An Enzymatic Platform for Primary Amination of 1-Aryl-2-alkyl Alkynes

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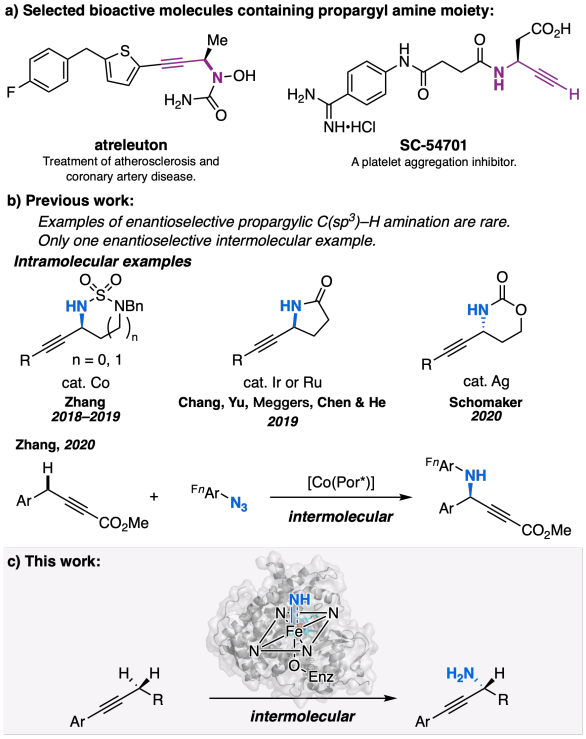
ABSTRACT: Propargyl amines are versatile synthetic intermediates with numerous applications in the pharmaceutical industry. An attractive strategy for efficient preparation of these compounds is nitrene propargylic C(sp^3)–H insertion. However, achieving this reaction with good chemo-, regio-, and enantioselective control has proven to be challenging. Here, we report an enzymatic platform for the enantioselective propargylic amination of alkynes using a hydroxylamine derivative as the nitrene precursor. Cytochrome P450 variant **PA-G8** catalyzing this transformation was identified after eight rounds of directed evolution. A variety of 1-aryl-2-alkyl alkynes are accepted by **PA-G8**, including those bearing heteroaromatic rings. This biocatalytic process is efficient and selective (up to 2610 total turnover number (TTN) and 96% *ee*) and can be performed on preparative scale.

Propargyl amines are an important class of molecules in organic synthesis and medicinal chemistry.[1–3] This motif is widely observed in many bioactive molecules,[2,3] such as drugs and their analogs (Scheme 1a). The functional group combination of an alkyne and an amine also makes them versatile synthetic intermediates toward complex structures.[1,4] Popular methods to prepare propargyl amines include propargylic substitution with nitrogen nucleophiles, reductive amination of alkynyl ketones, and imine alkynylation.[1,5] While efficient, these methods require the use of hazardous organometallic reagents or prefunctionalization of the alkyne substrates at the propargylic positions. Direct nitrene C–H insertion reactions represent an attractive alternative strategy to make propargyl amines from simple alkynes;[6] however, selective C–H amination of these substrates is extremely challenging. On the one hand, the highly reactive nitrene species are usually undiscriminating toward different functionalities (e.g., the triple bonds of alkynes are known to react with nitrenes[7]) and C–H bonds, leading to low chemo- and regioselectivity and uncontrolled side reactions. On the other hand, the relatively small steric effect imposed by the alkynyl group can be a problem for enantiotopic differentiation in small-molecule catalysis.[6e,6f] Nonetheless, several catalytic systems based on cobalt,[6c,6d] iridium,[6e,6h] ruthenium[6f,6g] or silver[6i] have been developed for asymmetric intramolecular propargylic amination reactions since 2018 (Scheme 1b). To the best of our knowledge, however, there is only one example of an enantioselective intermolecular propargylic C(sp^3)–H amination in the literature, which used perfluoroaryl azide as the nitrene precursor.[8]

