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Synthesis of Imidazolidin-4-ones via a Cytochrome P450-Catalyzed Intramolecular C−H Amination

Xinkun Ren,[†,¶] Jack A. O'Hanlon,[‡,¶] Melloney Morris,[§] Jeremy Robertson,[*,‡] and Luet Lok Wong[*,†]

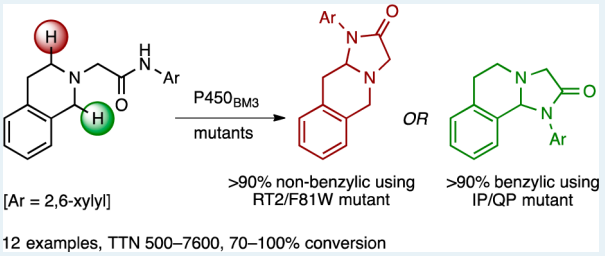
[†]Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, United Kingdom

[‡]Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, United Kingdom

[§]Chemistry Department, 100/79, Syngenta, Jealott's Hill International Research Centre, Bracknell RG42 6EY, United Kingdom

Supporting Information

ABSTRACT: Expanding Nature's catalytic repertoire to include reactions important in synthetic chemistry opens new opportunities for biocatalysis. An intramolecular C−H amination route to imidazolidin-4-ones via α-functionalization of 2-aminoacetamides catalyzed by evolved variants of cytochrome $P450_{BM3}$ (CYP102A1) from *Bacillus megaterium* has been developed. Screening of a library of ca. 100 variants based on four template mutants with enhanced activity for the oxidation of unnatural substrates and preparative scale reactions in vitro and in vivo show that the enzymes give up to 98% isolated yield of cyclization products for diverse substrates. 2-Aminoacetamides with one- and two-ring cyclic amines bearing substituents and aliphatic, alicyclic, and substituted aromatic amides are cyclized. Regiodivergent C−H amination was achieved at benzylic and nonbenzylic positions in a tetrahydroisoquinolinyl substrate by the use of different mutants. This C−H amination reaction offers a scalable route to imidazolidin-4-ones with varied functionalized substituents that may have desirable biological activity.



KEYWORDS: *P450, heme monooxygenases, C−H amination, protein engineering, C−H activation*

INTRODUCTION

Nitrogen heterocycles play vital roles in numerous drugs, driving the search for efficient and selective methods for C−N bond formation. Late-stage cyclization via oxidative amination is an attractive strategic choice in this regard. The two main approaches utilize a variety of oxidants ($KMnO_4$, $Hg(OAc)_2$, $K_3[Fe(CN)_6]$, etc.) to create an electrophilic carbon center that is trapped by nitrogen-based nucleophiles,[1] or an electrophilic nitrenoid intermediate formed by transition-metal catalysts (Rh, Ru, Mn, Co, Fe, etc.) reacts with electron-rich alkenes, heteroatoms, or C−H bonds.[2] These chemical routes require the presence of specific functional groups in the substrate and, conversely, may also be relatively harsh and intolerant of sensitive functionality; although control of enantioselectivity of C−N bond formation is possible, the regioselectivity of these processes is determined by innate chemical reactivity trends in the substrate and is usually not controllable by modifying the reagent.

As an alternative to chemical reagents, enzymes are known that catalyze C−N bond formation. For example, transaminases,[3] ammonia lyases,[4] the nitrating enzyme P450TxtE,[5] and amino acid dehydrogenases[6] target oxidized or chemically activated carbon centers. As developed from Breslow's pioneering studies,[7a,b] engineered P450 enzymes and myoglobin have been shown to activate azido groups in azidoformates and arylsulfonyl azides to effect nitrenoid formation and intramolecular C−H insertion (Scheme 1A).[7c,d] Regioselectivity was achieved in the C−H amination of 2,5-dipropylbenzenesulfonylazide at either the benzylic or homobenzylic position by different P450 mutants, with substrate binding overcoming the preference for insertion into the weaker C−H bond (Scheme 1B).[8]

