Highly Enantioselective Construction of Oxazolidinone Rings via Enzymatic C(sp^3)−H Amination

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ABSTRACT

Oxazolidinones are important heterocycles widely utilized in medicinal chemistry for the synthesis of antifungals, antibacterials, and other bioactive compounds and in organic chemistry as chiral auxiliaries for asymmetric synthesis. Herein, we report a biocatalytic strategy for the synthesis of enantioenriched oxazolidinones through the intramolecular C(sp^3)-H amination of carbamate derivatives using engineered myoglobin-based catalysts. This method is applicable to a diverse range of substrates, with high functional group tolerance, providing enantioenriched oxazolidinones in good yields and with high enantioselectivity. The synthetic utility of this methodology is further highlighted by the development of enantiodivergent biocatalysts for this transformation and through the preparative-scale synthesis of key oxazolidinone intermediates for the production of the cholesterol-lowering drugs Ezetimibe and CJ-15-161. An outer sphere mutation, Y146F, was found to be beneficial to favor the productive C–H amination reaction over an unproductive reductive pathway commonly observed in hemeprotein-catalyzed nitrene transfer reactions. This study demonstrates a biocatalytic, enantiodivergent synthesis of oxazolidinones via C-H amination of carbamate derivatives, offering an attractive strategy for the synthesis of these valuable intermediates for applications in medicinal chemistry, target-directed synthesis, and asymmetric synthesis.

INTRODUCTION

The oxazolidinone framework is an important structural and functional motif found in many biologically active molecules and natural products, including fungicides, antibacterials, and antimicrobial agents (**Figure 1a**, left).[1-4] As exemplified by Evans' chiral auxiliaries, substituted oxazolidinones have also played a pivotal role in the development of asymmetric methodologies in modern organic synthesis (**Figure 1a**, right).[5-8] Indeed, enantiopure oxazolidinones have found widespread use as chiral ligands for enabling a broad spectrum of asymmetric transformations.[9, 10] Oxazolidinones also represent highly valuable synthetic intermediates, serving as precursors to *β*-amino acids and as key building blocks for the synthesis of pharmaceuticals, including FDA-approved drugs Linezolid,[11-13] ezetimibe,[14] CJ-15161,[15] and zolmitriptan.[16]

Given the prominent importance and versatility of oxazolidinone motifs, the stereocontrolled synthesis of enantiopure oxazolidinones has garnered significant attention in the synthetic community.[17] In this context, traditional methods often rely on chiral pool reagents, particularly *β*-amino alcohols, for carbonylation reactions. However, the optically active *β*-amino alcohol precursors are not readily available, and the overall process typically requires toxic phosgene reagents under harsh conditions.[18, 19] Additionally, the formation of key stereocenters in *β*-amino alcohols poses significant challenges, especially when they are not derived from naturally occurring enantiopure amino acids.[20, 21] Metal-catalyzed asymmetric reduction of unsaturated heterocyclic compounds[22-25] such as oxazolones has provided another route to the synthesis of optically active oxazolidinones.[26-28] More recently, halohydrin dehalogenases (HHDHs) have been

investigated as biocatalysts for synthesizing enantiopure 2-oxazolidinones through the epoxide ring-opening reaction with cyanate (**Figure 1b**, left).[29-34]

In this context, the intramolecular C–H amination of carbamates *via* nitrenoid intermediates[35, 36] represents a very attractive and most direct approach for the formation of chiral oxazolidinones.[37-39] In this regard, transition metal-catalyzed enantioselective reactions have been developed recently; however, their enantioselectivity remains generally low to moderate.[40-47] In addition, in the context of drug synthesis, the use of these precious metals entails important safety concerns and downstream processes associated with the need of removing metal contaminants.[48]

