselectivity (88:12 er), albeit in more modest yield (32%). The reactions with aniline derivatives carrying electron-withdrawing groups or large substituents in the para position generally, albeit not always (e.g., 4f,m), displayed higher levels of enantioselectivity as indicated by the results with 4e,f (94:6 er), 4h (93:7 er), and 4i (95:5 er) in comparison to 3c and 4b. An opposite trend was observed for meta-substituted anilines (4n vs 4c). Notably, keto- and nitrile-functionalized anilines could be readily converted to the desired α-trifluoromethyl amine derivatives (4l,m), despite the potential reactivity of the keto group with the amine substrate and the tendency of nitrile compounds to bind metal centers, respectively. Substrates 1j,k were also tested to explore the scope of the reaction across other aromatic amines. These naphthyl- and benzo[d][1,3]dioxole-substituted amines were both accepted to give the desired N−H insertion products 4j,k, respectively, in high yields (68–99%) and in high enantiomeric ratios (91:9 to 92:8 er), further demonstrating the broad substrate profile of the engineered Ht-Cc552-based biocatalyst.

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of the engineered Ht-Cc552 biocatalyst toward obtaining α-trifluoromethyl-α-amino esters in both enantiomeric forms.

**Origins of Enzyme-Controlled Enantioselectivity.**

Computational studies were performed to better understand the role of the metalloprotein scaffold in controlling the enantioselectivity of the reaction as well as the nature of the carbene donor reagent-induced switch in enantioselectivity. Similarly to carbene S−H insertion,29 heme-catalyzed carbene N−H insertion was previously proposed to proceed via the formation of an iron ylide complex generated by nucleophilic attack of the amine substrate to the iron porphyrin carbene intermediate,41 followed by protonation of the ylide to give the N−H insertion product.49 By analogy with other metal-catalyzed N−H insertions,50−54 the stereochemical outcome of this reaction can be determined by the facial selectivity of amine attack to the metallo-carbene species during formation of the metal-bound ylide intermediate and/or during protonation of the dissociated ylide. In the present system, divergent enantioselectivity was obtained using the same enzyme variant in the presence of diazo reagents with varying steric bulk, indicating that enantioselectivity is primarily dictated during the formation of the heme-bound ylide intermediate, followed by a conserved mechanism for protonation of the proR or proS heme-ylide complex on the solvent-exposed side of these species (Figure 3A). Reasonably, the protonation step could be mediated by the protein matrix (i.e., by amino acid residues proximal to the heme cofactor) or directly from the solvent.

According to this mechanistic scenario, we performed density functional theory (DFT) calculations on the iron porphyrin bound ylide intermediate formed by the reaction with the 2c-derived iron porphyrin carbene intermediate and p-methoxyaniline (1a). We computed the structures and energies for four conformations of the iron porphyrin ylide complex leading to either proR or proS enantioselectivity, and the orientation of the ester moiety (indicated by + or −) with respect to the porphyrin group plane (see the Experimental Procedures, Figure 3B, and Figure S10). The computed energy

### Table 2. Rosetta-Calculated Energies of the Engineered Ht-Cc552(G50T,M59G,P60E,Q62R) Variant Complexed with 2c/1c-Derived Heme Ylide Intermediates in the proR or proS Configurations and +/− Conformations

| entry | diazo | yield (%) | er (R:S) | proR-rot2 (−) | proR-rot3 (−) | proS-rot1 (+) | proS-rot1 (−) |
|-------|------|-----------|----------|--------------|--------------|--------------|--------------|
| 1     | 2a   | 93        | 81:19    | −202.46      | −220.89      | −210.93      | −211.69      |
| 2     | 2c   | 83        | 91:9     | −183.27      | −207.24      | −205.47      | −193.99      |
| 3     | 2e   | 45        | 12:88    | −189.69      | −187.12      | −204.22      | −196.13      |
| 4     | 2f   | 61        | 5:95     | −190.77      | −122.31      | −206.28      | −130.91      |
| 5     | 2g   | 62        | 0.5:99.5 | −184.77      | −157.02      | −205.61      | −198.40      |
| 6     | 2h   | 11        | 31:69    | −169.76      | −110.24      | −185.70      | −68.12       |

The lowest energy value is highlighted in boldface, while the energy of the most competitive state is underlined. For complete data, see Table S2. Energies are reported in Rosetta energy units (REUs).
differences between the different conformations (proR(+), proR(−), proS(+), and proS(−)) of the porphyrin-bound ylide intermediate are within 1.7 kcal/mol (Figure S10), confirming that the experimentally observed enantioselectivity of the enzymatic reactions is derived from differential stabilization of these conformations within the enzyme active site.