As part of a larger study on drug metabolism by mutant cytochrome $P450_{BM3}$ (CYP102A1) enzymes, we reported the high yield oxidative cyclization of lidocaine to an imidazolidin-4-one (Scheme 1C).[9] The formation of an imidazolidinone, by N-cyclization, rather than an oxazolidinimine, by O-cyclization, was confirmed spectroscopically and by comparison with a sample prepared by condensation with acetaldehyde.[10] This outcome accords with the instability of oxazolidinimines in aqueous solution and their conversion into the isomeric imidazolidinones by heating in pyridine.[11] In the proposed reaction pathway (Scheme 2), an iminium intermediate is formed by oxidation of an aminyl α-radical (pathway **A**), or via α-hydroxylation of the amine (pathway **B**), and is then trapped

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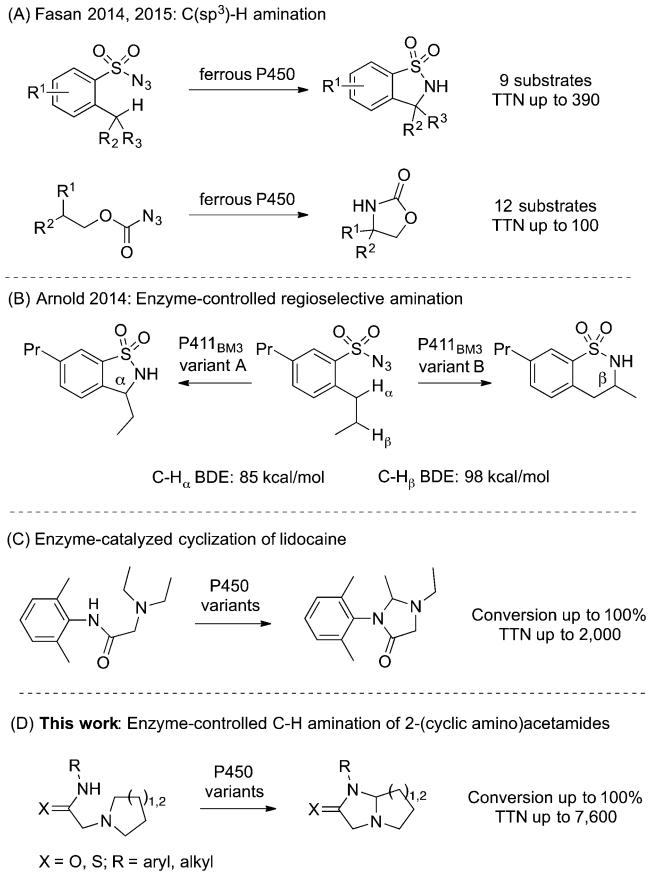
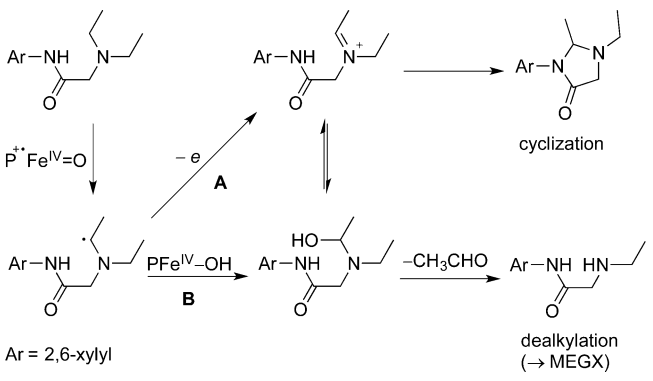
Scheme 1. Enzyme-Catalyzed C−H Amination



Scheme 2. Proposed Mechanism of P450-Catalyzed Amination of Lidocaine



by the amide nitrogen. The overall transformation constitutes an intramolecular amination of an sp^3 C−H bond.