Among its growing impact and applications for organic chemistry,[49-55] biocatalysis has emerged as a promising approach for achieving asymmetric C-N bond formation *via* nitrene transfer chemistry.[56-58] In particular, we and the Arnold group have demonstrated that engineered cytochrome P450 enzymes can catalyze intramolecular C–H amination reactions *via* nitrene transfer, producing sultams, cyclic carbamates, and sulfamides,[59-64] whereas a natural P450 with putative nitrene transfer activity was also identified.[65] Artificial metalloenzymes useful for the synthesis of sultams *via* intramolecular C-H amination have also been reported,[66, 67] More recently, an efficient method was developed for the enantioselective synthesis of lactams through myoglobin-catalyzed intramolecular C–H amidation of dioxazolones.[68] Despite these advances, the enantioselective synthesis of oxazolidinones by biocatalytic means has remained elusive. Indeed, while we previously reported the formation of oxazolidinones through P450-catalyzed cyclization of carbonazidates (**Figure 1b**, right),[69] these reactions lacked of stereoselectivity (<5% *ee*), which limited their synthetic utility. Here, we report the development of an efficient biocatalytic strategy involving engineered myoglobin-based catalysts for the asymmetric synthesis of oxazolidinones *via* intramolecular C(sp^3)–H amination of readily accessible carbamate reagents

(**Figure 1c**). This method offers broad substrate scope, high enantioselectivity, and enantiocomplementary selectivity for benzylic C–H amination reactions. In addition, its synthetic utility is further demonstrated through the preparative-scale synthesis of key oxazolidinone building blocks useful for the preparation of drug molecules.

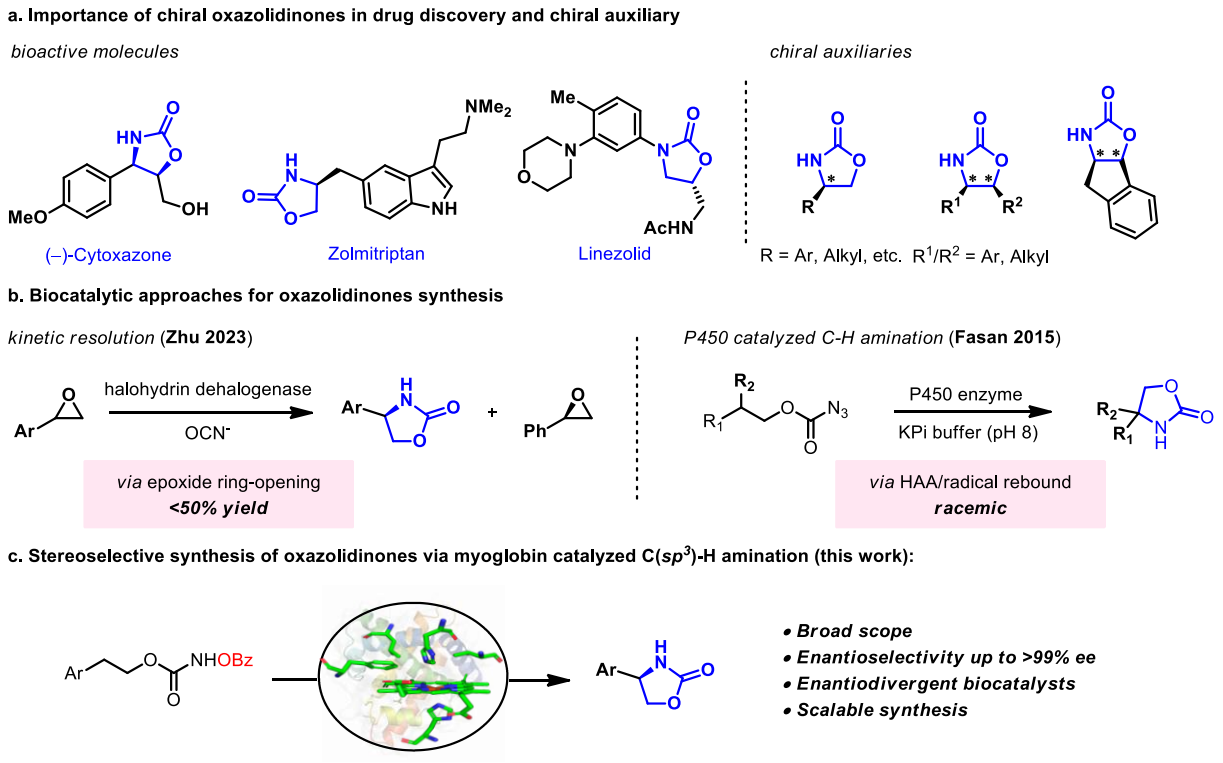

Figure 1. Synthesis and applications of oxazolidinones. (a) Applications of optically active oxazolidinones in medicinal and organic chemistry. (b) Previously reported biocatalytic methods for oxazolidinone synthesis. (c) Enantioselective method for oxazolidinone synthetic *via* myoglobin-catalyzed intramolecular C−H amination of carbamates reported here.