Since 2013, biocatalytic nitrene transformations have emerged as powerful tools for the construction of nitrogen-containing molecules. Several research groups have demonstrated that enzymatic nitrene C–H insertion reactions can be achieved with excellent efficiency and selectivity on various substrates.[9] In 2020, we reported an unprecedented primary amination reaction of benzylic and allylic C–H bonds using P411 enzymes (cytochromes P450 with serine in place of cysteine as their axial ligand).[10] The remarkable ability of P411 enzymes to tame highly reactive unprotected nitrene species while also allowing exquisite stereocontrol demonstrates their catalytic potential for other challenging transformations. We therefore hypothesized that P411 enzymes could be suitable catalysts for propargylic amination. In this work, we show that an engineered P411 variant, namely **PA-G8**, derived from cytochrome $P450_{BM3}$ and improved by directed evolution, catalyzes an intermolecular propargylic amination reaction (Scheme 1c) with excellent enantioselectivity and catalytic efficiency (up to 79% yield, >2000 TTN, and 82–96% *ee*).

We commenced this investigation of enzymatic propargylic amination by focusing on the reaction between 1-phenyl-butyne **1a** and *O*-pivaloylhydroxylamine triflic acid[11] **2**. The hydroxylamine-based nitrene precursor is more stable and safer to handle compared to the azide precursors used in our previous work. The expected primary amine product **3a** is of biological interest[12] and can be converted easily to other valuable compounds through downstream transformations.[1] The reaction was first evaluated with a panel of hemoproteins, previously evolved for different nitrene and carbene transformations,[13] in the form of whole *Escherichia coli* (*E. coli*) cell catalysts. Although most variants did not show

Scheme 1. Background and Project Synopsis



detectable activity toward this reaction, a few of the P411 enzymes from the allylic primary amination lineage indeed showed promising initial activities.[10] The best hit (P411-b4) among them could catalyze the standard reaction with 64% yield, albeit with negligible enantioselectivity (–1% *ee*). The heme domain of P411-b4 has four mutations (A78M, L263M, G268P, and L437G) with respect to our previously reported "**E10**" variant of $P450_{BM3}$ for benzylic C–H tosylamidation, which has a solved crystal structure (PDB ID: 5UCW).[9b]

Variant P411-b4 (named **PA-G0** in this new lineage) was chosen as the parent for a directed evolution campaign (Figure 1a). Site-saturation mutagenesis (SSM) libraries were generated and screened by revisiting beneficial sites for previous P411-catalyzed transformations (see Table S1 and S2 in *Supporting Information* (SI) for all the targeted sites in directed evolution). To reduce the screening burden in SSM rounds, only variants which displayed more than 60% activity compared to the parent were assayed for enantioselectivity. Gratifyingly, by introducing a single mutation E267D, we obtained a new variant **PA-G1**, which could catalyze the model reaction with nearly quantitative yield and 28% *ee*. Seven additional rounds of SSM and screening generated the final variant **PA-G8**, which could perform propargylic amination in excellent yield and enantiocontrol (79% analytical yield and 88% *ee*). Mutations E267D, G437Q, and S438G, which reside on the flexible loop and the α-helix directly above the heme cofactor (Figure 1b), provide the most significant enantioselectivity boosts. All the mutations (E267D, N395C, G437Q, S72T, S438G, T269V, H266S, and A74K) were selected for enhancing enantioselectivity, even though some of them reduce the reaction yield (N395C, G437Q, S438G, and H266S).