Lidocaine oxidation was screened with a library of ca. 100 variants of $P450_{BM3}$ (Tables S1 and S2). These were based on four mutants, A330P (AP), A191T/N239H/I259 V/A276T/L353I (KT2), I401P (IP), and F87A/H171L/Q307H/N319Y (KSK19), which possess increased oxidation activity for a wide range of organic compounds,[12] and were generated by adding mutations at two or more of the active-site residues Arg47, Tyr51, Ser72, Ala74, Val78, Phe81, Ala82, Phe87, Thr88, Ala184, Leu188, Ala328, Pro329, Ile263, Glu267, and L437, to create a diversity of substrate pocket topology. The partition between lidocaine dealkylation (to MEGX, norlidocaine, Scheme 2) and intramolecular C−H amination is controlled by the mutations; for example, the RP/FV/EV/F81W mutant gave 96% dealkylation, whereas RT2/A330W only showed cyclization activity.[9] Therefore, the $P450_{BM3}$ library appeared to encompass variants with an inherent bias away from the trivial activity of dealkylation and toward C−H amination.

We envisaged the possibility of exploiting these P450 catalysts for engaging 2-aminoacetamides derived from cyclic amines in this intramolecular C−H amination process (Schemes 1D and 3). The construction of the so-formed imidazolidin-4-ones would be of interest as these compounds are 3D templates with polar groups, hydrogen bond acceptors, and potentially donors within the core. Further structural and functional group diversity around these cores may give compounds with varied biological activity. Such diversity covers more chemical space (e.g., for drug discovery) but requires the cyclization catalyst to tolerate varied substituents at both the amine and amide ends of the substrate. Furthermore, the reduced pyrrolo- and pyrido-imidazolone motifs are validated biologically active cores found in the nootropic analgesic dimiracetam and the mannosidase I inhibitor kifunensine, respectively.

EXPERIMENTAL METHODS

General Methods. DNA and microbiological manipulations were carried out by standard methods. Site-directed mutagenesis was carried out by a polymerase chain reaction (PCR)-based method using the KOD Hot Start Polymerase kit from Merck Bioscience, U.K. Heterologous production of $P450_{BM3}$ enzyme variants in *Escherichia coli* BL21 (DE3), and their purification by anion exchange chromatography have been reported previously.[12a,d] Synthesis of the 2-aminoacetamide substrates is reported in the Supporting Information.

Screening of 2-Aminoacetamides for Oxidation by the $P450_{BM3}$ Mutant Library. Screening reactions were carried out in 0.2 M phosphate buffer, pH 7.5, at an assay volume of 0.5 mL in 14 mL glass vials using a NADPH regeneration system. The $P450_{BM3}$ mutant was added as a 10 μM stock in phosphate buffer, pH 7.5 (final concentration = 1 or 2 μM), the substrate as a 100 mM methanol or ethanol stock (final concentration = 1 or 2 mM), glucose dehydrogenase as a 2 units/μL stock in 0.2 M phosphate buffer, pH 7.5 (final concentration =2 units/mL), and glucose as a 1 M stock in 0.2 M phosphate buffer, pH 7.5 (final concentration = 0.1 M). $NADP^+$ monosodium salt was added as a 4 mM stock in 0.2 M phosphate buffer, pH 7.5 (final concentration = 80 μM), to initiate the reaction. The reaction mixtures were shaken at 200 rpm for 16 h at ambient temperature. The aqueous phase was extracted with 300 μL of ethyl acetate after adjusting the pH to 11 with 2 M KOH. The phases were separated by centrifugation at 14 300*g* for 2 min, and the organic phase was analyzed by GC.