RESULTS AND DISCUSSIONS:

As noted earlier, our previous attempts to develop a biocatalytic strategy for oxazolidinone formation *via* nitrene-mediated intramolecular C–H amination of carbonazidate substrates engineered P450 enzymes were faced with a lack of enantioselectivity along with low catalytic activity.[69] Factors contributing to the modest catalytic efficiency of this system included reduction of the heme-bound amidyl intermediate to give a carbamate byproduct, a side-reaction observed in various other hemoprotein-catalyzed nitrene transfer reactions reported by us and others.[61, 70] This undesired pathway is believed to arise from over-reduction and protonation of the iron-amidyl intermediate, a process favored by the native single-electron/proton transfer mechanism operating in P450s as required for their monooxygenase activity.[64] In addition, this C-H amination reaction was plagued by a competing decarboxylation (or decarbonylation) reaction, resulting in the formation of an alcohol byproduct.[64] Collectively, these side reactions were found to account for 90% of the consumed carbonazidate substrates, limiting the synthetic utility of this methodology. Importantly, and unlike other P450-catalyzed C-H amination reactions,[69] negligible enantioinduction (<5% enantiomeric excess (*ee*)) was observed in these P450-catalyzed transformations, further highlighting the challenges associated with the enzymatic construction of enantioenriched oxazolidinones using this chemistry.

Motivated by our recent progress in asymmetric lactam synthesis *via* myoglobin-catalyzed cyclization of dioxazolones,[68] we envisioned the possibility of exploiting this metalloprotein catalyst for the asymmetric synthesis of oxazolidinone rings *via* the cyclization of carbamate-derived nitrene precursors. Accordingly, a series of phenyl ethyl carbamate substrates, including *N*-hydroxy (**1aa**), *N*-pivaloyl-(**1ab**), *N*-benzoyl (**1ac**), and *N*-tosyl (**1ad**) carbamate derivatives along with carbonazidates (**1ae**) were prepared and tested for reactivity in the presence of

Mb(H64V,V68A) (a.k.a. Mb*) as the catalyst (**Figure 2a**). Compared to azide reagents, which were explored in prior biocatalytic nitrene transfer reactions,[59-64] the non-azide nitrene precursors were chosen because of their more desirable properties in terms of chemical stability and non-explosive nature. On the other hand, Mb* was chosen because of its best performance as biocatalyst previously reported for lactam synthesis from dioxazolones.[68] These experiments showed that all of the reactions gave the desired oxazolidinone product **2a** in low yields but with excellent enantioselectivity (1-7% yield and 99:1 er). Among them, the *N*-benzoyl substrate (**1ac**) emerged as the most promising reagent (7% yield), followed by the *N*-tosyl carbamate substrate (**1ad**) (6% yield). Interestingly, and unlike the reaction with engineered $P450_{BM3}$ variants investigated previously,[69] the carbonazidate substrate **1ae** could be also converted to **2a** in high enantioselectivity, albeit in modest yield using this catalyst. In each case, the low yields of these reactions could be attributed to the formation of the carbamate byproduct **2aa**, resulting from the reduction of the reactive nitrenoid species mediating these transformations.[64]

Based on the promising results with the *N*-benzoyl carbamate (**1ac**) reagent, we extended our screening to an in-house panel of engineered myoglobin variants (~200) targeting the cyclization of **1ac** as the model reaction and using whole cell reactions. This library included a series of Mb*-based variants containing Tyr→Phe substitutions at various Tyr positions near the heme cofactor (i.e., Y103F, Y146F, Y151F), which were designed with the goal of suppressing unproductive electron/proton transfer pathways known to affect the efficiency of hemoprotein-catalyzed C-H amination reactions as mentioned above. Inspired by our previous work in enhancing P450-catalyzed C-H aminations by suppressing their native proton relay pathways,[64] these mutations were selected in view of the well known role of tyrosines in mediating proton-coupled electron transfers (PCET) in proteins,[71-74] including hemoproteins.[74]

