BIOCATALYSIS

Site-selective enzymatic C–H amidation for synthesis of diverse lactams

Inha Cho*, **Zhi-Jun Jia***, **Frances H. Arnold**†

A major challenge in carbon–hydrogen (C–H) bond functionalization is to have the catalyst control precisely where a reaction takes place. In this study, we report engineered cytochrome P450 enzymes that perform unprecedented enantioselective C–H amidation reactions and control the site selectivity to divergently construct β-, γ-, and δ-lactams, completely overruling the inherent reactivities of the C–H bonds. The enzymes, expressed in *Escherichia coli* cells, accomplish this abiological carbon–nitrogen bond formation via reactive iron-bound carbonyl nitrenes generated from nature-inspired acyl-protected hydroxamate precursors. This transformation is exceptionally efficient (up to 1,020,000 total turnovers) and selective (up to 25:1 regioselectivity and 97%, please refer to compound 2v enantiomeric excess), and can be performed easily on preparative scale.

Functionalization of unactivated carbon–hydrogen (C–H) bonds represents a sought-after strategy to construct and diversify molecules, markedly reshaping the logic of synthetic chemistry (*1*–*5*). Because organic molecules often contain multiple, similar C–H bonds, a major challenge is to control the site at which a reaction takes place. Current approaches to targeting specific C–H bonds rely mainly on substrate control, wherein the substrate guides the catalyst with directing groups (*6*, *7*) or bears one C–H bond that is inherently more reactive (*8*–*12*). The former necessitates extra synthetic steps to manipulate directing groups, and both approaches limit the breadth of potential substrates. An appealing approach is to use a modular catalyst platform that could be tuned to deliver different site selectivities, thereby enabling regiodivergent C–H functionalization of a broad range of substrates (*13*–*16*).

Enzymes, the catalytic machinery of the biological world, exert exquisite control over selectivity in biochemical transformations (*17*). This is exemplified by cytochrome P450 enzymes, which can hydroxylate a specific C–H bond within a complex molecule (*18*, *19*). Furthermore, this site selectivity can be tuned by protein engineering techniques such as directed evolution (*20*–*22*). In the past few years, our group and others expanded the boundaries of biocatalytic C–H functionalization by repurposing native cytochrome P450s to perform carbene and nitrene transfer reactions (*23*–*25*). These enzymes have not yet been engineered to control the site selectivity of the nonnatural reactions in a manner comparable to what has been achieved with P450-catalyzed hydroxylation.

C–H insertion of sulfonyl nitrenes can be catalyzed by variants of cytochrome $P450_{BM3}$ having the axial heme-ligating cysteine amino acid residue substituted with serine (so-called cytochrome P411s) (*26*, *27*). As analogs of sulfonyl nitrenes, carbonyl nitrenes are synthetically more useful owing to their ease of manipulation and wide applicability of the resulting amide products. However, carbonyl nitrenes are difficult to use for C–H functionalization due to their facile decomposition to isocyanates through a well-known Curtius-type rearrangement (*28*). Chang and co-workers reported that an iridium-based catalytic system could harness carbonyl

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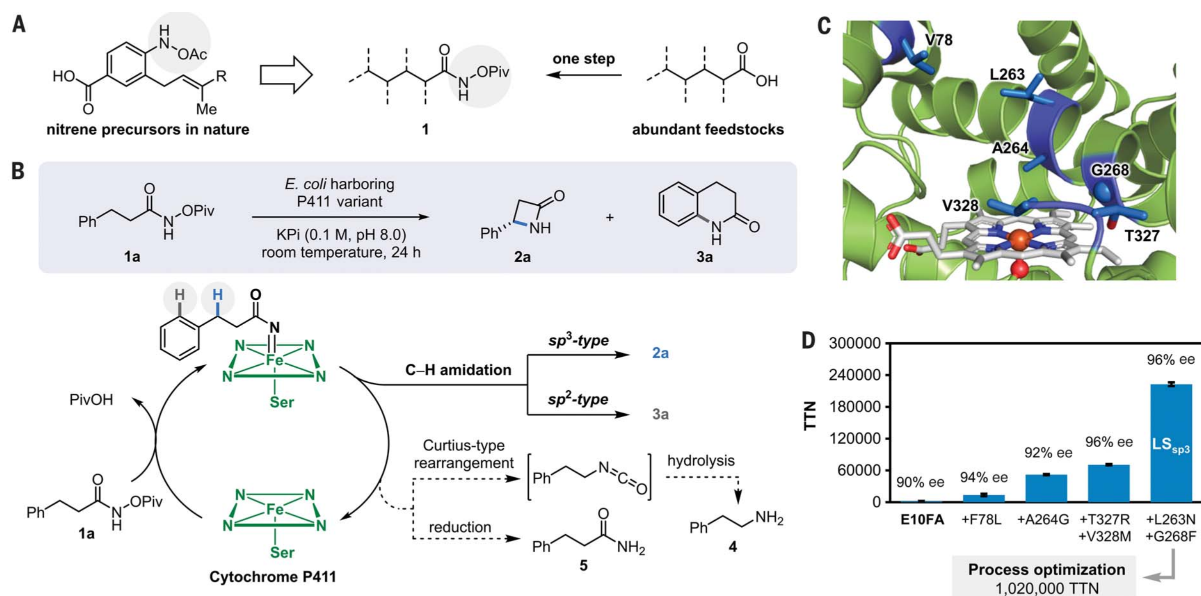