Preparative Scale Enzymatic 2-Aminoacetamide Oxidation in Vitro. Preparative scale reactions were performed in phosphate buffer (0.2 M, pH 7.5) in a final volume of 200 mL. In a typical reaction, to phosphate buffer (167 mL) in a 500 mL beaker was added sequentially glucose in phosphate buffer (20 mL of a 1.0 M stock solution, 20 mmol, final glucose concentration = 0.1 M), the $P450_{BM3}$ mutant enzyme in phosphate buffer (5.0 mL of a 40 μM stock solution, 0.2 μmol, final enzyme concentration =1.0 μM), the amide in methanol (4.0 mL of a 0.1 M stock solution, 0.4 mmol, final substrate concentration = 2.0 mM), and glucose dehydrogenase (GDH) in phosphate buffer (200 μL of a 2 unit/μL stock solution, 400

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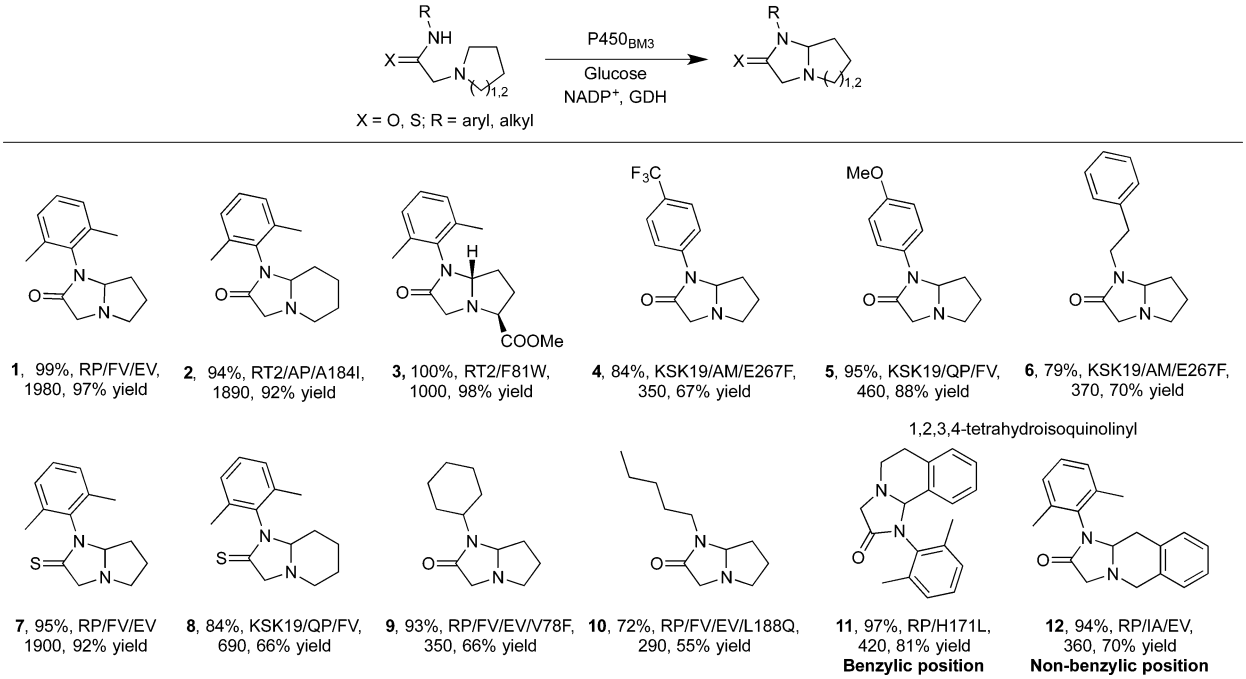
Scheme 3. $P450_{BM3}$-Catalyzed Intramolecular C−H Amination of 2-Aminoacetamides to Imidazolidin-4-ones[a]



[a]The data are given as % substrate conversion, the $P450_{BM3}$ mutant, total turnover number (TTN), isolated yield for reactions in vitro. TTN is the concentration of cyclization product formed per unit enzyme concentration. Where conversion exceeds ca. 95%, the enzyme is capable of further substrate conversion and the actual TTN is higher than the value given, e.g. for **1** under unoptimized conditions, a TTN of 7600 (76% conversion, 10 mM substrate, 1 μM enzyme) was observed.

units, final GDH concentration = 2 units/mL). $NADP^+$ monosodium salt in phosphate buffer (4.0 mL of a 4.0 mM stock solution, 16.0 μmol, final $NADP^+$ concentration = 80 μM) was added to initiate the reaction. The mixture was stirred at 500 rpm at room temperature for 2−6 h. The reaction mixture was extracted with ethyl acetate (3 × 200 mL), the combined organics were dried over $MgSO_4$, filtered, and the solvent was removed by rotary evaporation. The products were purified by silica gel chromatography, eluting with a mixture of petrol and ethyl acetate.