From these screening efforts, several variants were identified that exhibit improved yields compared to Mb*, while maintaining excellent enantioselectivity for the formation of the same enantiomeric product (**Figure 2b**), which was determined to have *R*-configuration by comparison with an authentic standard (see SI for details). In addition, Mb variants with *inverted* enantiopreference compared to Mb* (i.e. *R*-selectivity) were also identified within this library. These most promising variants were further characterized to evaluate their activity and enantioselectivity. Among these, Mb(H64V,V68A,Y146F) exhibited the highest activity for formation of the *R*-isomer of **2a**, whereas Mb(F43V, I107E) showed the best performance for formation of the opposite enantiomer with excellent enantioselectivity (99% *ee*; **Figure 2b**). While most mutations were localized in the active site (**Figure 2c**), Mb(H64V,V68A,Y146F) also contains a remote mutation (i.e., Y146F) which proved beneficial for catalytic activity compared to Mb(H64V,V68A) (14% vs. 7% yield).

Following further reaction optimization (SI Figures S3-S6), best results were obtained at slightly alkaline pH (pH 9) and using a cell density (OD_{600}) of 20 in the presence of 5% acetonitrile (MeCN) as a co-solvent. Under these conditions, the Mb(H64V,V68A,Y146F)-catalyzed reaction with **1ac** produces *R*-**2a** in 82% yield and 99:1 er (**Figure 2d**), whereas the reaction of the enantiocomplementary biocatalyst Mb(F43V, I107E) afforded enantiopure *S*-**2a** in 48% yield and 92:8 er. Further experiments showed that the Mb(H64V,V68A,Y146F)-catalyzed C-H amination of **1ac** proceeds equally well (90% yield) in the presence of purified protein under reducing conditions (90% yield with 2.5 mM $Na_2S_2O_4$; **SI Figure S7-S8**). Interestingly, detectable activity (12 TON) was also obtained in the absence of reductant, indicating that the hemoprotein in its ferric state is also catalytically competent (**SI Figure S7-S8**). Under catalyst-limiting conditions, Mb(H64V,V68A,Y146F) was found to support a total turnover number (TTN) of 1,600 with **1ac**,

surpassing by more than two orders of magnitude the performance of the engineered $P450_{BM3}$ variants previously investigated for this transformation (**SI Figure S9**).[69] In addition, the Mb catalyzed reaction provides with high enantioselectivity (99:1 e.r.), whereas racemic products were obtained using the $P450_{BM3}$-based catalyst with both **1ac** and carbonazidate **1ae** (**SI Figure S9**). These results suggest that the Mb active site is more conducive of asymmetric induction in this intramolecular C-H amination reaction compared to the P450 system under investigation. Upon further comparison of the various nitrene precursor reagents **1aa-1ae** under these optimized catalytic conditions, these experiments further highlighted the superiority of N-benzoyl-carbamate **1ac** as the substrate for this reaction, although Mb(H64V,V68A,Y146F) shows good activity and enantioselectivity also toward cyclization of carbonazidate **1ae** (1,300 TON, 99:1 e.r.; **SI Figure S9**). In this case, the higher yield observed with **1ac** vs. **1ae** could be ascribed to more efficient C-H amination over the competing reaction leading to the reduced carbamate side-product (**SI Figure S9**).