To gain insight into the origin of the enantiopreference endowed by the engineered Hi-Cc552 biocatalyst, we generated models for the hemoprotein-bound ylide complexes in the various Hi-Cc552 variants using the Rosetta software suite. Specifically, we superimposed DFT-calculated Fe ylide models onto the available crystal structure of Hi-Cc552, introduced amino acid substitutions present in Hi-Cc552(G50T,M59G,P60E,Q62R), and optimized the structure and energy of the resulting protein–ligand complexes. Carbene from the diazo reagents gaining high activity (>50% yield) in the N–H insertion reaction were included in our calculations (Table 2, column 2) and used in the modeling. As the DFT calculations were performed with the porphyrin, which is C4k symmetric (unlike heme), each of the four DFT-generated porphyrin-bound ylide intermediates (i.e., proR(+), proR(−), proS(+), and proS(−)) can be superimposed into the protein active site in four ways by aligning to one of four heme group rings (A–D, Figure 4), resulting in 16 different arrangements for each substrate (named proR-rot1(+), proR-rot1(−), proS-rot1(+), proS-rot1(−), proS-rot2(+), etc.; see the Experimental Procedures for details). The lowest energy arrangement is predicted to correspond to the preferred enantiomer generated by the enzyme. As shown in Table 2, Rosetta-based energy calculations are able to qualitatively recapitulate the enantioselectivity of the engineered Hi-Cc552-catalyzed N–H insertion in the presence of the different diazo reagents. For both 2c and 2a, the proR configurations of the corresponding protein ylide complexes lie 2–10 Rosetta energy units (REUs) lower in energy than the proS configurations, which recapitulate well the R selectivity of the enzyme in the reactions with these diazo compounds. Similarly, the protein ylide complexes derived from 2e–g in the proS configurations are favored by 15–22 REU in comparison to the proR counterparts, which is consistent with the S enantiopreference of the Ht-Cc552(G50T,M59G,P60E,Q62R)-catalyzed reactions in the presence of these diazo reagents. The S enantiopreference of the N–H insertion reaction with 2h was also correctly predicted from a qualitative standpoint (Table 2), even though the energy differences between the various binding poses corresponding to the different proR and proS configurations do not fully correlate with the lower enantioselectivity observed experimentally for this reaction in comparison to those with the related diazo reagents 2e–g (i.e., 3h vs 3e–g; Scheme 2). Notably, however, the calculated energy values of all configurations of the 2h-derived complexes were found to be significantly higher in comparison to the corresponding complexes with 2e–g (Table 2). This uniform destabilization suggests a less favorable fit of the 2h-derived ylide, in comparison to the analogous ylide complexes derived from 2e–g, into the near-native conformation of the protein used in our modeling. This general destabilization may explain, at least in part, the lower yield observed in the N–H reaction with this reagent (11%) in comparison to the reactions with 2e–g (11% vs 45–62% yield; Scheme 2).

Having established that Rosetta-based energy calculations can recapitulate the enantioselectivity of Hi-Cc552(G50T,M59G,P60E,Q62R), we further inspected the protein and ylide complex models to gain insights into the structural features underlying its higher catalytic activity and enantioselectivity in comparison to the wild type protein. In wild-type Ht-Cc552 (Figure 1), the sulfur atom of the Met59 side chain coordinates to the iron center of the heme cofactor, which likely prevents an interaction of the diazo reagent with the heme center, resulting in the lack of catalytic activity observed experimentally (Figure 2). In contrast, the M59G mutation in the engineered Hi-Cc552 variant creates an open coordination site at the heme iron center, which enables binding and activation of the diazo reagent to form the heme carbene intermediate, inducing a major increase in N–H insertion reactivity (Figure 2 and Table 1, entry 5 vs entry 4).

The M59G mutation also creates a cavity above ring B of the heme c cofactor (Figure 4A,B), so that the aniline substrate can more readily approach the Si and Re faces of the rot3 and rot1 2c-derived carbene, respectively, to form proS-rot1(+) or proS-rot1(+) heme ylide complexes. In combination with the M59G mutation, the P60E mutation increases the backbone flexibility of the active site loop to better accommodate the aniline substrate.