Whole Cell Oxidation of 2-Aminoacetamides. A single colony of *E. coli* BL21 (DE3) harboring the plasmid containing the gene encoding the relevant $P450_{BM3}$ mutant was inoculated into 250 mL LB media containing 34 mg/L kanamycin and grown for 16 h at 37 °C with shaking at 120 rpm. Protein production at 20 °C was induced by adding IPTG to 0.05 mM. After shaking for a further 24 h, 200 mL of culture was taken for the in vivo reaction while 50 mL of culture was used for enzyme quantitation. Cells were harvested from 200 mL of culture by centrifugation at 9250*g* for 5 min at 4 °C and resuspended in 200 mL of *E. coli* minimal media (EMM). The substrate was added to a final concentration of 2.0 mM from a 100 mM methanol stock, along with glucose to a final concentration of 100 mM, from a 1.0 M stock in phosphate buffer. Samples (600 μL) were taken in duplicate every 4 h and centrifuged, after which 500 μL of the supernatant was extracted with 300 μL of ethyl acetate or chloroform for GC analysis of the soluble organics to determine the substrate conversion. More aliquots of substrates could be added, typically to a total of 10 mM. The whole in vivo biotransformation mixture was extracted with ethyl acetate, and the combined organics were dried over $MgSO_4$. Solvent was removed by rotary evaporation after filtration. The crude residue was purified by silica gel column chromatography to isolate the product using the same procedure as for the in vitro reactions.

Computation. For each diastereomer of **3**, conformers were obtained in Spartan 14 following a Monte Carlo search (MMFF). Each conformer was then submitted for an equilibrium geometry calculation (B3LYP/6-31G**) with a water solvation model, and a Boltzmann-weighted G^o was obtained from a thermodynamics calculation.[13] The obtained $\Delta G^o_{298\ K}$ = 2.46 kcal mol^{-1} corresponds to an ∼63:1 ratio of *exo*- to *endo*- diastereomers at equilibrium.

RESULTS AND DISCUSSION

Selected synthetic 2-aminoacetamides bearing cyclic amino moieties were screened in vitro for oxidation by the enzyme library (Scheme 3) using glucose dehydrogenase/glucose to regenerate the NADPH cofactor. At the end of each reaction, the organic-soluble extract was analyzed by gas chromatography. Reactions that showed high substrate conversion and product selectivity were scaled up (50−100 mg) in vitro for product purification and characterization. Pleasingly, 5,5- and 5,6-bicyclic imidazolidin-4-ones were formed from lidocaine-like 2-aminoacetamides with pyrrolidinyl (Scheme 3, entry **1**, Figure S1, Table S3) and piperidinyl (entry **2**, Figure S2, Table S4) groups (all characterization data are in the SI). The 2-aminoacetamides **1** and **2** are challenging substrates. Only the RP/FV/EV (for **1**) and RT2/AP/A184I (for **2**) mutants showed >90% conversion (total turnover number, TTN ∼ 2000) and isolated yields for reactions in vitro, although more mutants had TTN > 300 (0.33 mol % catalyst loading). The reactions were also readily carried out in whole-cells in shake flasks where substrates were added in 2 mM aliquots up to 10 mM total concentration. The results suggest that 2-amino-

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acetamides and imidazolidin-4-ones readily cross the *E. coli* cell wall. Higher product concentrations are likely to be feasible at higher cell densities and more efficient mass transport (e.g., in a bioreactor vessel).