After identifying the best variant **PA-G8** for propargylic amination of the model substrate **1a**, we next evaluated the enzyme's activity on an array of alkyne substrates (**1b–1n**) under the standard whole-cell reaction conditions (Figure 2a). In general, **PA-G8** accepts many 1-aryl-2-alkyl alkynes, delivering the desired products with good to excellent enantioselectivity (82–96% *ee*). Alkynes bearing longer alkyl chains could be transformed to the corresponding propargyl amines by **PA-G8**, albeit in lower yields (**3b** and **3c**). The absolute configurations of **3b** and **3c** were determined as *R* (see section VIII in the SI for details). Interestingly, increased chain length correlated with improved enantioselectivities (91% and 96% *ee* for **3b** and **3c**, respectively). Furthermore, aryl groups with various substitution patterns were well tolerated in this reaction, including substituents with different electronic properties at *ortho*-, *meta*-, and *para*-positions of the aromatic ring (**3d–3i**). Alkynes containing heteroaromatic rings were competent substrates as well (**3j–3l**). This is notable since many transition-metal-based catalytic systems are not compatible with heterocycles, especially pyridines, due to their tendency to deactivate transition-metal catalysts.[14] Besides nitrene insertion into secondary C(sp^3)–H bonds, **PA-G8** could also catalyze the amination reaction of primary and tertiary C–H bonds (**3m** and **3n**). Although the reaction yields were relatively low for some of the alkynes,[15] we believe they can be improved through further optimization of these "propargylic aminases" (see Scheme S1 in SI for some preliminary efforts). We also tested **PA-G8** with dialkyl alkynes, for example, 3-hexyne. Unfortunately, only trace yields were obtained with such substrates (see Figure S2 in SI for details).

To demonstrate the synthetic utility of this biocatalytic platform, we performed the enzymatic reactions at 1-mmol scale with alkyne substrates **1a** and **1d** (Figure 2b). To our delight, propargyl amine **3a** was obtained in 64% isolated yield, 1150 TTN, and 88% *ee*. Similarly, product **3d** was obtained in 64% yield, 1570 TTN, and 91% *ee*. After benzoyl protection, the optical rotation of amide **4a** was measured as +49.0 (c = 0.83, in $CHCl_3$), which is consistent with the reported value of (*R*)-**4a**, thus establishing the absolute configuration of **3a**.[5b]