Fig. 1. Hemeprotein-catalyzed intramolecular C–H amidation. (**A**) Design of acyl-protected hydroxamate **1** as nitrene precursor, inspired by naturally occurring nitrene precursors in the biosynthesis of benzastatins (*30*) (Ac, acetyl; Piv, pivaloyl). (**B**) Reaction scheme and proposed catalytic cycle of intramolecular C–H amidation catalyzed by the P411 variant. (**C**) Crystal structure of a variant closely related to P411 **E10** (Protein Data Bank ID: 5UCW) (*27*), with mutated residues marked in blue. (**D**) Directed evolution of E10FA to LS_{sp3} for the synthesis of β-lactam **2a** and further improvement of TTN by process optimization. Experiments were typically performed at analytical scale using suspensions of *E. coli* cells expressing E10FA variants [optical density at 600 nm (OD_{600}) = 2.5] in KPi (0.1 M, pH 8.0) buffer, 50 mM substrate **1a**, 5 volume % organic cosolvents, and 400 μl of reaction volume at room temperature under anaerobic conditions for 36 hours. See supplementary materials for further details. TTN is the total desired product as quantified by comparison to standards by gas chromatography or HPLC, divided by total cytochrome P411. Single-letter amino acid abbreviations (here and in Fig. 3): A, Ala; E, Glu; F, Phe; G, Gly; L, Leu; M, Met; N, Asn; P, Pro; R, Arg; T, Thr; V, Val; Y, Tyr.

nitrenes for intramolecular C–H amidation; however, these catalysts were not enantioselective and formed only the thermodynamically favored products (*29*). We hypothesized that cytochrome P450s and other hemeproteins could be engineered to generate reactive carbonyl nitrenes, direct them to C–H amidation over competing decomposition pathways, and precisely control the enantioselectivity and site selectivity of the reaction.

Rather than using the carbonyl analogs of the sulfonyl azides to generate enzyme-bound nitrenes, we sought a more easily accessible and biocompatible nitrene precursor. Tsutsumi and co-workers identified a naturally occurring nitrene-transfer reaction catalyzed by a cytochrome P450 enzyme in which the nitrene is generated from an acyl-protected hydroxylamine (*30*). We reasoned that acyl-protected hydroxamates **1**, which are prepared from carboxylic acids in one step (Fig. 1A), could act as nitrene sources in enzyme-catalyzed reactions (*31*–*33*).

Motivated by the extensive pharmaceutical applications of β-lactams (*34*), we challenged hemeproteins to construct these strained, four-membered rings through intramolecular C–H amidation (*35*–*37*). We chose as the model substrate hydroxamate **1a** with two sets of reactive C–H bonds, potentially affording β-lactam **2a** through C(sp^3)–H amidation or δ-lactam **3a** through C(sp^2)–H amidation (Fig. 1B). We first tested whether free heme could catalyze C–H amidation in biocompatible conditions and observed alkyl amine **4** as the sole product, presumably from Curtius-type rearrangement of the carbonyl nitrene followed by hydrolysis (table S1). We then tested a panel of hemeprotein variants, including P450s, P411s, cytochromes c, and globins, under anaerobic conditions. Most evaluated hemeproteins, including the P411 variants previously engineered for C–H insertion of sulfonyl nitrenes (*26*, *27*), only generated amide **5,** presumably through reduction of the carbonyl nitrene intermediate. A few hemoproteins of various lineages, however, produced small amounts of the lactam products (tables S1 and S2). The P411 variant **E10**-V78F S438A (E10FA), originally engineered to catalyze carbene transfer to alkynes (*38*), was able to transform substrate **1a** to the desired β-lactam (**2a**), giving only 1.5% yield but 90% enantiomeric excess (ee). The δ-lactam **3a** was also detected, with 1.0% yield. In stark contrast to the reaction catalyzed by free heme, amine **4** was not observed in the reaction using this hemeprotein, supporting our hypothesis that an enzyme could divert highly unstable carbonyl nitrenes to the desired C–H amidation and bypass the Curtius-type rearrangement.