In the proR configuration of the 2c-derived ylide complex of Ht-Cc552(G50T,M59G,P60E,Q62R), the benzyl ester group projects inward into the buried side of the distal cavity of the heme c cofactor (Figure 4A), whereas the trifluoromethyl group is oriented outward. The aliphatic side chain of Arg62 (introduced via the beneficial Q62R mutation, Figure 2) is packed against the triazole group is oriented outward. The aliphatic side chain of Arg62 projects inward into the buried side of the distal cavity of the heme c cofactor (Figure 4A). In the proS arrangement of the same complex, the benzyl ester group is directed outward and toward the solvent, whereas the trifluoromethyl group is oriented inward (Figure 4B). In this arrangement, Thr50 makes no contacts with the ylide complex and the side chain of Arg62 is “pushed away” from the benzyl group. The latter group also physically separates the Arg62/Trp60 ionic pair and brings their ionized side-chain groups at a greater distance than in the proR complex (∼10 vs 5 Å). In both complexes, the aniline group adopts a similar position, making close contacts with the stem region of the long Met60-bearing loop (i.e., residues 49–50 and 58–59; Figure 4A,B). Altogether, these analyses suggest that (a) preorganization of the heme-bound carbene is critical for dictating facial selectivity of amine attack to this species to generate the ylide intermediate and (b) the proR configuration is favored by the combination of steric effects and favorable van der Waals and electrostatic interactions mediated by Arg62, Glu60, and Thr50, which are disrupted (or absent in the case of Thr50) in the proS configuration. Consistent with these conclusions, a per-residue energy decomposition comparison of the ylide complex models in the preferred R-rot3(−) arrangement versus the competing proS-rot1(+) state revealed that the largest energy differences favoring the proR vs proS arrangement reside in contacts mediated by Arg62, Glu60, and Thr50 (Figure S12A,B), all of which have contributed to the enhancement of the R
Scheme 5. Chemoenzymatic Synthesis of Enantioenriched α-Trifluoromethyl Amine Derivatives

**Origins of Reagent-Induced Enantioselectivity Switch.** We next investigated how the enantiopreference of the **Ht-Cc552**-based catalyst could be completely switched to favor the S-configured N–H insertion product in the presence of diazo reagent 2g (0.5:99.5 R:S) in comparison to the structurally related diazo reagent 2c (91:9 R:S). In contrast to the 2c-derived ylide (Figure 4A,B), the 2,5-dimethyl-substituted benzyl ester moiety of the 2g-derived ylide in the proR-rot3(−) configuration can no longer be accommodated by the inner pocket region above ring D of the heme cofactor. This state is largely destabilized by clashes with residue Thr50 (i.e., the site of **G50T** substitution), Leu42 and Lys45 side chains, and the propionic groups of the heme ε cofactor (Figure 4C and Figure S12C,D). Other proR-configuration states of the 2g-derived ylide also cannot rival proS-rot1(+) (Figure 4D), which can thus explain the high S enantioselectivity of the **Ht-Cc552** (**G50T,MS9G,P60E,Q62R**) catalyzed N–H insertion reaction with 2g. For example, the most competitive state proR-rot2(−) has its aniline moiety occupying an alternative cavity above ring A, breaking the hydrogen bond between the backbone atoms of Lys47 and Arg62 (Figure S11C). Its benzyl ester moiety is also destabilized by unfavorable interactions with Glu60 and Arg62 (Figure S11C), as evidenced by a per-residue energy decomposition analysis of these complexes (Figure S12E,F). In addition to enantiodivergence, the lower energy of the dominant state of the ylides derived from the less bulky diazo esters (**2a–c,h** vs **2e–g; Table 2**) tend to correlate with the higher yields of the corresponding reactions (Scheme 2), although other factors can contribute to these differences. Thus, these analyses show that a combination of structural and energetic factors, mediated by all four beneficial mutations as well as other residues surrounding the heme ε cofactor, contribute to destabilize the proR configurations of the 2g-derived ylide over the proS state, resulting in the dramatic switch in enantioselectivity observed experimentally in the **Ht-Cc552** (**G50T,MS9G,P60E,Q62R**) catalyzed N–H insertion reactions with 2g.