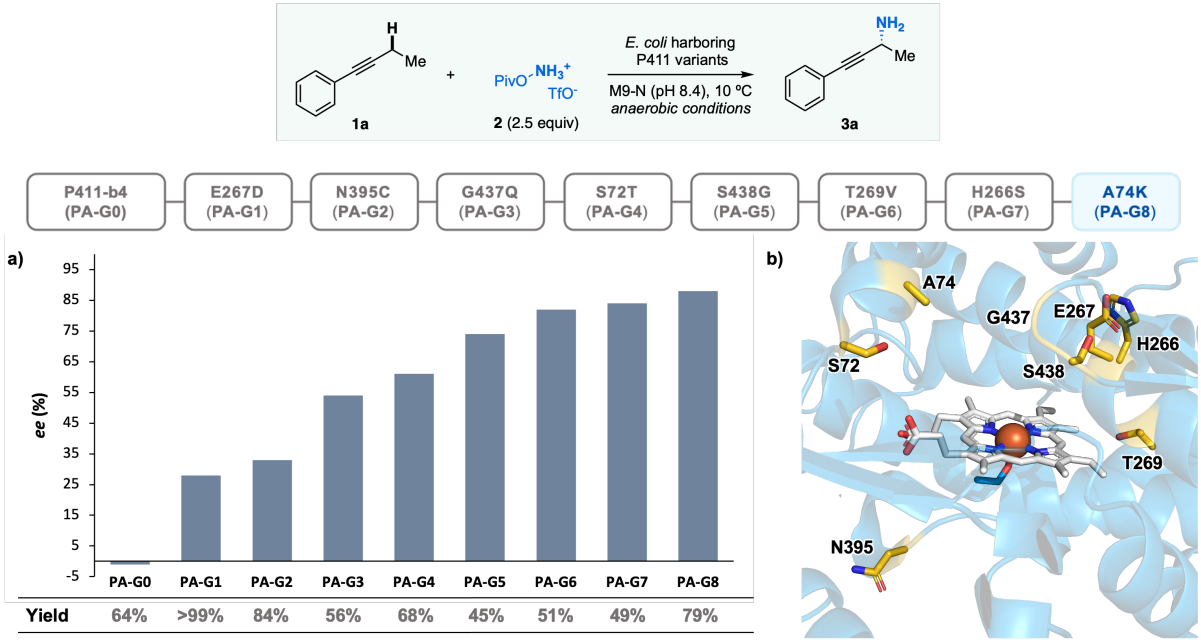


Figure 1. Directed evolution for enantioselective propargylic amination. a) Evolutionary trajectory of **PA-G8** for the synthesis of propargyl amine **3a**. **PA-G8** was evolved through eight rounds of SSM and screening starting from **PA-G0**. Indicated mutations are relative to **PA-G0**. The experiments were performed using *E. coli* (OD_{600} = 20) that expressed P411 enzymes with 2 mM substrate **1a** and 5 mM substrate **2** in M9-N buffer (pH = 8.4) at 10 °C under anaerobic conditions. Performing reactions at 10 °C was found to give higher yields compared to running them at room temperature. Yields were quantified by LC-MS based on the calibration curve of **3a**. Enantioselectivities were measured by HPLC on a chiral phase after benzoyl protection. See SI for details. b) The mutated residues (S72, A74, H266, E267, T269, N395, G437, and S438) are highlighted in the active site of P411 variant **E10** (PDB ID: 5UCW).