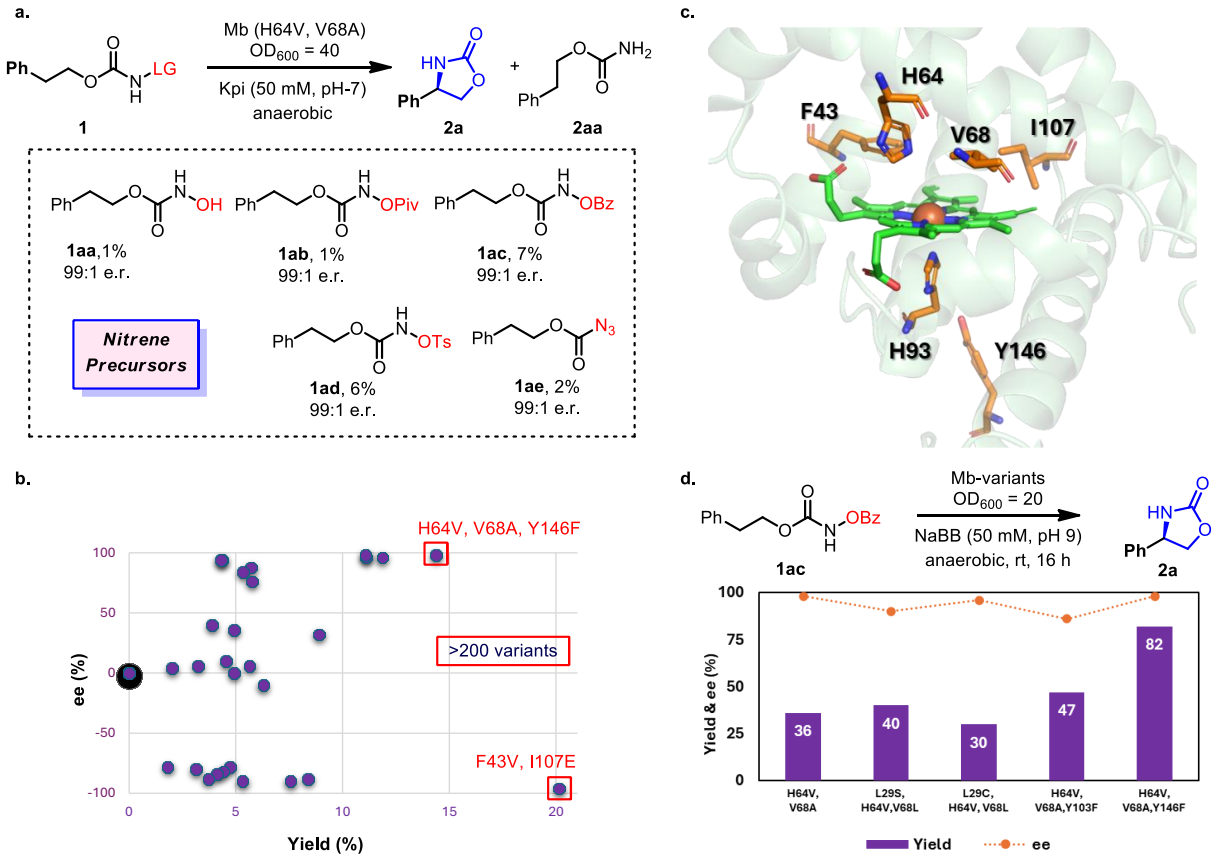

Figure 2. Myoglobin-based biocatalysts for asymmetric intramolecular C–H amination of carbamates. (a) Activity and enantioselectivity of Mb* toward cyclization of **1a-e** under unoptimized reaction conditions. KPi, potassium phosphate; rt, room temperature. LG = leaving group; (b) Yield and enantioselectivity of 200 engineered Mb variants from screening in 96-well plates as whole cells in the presence of **1ac**. The most active and selective variants for formation of either enantiomer of **2a** is highlighted. (c) Crystal structure of sperm whale myoglobin (PDB 1MBI), where the mutated residues in the two enantiocomplementary biocatalysts are highlighted as stick models (orange). (d) Activity and enantioselectivity of the most active Mb variants under optimized reaction conditions: 1 mM **1ac** in sodium borate buffer (NaBB) (50 mM, pH 9), Mb (OD_{600} = 20), 16 h, room temperature, anaerobic conditions. The yields were determined by GC using calibration curves of the isolated product.