We chose E10FA as the parent for directed evolution of lactam synthases (LS). To improve catalytic activity as well as site- and enantioselectivity of C–H amidation for the synthesis of β-lactam **2a**, we targeted amino acid residues located near the heme iron in a closely related crystal structure (P411 **E10**) for sequential rounds of site-saturation mutagenesis and screening (Fig. 1C). In each round, the libraries of enzymes were expressed and screened as whole-cell catalysts in 96-well plates for increased product formation and/or enantioselectivity. Two rounds of site-saturation mutagenesis and screening introduced the mutations F78L and A264G, leading to a 28-fold improvement in total turnover number (TTN) to 52,000 for β-lactam **2a** while almost eliminating formation of δ-lactam **3a** (Fig. 1D). Double site-saturation mutagenesis and screening subsequently added mutations T327R and V328M, which further increased TTN to 71,000 and ee to 96%. Another round of double site saturation led to the discovery of the final variant, LS_{sp3}, containing mutations L263N and A268F, which affords the desired β-lactam **2a** with 223,000 TTN (96% yield) and 96% ee. Adding both the *E. coli* expressing LS_{sp3} and precursor **1a** portionwise to a reaction mixture boosted the TTN for β-lactam **2a** to 1,020,000 (see supplementary materials for details).

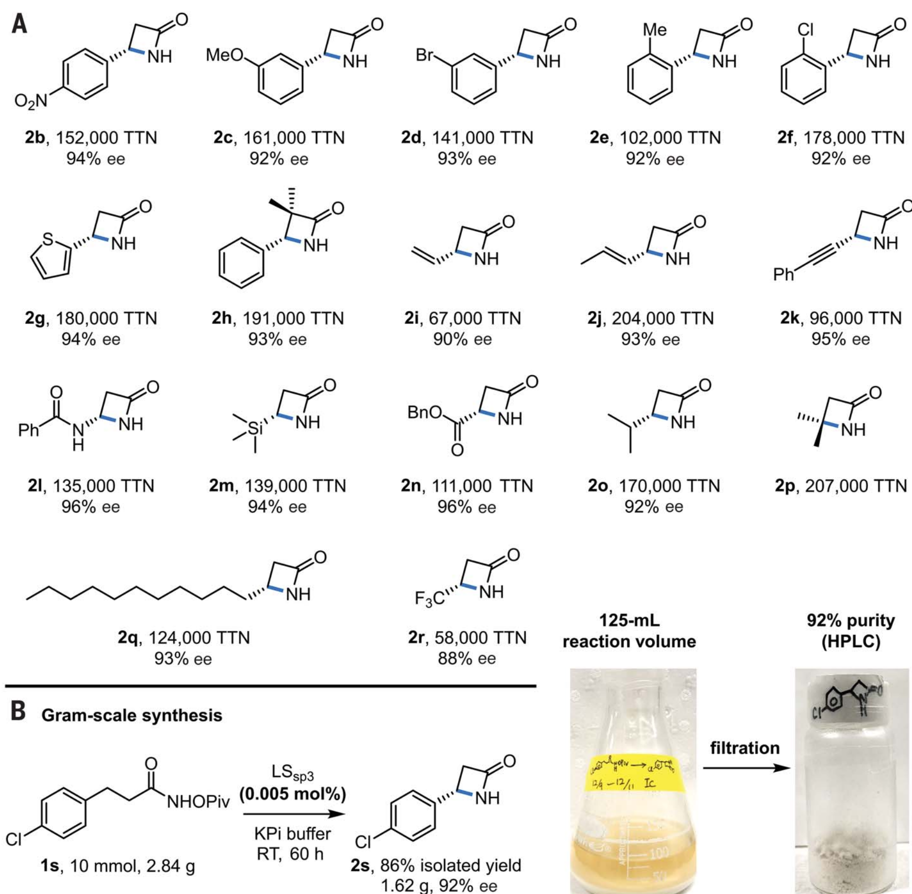

Fig. 2. Scope of β-lactam products. (**A**) Synthesis of β-lactams from corresponding hydroxamate substrates using LS_{sp3}. Experiments were typically performed at analytical scale using LS_{sp3}-expressing *E. coli* cells resuspended to OD_{600} = 2.5 or 3.0 in KPi (0.1 M, pH 8.0) buffer, 25 mM or 50 mM substrate **1**, 5 volume % organic cosolvents, and 400 μL of reaction volume at room temperature under anaerobic conditions for 36 hours. (**B**) Gram-scale synthesis of β-lactam **2s** using cell lysate of *E. coli* expressing LS_{sp3}. See supplementary materials (table S5) for details and more scale-up reactions. In all cases, rr > 20:1.