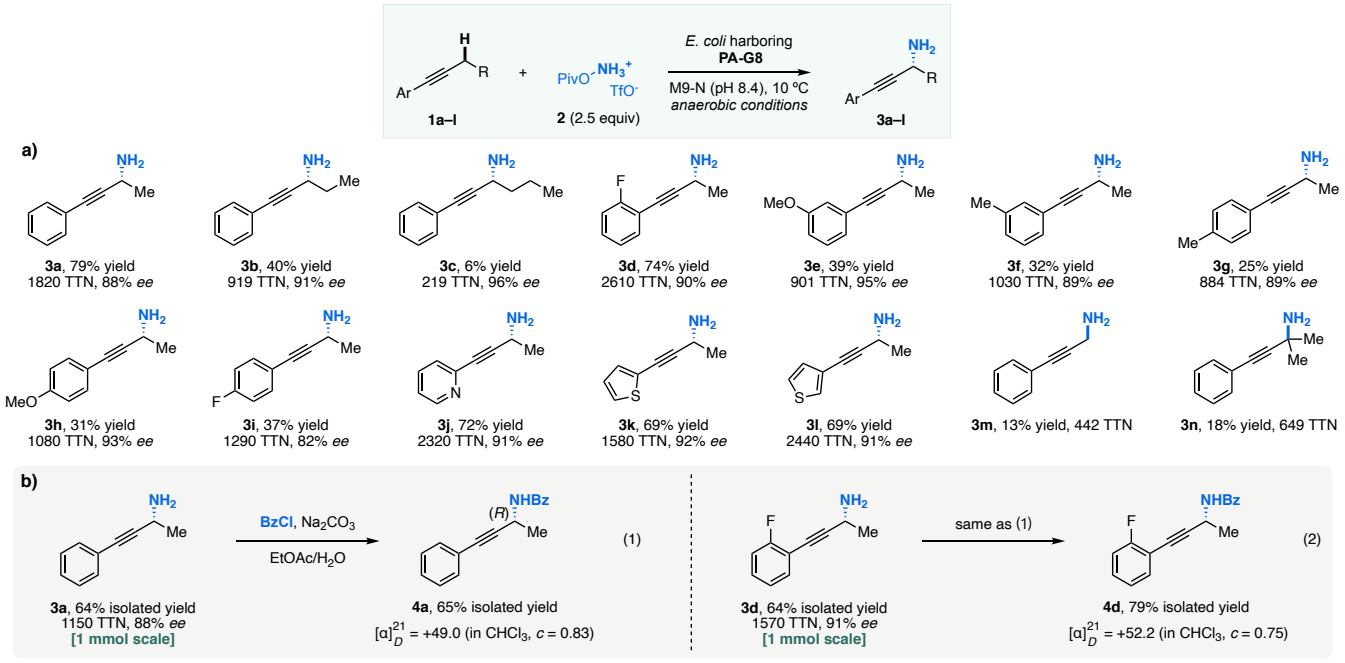


Figure 2. a) Substrate scope of enantioselective propargylic amination. The experiments were performed at analytical scale using *E. coli* (OD_{600} = 20) that expressed the **PA-G8** enzyme with 2 mM substrate (**1a–l**) and 5 mM substrate **2** in M9-N buffer (pH = 8.4) at 10 °C under anaerobic conditions. Yields were quantified by LC-MS based on the calibration curves of the corresponding reference products. Enantioselectivities were measured by HPLC on a chiral phase after benzoyl protection of the propargyl amine products. See SI for details. b) Preparative-scale synthesis and product derivatization for the determination of absolute stereochemistry. Chiral amides **4a** and **4d** were obtained from benzoyl protection of amines **3a** and **3d**. Compound **4a** was used to determine the absolute stereochemistry by comparing the optical rotation with the reported value of (*R*)-**4a**.[5b]

Finally, density functional theory (DFT) calculations with a truncated system were carried out to explore the nature of the nitrene insertion into propargylic C(sp^3)–H bond using model substrate **1a** (see SI for computational details). DFT calculations describe the iron-nitrenoid active species as a triplet ground state, in line with similar reported iron-nitrenoid species.[16] Calculations indicate that nitrene insertion proceeds through a hydrogen atom transfer (HAT) step (TS1 $\Delta G^{\ddagger}$ = 14.7 kcal·mol^{-1}, in the triplet state) to initially form a propargyl radical intermediate, which then undergoes a radical rebound step (TS2 $\Delta G^{\ddagger}$ = 8.2 kcal·mol^{-1}, in the triplet state) generating the final propargyl amine product (Figure 3, and Figure S3 in the SI). This is similar to what was previously described for inter- and intramolecular nitrene C(sp^3)–H insertion based on sulfonamide and sulfamide Fe-nitrenoids,[16a,17] and acyl Fe-nitrenoids.[16b]