**Diversification of α-Trifluoromethyl Amine Products.** To further demonstrate the synthetic value of the present biocatalytic strategy, asymmetric **Ht-Cc552**-catalyzed N–H carbene insertion with DTPs was leveraged to enable the chemoenzymatic synthesis of various α-trifluoromethylated amine derivatives (Scheme 5), which are highly sought after motifs for medicinal chemistry and drug discovery. For example, benzyl-protected α-trifluoromethylamino acid 7 was synthesized in high yields and in a highly enantioenriched form (86% yield, 90:10 er) by treating enzymatically produced 3c with ceric ammonium nitrate (CAN) (Scheme 5). α-Trifluoromethylated amino acids are valuable noncanonical amino acids and find applications in the design of peptidomimetics and peptide-based drugs. On the other hand, medicinally valuable β-trifluoromethyl-β-amino alcohols such as 8 and 9 could be obtained via reduction of the enzymatic N–H insertion product 3c in the presence of lithium aluminum hydride (LAH) to give **8** in good yield and enantioenrichment (54% yield, 86:14 er) and via nucleophilic arylation of 3c with Grignard reagents to give **9** with no erosion in enantiopurity (9:1 er) (Scheme 5). Finally, LAH reduction of 3c followed by exposure to **XtalfLFluor-E** and tetraethylammonium bromide afforded the trifluoromethylated β-amino alkyl bromide **10** in enantioenriched form (86:14 er; Scheme 5).

**CONCLUSIONS**

In conclusion, we developed a biocatalytic platform for the asymmetric synthesis of α-trifluoromethyl amines via an abiological N–H carbene insertion. Cytochrome **c552** from *Hydrogenobacter thermophilus* was engineered into a selective biocatalyst for the enantioselective N–H insertion of aryl amines with acceptor–acceptor 2-diazotrifluoropropanoates, a reaction with no reported chemocatalytic counterpart. While active site mutations around the heme ε cofactor have proven useful to improve the activity and enantioselectivity of this biocatalyst, a further boost in enantioselectivity as well as complete inversion of its enantiopreference could be achieved through engineering of the diazo reagent. In combination with DFT calculations, Rosetta-based molecular modeling studies provided insights into the origin of protein-mediated control of enantioselectivity in the N–H insertion reaction, along with the factors underlying the enantioselectivity switch upon variation of the ester group in the diazo compound. The enzymatic products can be diversified to obtain a variety of medicinally relevant chiral α-trifluoromethylated amine building blocks. These studies expand the scope of abiological carbene transfer reactions catalyzed by metalloproteins and pave the way to the further development of biocatalytic strategies for the synthesis of chiral organofluorines.

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EXPERIMENTAL PROCEDURES

Reagents and Synthetic Procedures. Synthetic procedures, analytical procedures, and characterization data for the dioxo reagents, N–H insertion products, and chemoenzymatic products are described in the Supporting Information.

Cloning and Plasmid Construction. Plasmid pET22 (Novagen) was used as a cloning vector, and cloning was performed using overlap extension PCR or a modified QuikChange mutagenesis protocol.59 The cytochrome $c_{552}$ Variants were expressed from pET22 vectors in the presence of a second plasmid (pEC8668) for the coexpression of the cytochrome c maturation system. Primer sequences are given in Table S3. Phusion DNA polymerase, dNTP mix, and Dpn I restriction enzyme were purchased from New England Biolabs. Chemically competent E. coli DHSR cells were used for molecular cloning, and E. coli C41(DE3) cells were used for protein transformation. Expression cultures were grown using Terrific Broth medium supplemented with 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol (TBamp/cm). The HtCc552 variants discussed in this work were cloned and expressed using a pET22(b) + vector (Novagen). A plasmid encoding for Hydrogenobacter thermophilus cytochrome $c_{552}$ (Ht-Cc552) was a gift from Prof. Kara Bren (University of Rochester). Ht-Cc552 was subcloned into pET22(b) + using overlap length extension PCR between restriction sites NdeI and XhoI with an N-terminal peptide leader sequence from T. versutus cytochrome c550 (MKISIYATLALSALPAVA) to ensure proper periplasmic maturation59 and a C-terminal 6xHis-tag. This recombinant plasmid was cotransformed with the cytochrome c maturation plasmid pEC86 into E. coli C41(DE3) chemically competent cells.

Library Construction. Site-saturation mutagenesis libraries were constructed using a modified “smart-intelligent” focused mutagenesis protocol.60 To create a library for a targeted amino acid residue site, a forward mutagenizing primer and the reverse partially complementary mutagenizing primer were designed containing the degenerate codons NDT, VMA, ATG, and TGG. The forward primers containing the degenerate codons NDT, VMA, ATG, and TGG were mixed together in a 12:6:1:1 ratio, respectively, and the reverse primers were separately mixed in the same ratio. The forward and reverse primer mixes were used to carry out site-saturation mutagenesis PCR using a modified QuikChange mutagenesis protocol. The PCR products were treated with Dpn I to digest the parental plasmid, and 5 μL of the reaction mixture was used to transform E. coli DH5α chemically competent cells. After the transformed cells were plated on a LBamp agar plate, 60 single colonies were individually picked and grown in 5 mL LBamp overnight cultures, and their plasmids were extracted and sequenced using a T7 forward universal primer (ACGT, Inc.).