With variant LS_{sp3} in hand, we evaluated a collection of hydroxamates for enantioselective β-lactam synthesis (Fig. 2A). Various aryl substituents were accepted to furnish β-lactams **2b**–**2g** with excellent TTN (up to 180,000) and ee (up to 94%); only trace amounts of δ-lactams through C(sp^2)–H amidation were detected. Introducing two methyl groups adjacent to the carbonyl group did not hamper the reaction, leading to the formation of product **2h**. Olefin-containing substrates could also undergo enantioselective C–H amidation (**2i, 2j**) with no competing aziridination observed, highlighting the enzyme's complete chemoselectivity. An α-alkynyl C–H bond was also readily amidated (**2k**). Substrates with heteroatom substituents, such as amide and silyl groups, reacted well to give the desired products **2l** and **2m**.

The reactive C–H bonds in all the preceding substrates were weakened by adjacent substituents. This is not required, however, as evidenced by the ester-substituted β-lactam **2n**, for which the turnover was not diminished. Substrates with secondary and tertiary aliphatic C–H bonds were also accepted, leading to products **2o**–**2p**.

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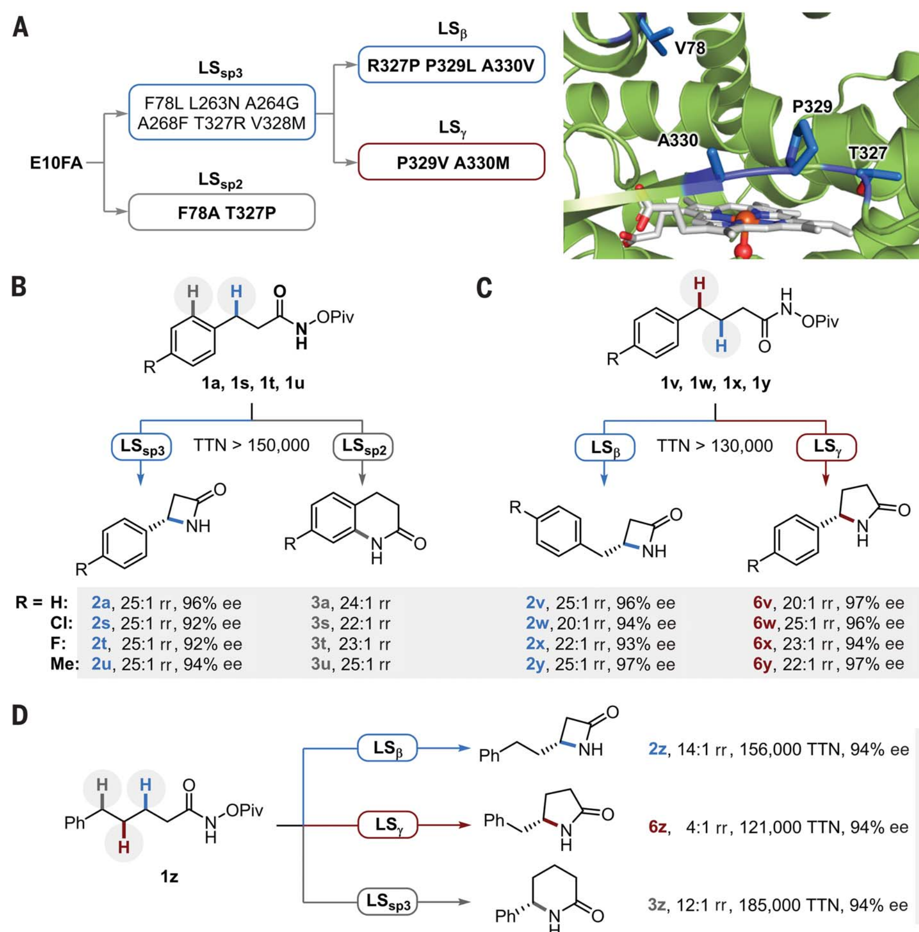