Next, we explored the scope of the methodology using a range of carbamate substrates containing a variety of electron-donating and electron-withdrawing groups at the *ortho*, *meta*, and *para* positions of the aryl ring (**Figure 3**). These experiments showed that Mb(H64V,V68A,Y146F) biocatalyst has high tolerance towards *para* substitutions with both electron-donating and electron-withdrawing groups, affording the desired oxazolidinone products **2b-d**, and **2g** in good yields (52-80%) and good to excellent enantiomeric ratios (86:14 to >99:1 e.r.) (**Figure 3**). As an exception, more moderate yield and enantioselectivity was observed for the trifluoromethyl-substituted product **2h**. Substitutions at the *ortho* and *meta* positions were also well tolerated by this enzyme, producing the corresponding oxazolidinone products (**2e** and **2f**) in good yields (65–86%) and excellent enantioselectivity (>99:1 e.r.). More sterically demanding substrates such as the bis-3,4-methoxy-phenyl- derivative **1i** and naphthyl-based substrate **1j** were also converted to the corresponding oxazolidinone products **2i** and **2j** in good yield, albeit with moderate enantioselectivity. Excellent tolerance for heteroaryl group was exemplified by the thiophene-containing product **2k**, which was obtained with good yield (85%) and excellent enantioselectivity (>99:1 e.r.). Gratifyingly, fused ring scaffold like the indene-containing product **2l** was produced in good yield (85%) and with excellent diastereo and enantioselectivity (>99 : 1 d.r. and >99:1 e.r.; **Figure 3**). Importantly, for all of these oxazolidinone products except **2h** and **2l**, the opposite enantiomer could be also obtained by performing the reactions in the presence of the Mb(F43V,I107E) variant as the catalyst. Although the yields of these reactions were generally lower than with Mb(H64V,V68A,Y146F), the large majority of the *N*-benzoyl substrates (10/12) could be cyclized with good to high enantioselectivity (up to 1:99 e.r.), highlighting the broad substrate scope and general enantiocomplementarity of the two Mb-based biocatalysts in this transformation.

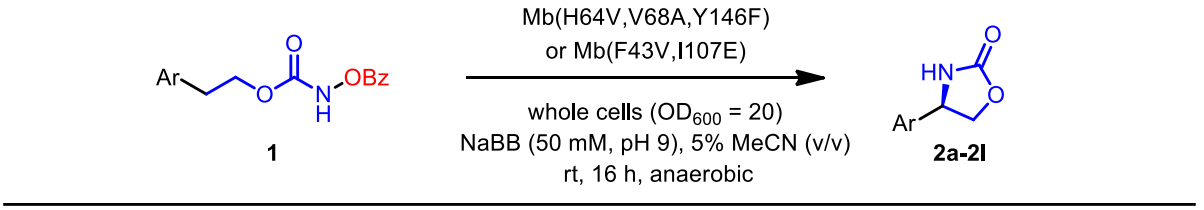

Figure 3 Substrates scope for Mb-catalyzed enantioselective cyclic carbamate synthesis in the presence of the enantiocomplementary biocatalysts Mb(H64V,V68A,Y146F) and Mb(F43V, I107E). Reaction conditions as in **Figure 2d**. Yields were determined by GC using calibration curves prepared with the isolated product. Enantioselectivity was determined by chiral HPLC. [a] Using Mb(F43V, I107E) as the biocatalyst.

To test the scalability of the method, a preparative-scale (100-150 mg) transformation of carbamate **1ac** was carried out using variants Mb(H64V,V68A,Y146F) and Mb(F43V,I107E) under optimized reaction condition. From these reactions, the *S*- and *R*-enantiomer of oxazolidinone **2a** were obtained in 41-61% isolated yields and comparably high levels of enantioselectivity (**Figure 4a**). Importantly, the enzymatic product ***S*-2a** obtained using the present method provides a more streamlined pathway to two different drug molecules, namely the cholesterol-lowering drug Ezetimibe[14] and opioid κ-receptor agonist CJ-15-161,[15] using established methods (**Figure 4a**). Overall, the high enantioselectivity and scalability of these reactions highlights the potential utility of the method for target-directed synthesis and medicinal chemistry, also providing a sustainable and cost-effective alternative to the use of related C-H amination methods involved rare metals.[35, 36, 39-47]

Based on previous investigations of hemoprotein-catalyzed C-H amination reactions by our and other groups,[62, 64] a plausible mechanism for this transformation is proposed in **Figure 4b**.[69] The *N*-benzoyl-carbamate substrate coordinates with the Fe(II) center of the protein (**I**), leading to complex intermediate **II**. Upon the elimination of benzoic acid (BzOH), intermediate **II** gives rise to the reactive intermediate **III**, which is likely in the form of a Fe(III)-imidyl radical species by analogy with Mb-catalyzed C-H amidation with dioxazolones.[68] *Via* a hydrogen atom transfer (HAT) pathway, intermediate **III** leads to the formation of the C-based radical intermediate **IV**, which undergoes a radical rebound step[68] to yield the oxazolidinone product and regenerate the biocatalyst (**I**). As observed in other hemoprotein-catalyzed C-H amination reactions[64, 66, 69, 75] the carbamate by-product (**2aa**) can be explained based on an unproductive pathway involving reduction of catalytic intermediate **III**.