The functional group tolerance of the $P450_{BM3}$ mutants at both the amine and amide ends of 2-aminoacetamides was then explored. Introduction of substituents to the cyclic amine had no adverse effect on turnover activity or chemoselectivity for C−H amination. The methyl-L-prolinate derivative (entry **3**) was converted into >97% of the corresponding cyclization product by, for example, the RT2/F81W mutant (Figure S3, Table S5). Interestingly, the presence of a substituent on the pyrrolidine ring increased the number of mutants showing high conversion and TTN (Table S5). The 1D NOE spectra (Figure S24) showed that **3** was generated solely as the (5*S*,7a*R*)-diastereoisomer by all mutants within the library. Calculations showed that this was the thermodynamically more stable diastereoisomer by 2.46 kcal mol^{-1}, suggesting rapid equilibration of the *N,N*-acetal center likely occurred during the reaction and isolation procedure. This is consistent with the observation that the unsubstituted compounds **1** and **2**, as well as the products from the other aminoacetamides in Scheme 3, were obtained in racemic form.

Electron-withdrawing (entry **4**) and electron-donating substituents (entry **5**) were introduced at the 4-position in place of the 2,6-dimethyl substituents on the phenyl group of 2-pyrrolidinylacetamides. The most selective mutants KSK19/A82M/E267F and KSK19/QP/FV provided the cyclized amination products with 84% and 95% conversion and 67% and 88% isolated yield of **4** and **5**, respectively (Figures S4 and S5). Notably, selectivity for the cyclization product **4** was reduced for some variants (as low as 33% for variants giving >60% conversion, Table S6) but remained high, often >85%, for **5** (Table S7). Reduced nucleophilicity of the amide nitrogen in the trifluoromethyl substrate presumably allows other pathways to compete more effectively with cyclization. Products from these other pathways were not isolated.

The mutant library also oxidized the 2-phenylethylamide derivative with up to 79% conversion and 70% isolated yield for the cyclized product (entry **6**). Selectivity for the cyclization product was higher than for **4** and as high as that for the lidocaine reaction (Figure S6, Table S8), supporting the importance of the nucleophilicity of the amide nitrogen. These results demonstrate that substitutions on the aromatic ring and different amine groups are tolerated by the enzyme. Entries **7** and **8** show that thioamides are also successful substrates for this reaction and the *N,N*-acetals were formed with high conversion (Figures S7 and S8, Tables S9 and S10).

The conversion of aliphatic and alicyclic amides to cyclization products **9** and **10** illustrates that an *N*-aryl substituent at the amide end is not required for 2-aminoacetamide binding or for achieving a binding orientation that facilitates α-amine functionalization and cyclization. For these reactions, high conversions were found, although with some mutants, there was some loss of selectivity for cyclization (Figure S9 and S10, Tables S11 and S12). The RP/FV/EV/V78F mutant converted 93% of the cyclohexyl amine derivative (entry **9**) and gave 66% isolated yield of the cyclization product, whereas the RP/FV/EV/L188Q mutant showed 72% conversion of the pentyl substituted 2-aminoacetamide (entry **10**) to the corresponding *N,N*-acetal in 55% isolated yield.

Cyclization of the tetrahydroisoquinoline (THIQ) derivative (entries **11** and **12**) to a tricyclic compound further highlights the tolerance of the $P450_{BM3}$ mutants for variations in the amine moiety and the ability to control the regioselectivity of C−H amination (Figure S11, Table S13). The RP/H171L mutant showed 91% conversion with 86% regioselectivity for C−N bond formation at the benzylic position (entry **11**), whereas RP/IA/EV showed 94% conversion with 77% selectivity for the nonbenzylic position (entry **12**). Mutants demonstrating near-complete shift between the two regioisomers were found from in vitro screening of the library. The IP/QP mutant showed 95% selectivity for **11** while RP/F81W gave 94% **12** although the conversions under screening conditions were low (~25%). Encouragingly, however, the conversion in both reactions was increased to >95% in whole-cell reactions without loss of chemo- or regioselectivity (IP/QP mutant, TTN = 800 for whole cells vs 110 in vitro for **11**; RP/F81W mutant, TTN = 1720 vs 125 in vitro for **12**), leading to isolated yields of >90% for each product. If an organic compound such as 2-aminoacetamides can cross the *E. coli* cell wall, large increases in total turnover for in vivo reactions over in vitro conditions are possible because the cytoplasmic enzyme is protected from high concentrations of organics and diffusion of products into the external medium reduces product inhibition.