Fig. 3. Engineering lactam synthases for regiodivergent intramolecular C–H amidation. (**A**) Evolutionary trajectory of variants LS$_{sp2}$, LS$_{sp3}$, LS$_{\beta}$, and LS$_{\gamma}$. (**B**) Selectivity and scope of LS$_{sp2}$- and LS$_{sp3}$-catalyzed intramolecular C–H amidation. (**C**) Selectivity and scope of LS$_{\beta}$ and LS$_{\gamma}$. (**D**) Regiodivergent amidation of aliphatic, homobenzylic, and benzylic C(sp^3)–H bonds catalyzed by LS$_{\beta}$, LS$_{\gamma}$, and LS$_{sp3}$. Regioisomeric ratio (rr) indicates the mole ratio of major product to combined minor regioisomers. See supplementary materials (tables S6 to S8) for details.

The site selectivity of the enzyme was highlighted by the formation of product **2q** from a substrate with multiple similar aliphatic C–H bonds. Even aliphatic C–H bonds adjacent to a trifluoromethyl group were amenable to C–H amidation, affording product **2r**. The transformation could be applied to preparative-scale synthesis without sacrificing yield or enantioselectivity (Fig. 2B). Isolation of most products was straightforward: product **2s**, for example, was simply filtered from the aqueous reaction mixture, resulting in 86% yield [1.62 g, 92% high-performance liquid chromatography (HPLC) purity] and 92% ee.

Having achieved β-lactam synthesis by intramolecular C(sp^3)–H amidation, we next investigated whether other types of C–H bonds could be amidated selectively. The parent enzyme E10FA showed trace reactivity toward C(sp^2)–H bonds; only two rounds of site-saturation mutagenesis and screening generated variant LS$_{sp2}$, with mutations F78A and T327P, that exclusively functionalized C(sp^2)–H bonds (Fig. 3A). Instead of forming the corresponding β-lactams (**2a**, **2s**–**2u**), LS$_{sp2}$ diverted the substrates to the corresponding δ-lactams (**3a**, **3s**–**3u**) with comparable TTNs (Fig. 3B).

To further demonstrate the tunability of enzyme-catalyzed C–H amidation, we synthesized precursor **1v** with two sets of C(sp^3)–H bonds, potentially affording β-lactam **2v** or γ-lactam **6v** (Fig. 3C). Variant LS$_{sp3}$ gave β-lactam **2v** as the major product, together with **6v** in a 2.2:1 ratio. Sequential site-saturation mutagenesis and screening delivered variant LS$_{\beta}$ bearing mutations R327P, P329L, and A330V, which produces β-lactam **2v** with a regioisomeric ratio (rr) up to 25:1 without diminishing TTN and ee. As shown in the synthesis of β-lactams **2w**–**2y**, this variant enforces high selectivity for the β-lactam products despite the presence of weaker benzylic C–H bonds and the option to form less-strained γ-lactams. Meanwhile, variant LS$_{\gamma}$ was also revealed, which has mutations P329V A330M and can selectively synthesize γ-lactam **6v** (Fig. 3C). The site selectivity of LS$_{\gamma}$ was largely insensitive to aromatic substitution, and γ-lactams **6w**–**6y** were afforded with up to 25:1 rr, 209,000 TTN, and 97% ee (Fig. 3C).

Finally, we challenged the enzymes with nitrene precursor **1z**, which has three sets of reactive C(sp^3)–H bonds. Variants LS$_{\beta}$, LS$_{\gamma}$, and LS$_{sp3}$ selectively formed β-lactam **2z,** γ-lactam **6z**, and δ-lactam **3z**, respectively. The site selectivity shown in this example further showcases the powerful regiocontrol of the enzymes that is tunable by directed evolution (Fig. 3D).

We have engineered a "lactam synthase" that can be tuned by directed evolution to convert individual substrates into different lactams through a catalyst-controlled C–H amidation process. Reactivity trends due to bond strength, inductive effects, steric accessibility, or ring strain could be completely overturned in this catalyst-controlled process. With these results, we propose that genetically tunable enzymatic catalysis may provide a general strategy to address the challenge of site selectivity in C–H functionalization.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Tables S1 to S8
NMR Spectra
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Site-selective enzymatic C?H amidation for synthesis of diverse lactams

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The right bond in the right place

Enzymes excel at specificity because of their constrained active sites. With appropriate evolutionary pressure, they can be made to differentiate between similar substrates or between positions on a single substrate. Cho *et al.* used directed evolution to generate cytochrome P450 variants that target different C–H bonds in substrates, forming lactam rings of varying size (see the Perspective by Hepworth and Flitsch). The enzyme directs amidation to the desired position and simultaneously prevents other side reactions.

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