To shed further light into the improved reactivity of Mb(H64V,V68A,Y146F) vs. Mb(H64V,V68A) (=Mb*) in the reaction with **1ac**, time course experiments were performed using these proteins in purified form and under catalyst-limiting conditions (0.1 mol%; **SI Figure S10**). Notably, these experiments indicated that the superior performance of Mb(H64V,V68A,Y146F) can be largely attributed to its improved ability to favor the productive C-H amination pathway (leading to oxazolidinone **2a**) over the unproductive reductive pathway leading to carbamate **1aa** (**SI Figure S10**). Since this effect is solely dependent upon the Y146F mutation, these results are consistent with the beneficial role of this substitution toward disfavoring unproductive electron transfer from the bulk solvent (e.g., sodium dithionite) to the heme center as required for formation of the reduction byproduct. In this regard, it is worth noting that Mb(H64V,V68A,Y103F), which bears another designer Tyr→Phe mutation near the heme cofactor as mentioned above, also shows improved C–H amination efficiency compared to Mb* (1.3 relative activity), albeit it remains inferior to Mb(H64V,V68A,Y146F) in terms of both yield (0.57 rel. act.) and enantioselectivity (86% vs. 98 *ee*). Overall, these results emphasize the value of mechanism-guided mutagenesis[64] in the context of new-to-nature enzymatic reactions.