Preparative-Scale Synthesis of 6c. The preparative-scale biocatalytic reaction of 6c was carried out with a 20 mL sample using 1.0 mol % of purified Ht-Cc552 variant, 1 equiv of aryl amine substrate, and 2 equiv of alkyl 2-diao-3,3,3-trifluoropropionate. Purified Ht-Cc552(G50T, MS95G, P60E, Q62R) (7.9 mL, 2.1 μmol) in KPI (50 mM, pH 7.0) was placed in a 50 mL round-bottom flask containing a Teflon-coated magnetic stir bar. In a separate 25 mL round-bottom flask containing a Teflon-coated magnetic stir bar were placed sodium dithionite (0.070 g, 0.40 mmol) and KPI (50 mM, pH 7.0), and both round-bottom flasks were purged in tandem for 3 min with Ar(g) using a cannula. The sodium dithionite solution was mixed with the HtCc552 variant via cannula, and the reaction was initiated by adding the arylamine substrate (5 μL, 400 μmol in EtOH) and alkyl 2-diao-3,3,3-trifluoropropionate (10 μL, 400 μmol in EtOH). The biocatalytic reaction mixture was stirred at 60 rpm for 16 h at room temperature under Ar(g) pressure. After 16 h, the reaction was quenched for product analysis by the addition of an internal standard (20 μL of 1,3-benzodioxole at 100 mM in EtOH), followed by extraction with dichloromethane (400 μL) in a 1.5 mL microcentrifuge tube and centrifugation at 14000 rpm for 5 min. The organic layers were collected and subjected to SFC analysis to calculate percent conversion, TON, and enantiomeric ratio.

Preparation and Purification of 6c. The crude reaction mixture was stirred at 60 rpm for 16 h at room temperature under Ar(g) pressure. The reaction was quenched by the addition of diethyl ether (3 mL) to precipitate the enzyme, and the resin was treated with an internal standard solution via cannula, and the reaction was initiated by adding the arylamine substrate (5 μL, 400 μmol in EtOH) and alkyl 2-diao-3,3,3-trifluoropropionate (10 μL, 400 μmol in EtOH). The biocatalytic reaction mixture was stirred at 60 rpm for 16 h at room temperature under Ar(g) pressure. After 16 h, the reaction was quenched for product analysis by the addition of an internal standard (20 μL of 1,3-benzodioxole at 100 mM in EtOH), followed by extraction with dichloromethane (400 μL) in a 1.5 mL microcentrifuge tube and centrifugation at 14000 rpm for 5 min. The organic layers were collected and subjected to SFC analysis to calculate percent conversion, TON, and enantiomeric ratio.
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imidazole porphyrin Fe ylide complex. DFT calculations were modeled (PCM) (diethyl ether) was used to mimic the dielectric permittivity in the enzyme active site. Coordinates of the optimized HtCc552 Figure 3A; porphyrin ring names can be found in Figure 4). To take into account these degrees of freedom, the Rosetta FastRelax mover was applied to the HtCc552 crystal structure aligned with DFT-generated Fe ylide models to make amino acid substitutions and refine the protein structures. The Rosetta score function used in modeling was REF2015 84 with the two additional score terms “fa_intra_atr_nonprotein” and “fa_intra_rep_nonprotein” to strengthen the intramolecular Lennard–Jones interactions in the Fe ylide intermediate. Coordinate constraints were applied to the protein backbone atoms to prevent large deviations of atomic positions from the crystal structure. Constraints and other Rosetta input files can be found in the Supporting Information. We ran 50 simulation trajectories for each complex, and the values reported in Table 2 are the minimum energy values with the coordinate constraint score subtracted from the total energy score over all 50 trajectories.

**ASSOCIATED CONTENT**

Supporting Information

Supporting Information includes supplementary The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c10750.

Accession Codes

CCDC 2113422 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

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**Notes**

Additional tables and figures as described in the text, chiral GC and SFC chromatograms, synthetic procedures, compound characterization data, NMR spectra, and atom coordinates of the DFT models (PDF)

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