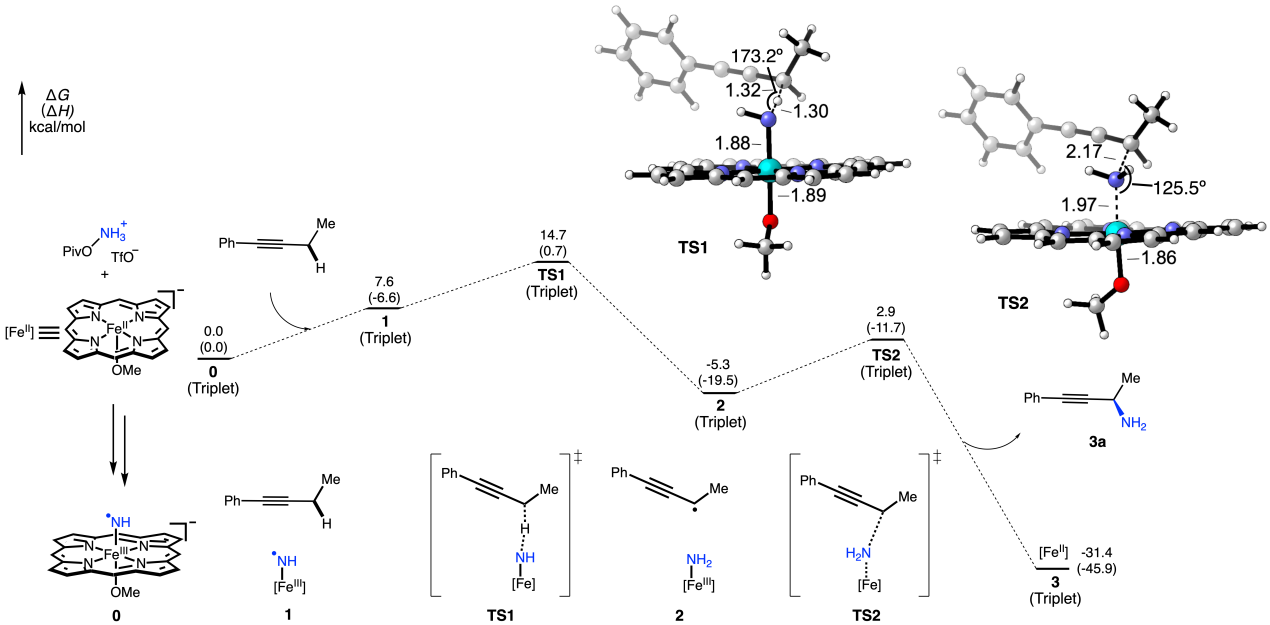


Figure 3. DFT computed free energy profile for the iron(II)-porphyrin catalyzed propargylic C(sp^3)–H amination using a truncated computational model and substrate **1a**. Gibbs free energies are obtained at the B3LYP-D3(BJ)/Def2TZVP/SMD(ε=4)//B3LYP-D3(BJ)/6-31G(d)-SDD(Fe) level of theory, and are given in kcal·mol^{-1}. Key distances and angles are given in Å and degrees, respectively.

The low energy barrier found for HAT TS1 ($\Delta G^{\ddagger}$ = 7.1 kcal·mol^{-1} from reactant complex, triplet state) reflects the enhanced stability of the propargyl radical being formed due to resonance and the high reactivity of the iron-nitrenoid. Higher activation barriers are found for the HAT step involving benzylic C(sp^3)–H bonds and Fe-acyl-nitrenoids.[16b] DFT calculations also describe that protonation of the axial serine would slightly increase the HAT barrier, favoring the quintet electronic state over the triplet for the HAT and radical rebound steps (see SI discussion and Figures S3 and S4).

The radical rebound step is calculated to be much faster than that for the previously reported enantioconvergent amination of tertiary C(sp^3)–H bond (ca. 10 kcal·mol^{-1} higher).[16a] The rapid capture of the propargyl radical suggests that the rebound process outcompetes radical reorientation and alkyl group rotation in the active site due to steric constraints. Consequently, the substrate (**1a**) and propargyl radical have similar orientations in the active site. Enantioselectivity of the amination process would be determined by the orientation of the propargyl radical during the rebound step. In effect, the selectivity of the amination process would be controlled by the binding pose of the substrate in the **PA-G8** active site relative to the reactive iron-nitrenoid species. This is consistent with the improved enantioselectivities observed for substrates with longer alkyl chains (**3b** and **3c**, Figure 2), which will be geometrically more constrained in the binding pocket.

In summary, we established a biocatalytic platform for highly enantioselective nitrene propargylic C–H insertion reactions to furnish a set of synthetically useful and biologically relevant propargyl amine products. This work represents the first example of an intermolecular propargylic C–H amination reaction via a putative unsubstituted nitrene intermediate. Through eight iterative rounds of directed evolution, we identified a P411 variant **PA-G8** which could catalyze the desired reaction in good efficiency and selectivity (up to 79% yield, >2000 TTN, and 96% *ee*). In addition, **PA-G8** could accept an array of different substituted alkyne substrates. DFT calculations provided mechanistic insights into the nitrene insertion, suggesting a key role of the enzyme active site in controlling the selectivity of the process by precisely positioning the substrate with respect to the Fe-nitrenoid species. Future work will be focused on engineering "propargylic aminases" for a broader scope of substrates, such as dialkyl alkynes, and investigating the origins of stereoselectivity based on the new mutations introduced during the laboratory evolution. Further characterization of the nitrene intermediate would be valuable for generalizing this process as well. We envision that this enzymatic primary amination system can be applied to the preparation of bioactive chiral amines for synthetic chemistry and drug discovery.

ASSOCIATED CONTENT

Supporting Information

Materials, experimental methods, compound characterization data, computational methods, and supplementary data. These materials are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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