This is the first report of selective direct C−H heterofunctionalization in simple THIQ derivatives. In addition to the many bond-forming processes at the 1-position, a small number of nonselective 1-/3-oxyfunctionalizations are known.[14] Selective C−C bond-forming processes at the nonbenzylic 3-position have been reported recently via two strategies: (1) for carbon substituents capable of supporting a negative charge (malonyl, cyano, nitroalkyl), enrichment of the fraction of 3-derivative can be achieved by thermal equilibration;[15] (2) the 3-C−H bond in *N*-(benzoxazol-2-yl)THIQ is more accessible to bulky Ir(I) catalysts that achieve direct alkylation at that position with terminal alkenes.[16] Both processes require high temperatures and offer limited scope; in contrast, the selective formation of compound **12** occurs under benign reaction conditions.

The activity profile of the mutant library (Table S3 to S13) suggests that the active-site residues F81, A82, F87, I263, E267, and A330 have important effects on 2-aminoacetamide cyclization via C−H amination. However, the base mutants with their enhanced activity for unnatural substrate oxidation are required because the single-site mutants such as F87A, among others, have very low activity. The data showed that the mutants RT2/F81W, RT2/A330P/A184I, RP/F87 V/E267 V, RP/I263A/E267 V, and KSK19/A82M/E267F (see Scheme 3), would have established the possibility of intramolecular C−H amination for all the tested 2-aminoacetamides.

CONCLUSION

In summary, by screening variants of $P450_{BM3}$ for activity on 2-aminoacetamides, we have developed a scalable enzymatic C−H amination process for the straightforward synthesis of bicyclic imidazolidin-4-ones under mild conditions for both 5,5- and 5,6- fused bicyclic systems. Enzymes offer advantages over transition-metal catalysts because they operate under mild conditions in aqueous solvent, are highly active, essentially inexhaustible, and can be evolved to be highly selective for the desired product. The increased conversion for in vivo C−H amination over reactions in vitro adds to the versatility of the system. The enzymes accept aromatic and aliphatic amides as well as substituents on the amine ring that also, by

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thermodynamic stereochemical relay, establish defined stereochemistry at the newly formed ring junction. Cyclization of the tetrahydroisoquinoline derivative (entries **11** and **12**, Scheme 3) to a tricyclic compound further highlights the tolerance of $P450_{BM3}$ mutants for variations in the amine moiety and the possibility of controlled site-selective C−H amination. Imidazolidin-4-ones are latent iminium ions, being amenable to Lewis acid-mediated ring-opening with subsequent nucleophilic attack offering new routes for α-functionalization of tertiary amines.[17] Ongoing engineering studies are directed at identifying $P450_{BM3}$ variants that will hydroxylate the imidazolidin-4-one products in a second step to introduce additional functional diversity and, potentially, biological activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.6b02189.

Materials and methods, substrate synthesis, enzyme activity screening method, in vitro and in vivo substrate conversion methods, oligonucleotides for mutagenesis, list of mutants, tables of substrate conversion and product selectivity for screened mutants, product distribution analysis by GC, NMR and MS data for substrates and products (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail for L.L.W.: luet.wong@chem.ox.ac.uk.

*E-mail for J.R.: jeremy.robertson@chem.ox.ac.uk.

Author Contributions

¶X.R. and J.A.O. contributed equally.

Notes

The authors declare no competing financial interest.

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