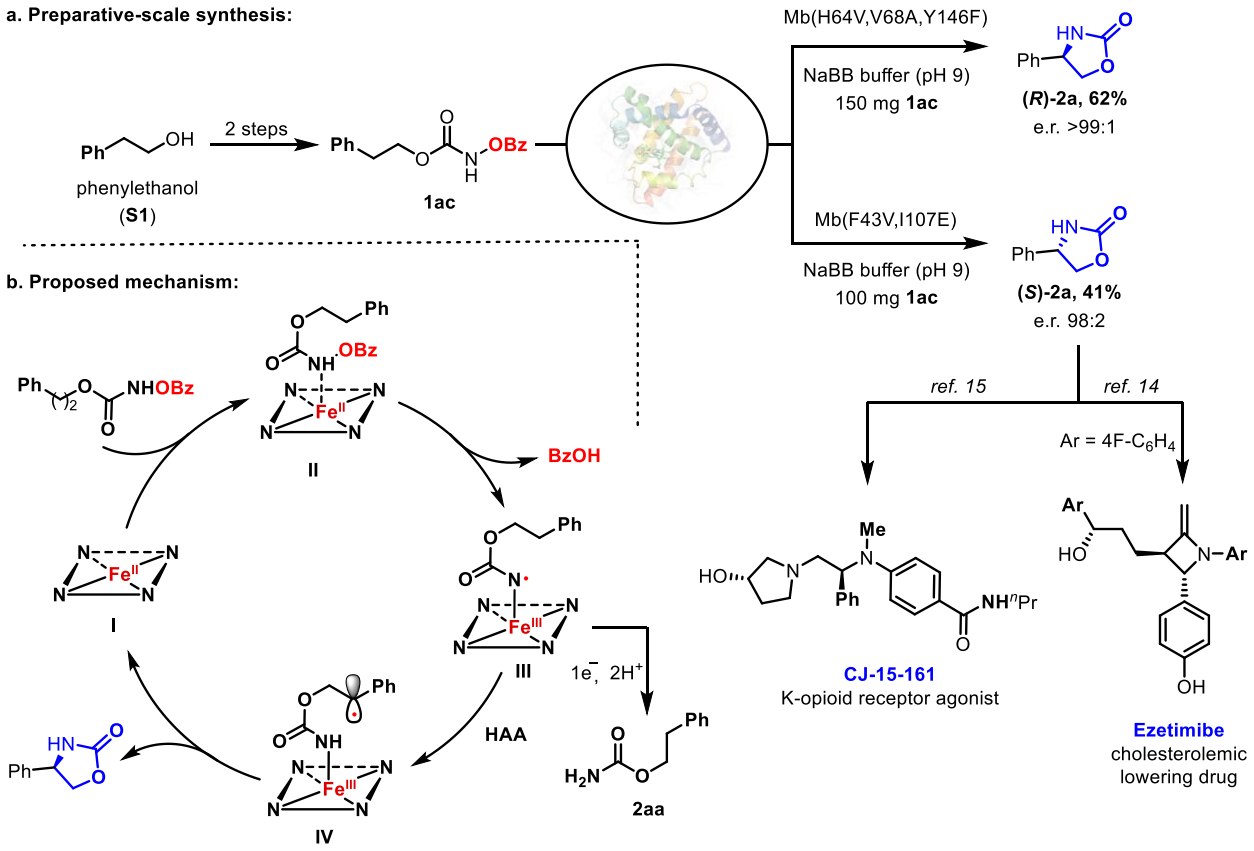

Figure 4. (a) Preparative-scale reaction and synthetic application of the oxazolidinone **2a** for the preparation of *Ezetimibe* and *CJ-15-161* (b) Proposed mechanism for the present Mb-catalyzed synthesis of oxazolidinones via intramolecular C-H amination of *N*-benzoyl carbamates.

CONCLUSION

In summary, we have developed a biocatalytic strategy for the enantiodivergent construction of oxazolidinone rings *via* myoglobin-catalyzed intramolecular C(sp^3)–H amination of readily accessible *N*-benzoyl carbamate reagents. In contrast to our previously reported P450-catalyzed cyclization of carbonazidates, which lacks enantioselectivity and suffered from limited scope and efficiency, the present strategy constitutes a first example of asymmetric oxazolidinone formation

via enzyme-catalyzed C(sp^3)-H nitrene insertion, offering good to excellent enantioselectivity across a range of diverse benzylic C–H substrates. Interestingly, an outer sphere mutation, Y146F, was found to be beneficial in the myoglobin scaffold to favor the productive C–H amination reaction over the unproductive reductive pathway. In addition, both enantiomers of the desired oxazolidinone products could be obtained by means of two enantiodivergent biocatalysts. Finally, the synthetic utility of the methodology was demonstrated through the enzymatic synthesis of a key precursors of two drug molecules on a preparative scale. This works provides an attractive and sustainable solution to the asymmetric synthesis of optically active oxazolidinones, which find broad applications in drug synthesis, as chiral auxiliaries, and as precursors of valuable 1,2-amino alcohols.[6, 10, 17]

METHODS

General Procedure for the Enzymatic Reaction: The reaction was carried out in an anaerobic chamber (Coy) at using *E. coli* C41(DE3) whole cells expressing the desired Mb variant. In a typical procedure, 200 μL of a stock solution of whole cells containing Mb variant (OD_{600}=40) resuspended in degassed buffer, are added to 24-well plates, followed by 180 μL of buffer. The reactions were initiated by the addition of 20 μL of carbamate **1ac** (20 mM stock solution in MeCN). The reaction mixture is shaken at 180 rpm for 16 hours at room temperature. The reactions were analyzed by adding 20 μL of internal standard (50 mM benzodioxole in MeOH) to the reaction mixture, followed by extraction with 400 μL of CH_2Cl_2 and analyzed by GCMS-FID using a Shimadzu GC-2010 gas chromatograph equipped with an FID detector, and a chiral Cyclosil-B column (30 m x 0.25 mm x 0.25 μm film). Separation method: 1 μL injection, injector temperature: 240°C, detector temperature: 300 °C. Gradient: column temperature set at 120 °C for 1 min, then

to 245°C at 10 °C/min for 6 min. Total run time: 19.50 min. Stereoselectivity determination was performed via chiral GC and HPLC.

ASSOCIATED CONTENT

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Author Contributions

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Conflicts of Interest

The authors declare no competing financial interests.

Supporting Information

Synthetic procedures, compound characterization data, NMR spectra, chiral HPLC, GC and SFC chromatograms. The Supporting Information is available free of charge at the journal website.

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