

Letter

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Diversity-Oriented Enzymatic Synthesis of Cyclopropane Building Blocks

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ABSTRACT: While biocatalysis is increasingly incorporated into drug development pipelines, it is less commonly used in the early stages of drug discovery. By engineering a protein to produce a chiral motif with a derivatizable functional handle, biocatalysts can be used to help generate diverse building blocks for drug discovery. Here we show the engineering of two variants of *Rhodothermus marinus* nitric oxide dioxygenase (*Rma*NOD) to catalyze the formation of *cis-* and *trans-* diastereomers of a pinacolboronate-substituted cyclopropane which can be readily derivatized to generate diverse stereopure cyclopropane building blocks.

Enzymes are increasingly used in industrial processes to produce value-added compounds and drugs.¹ As a drug moves further down the development pipeline, highly selective biocatalytic processes may become desirable alternatives to chemical ones for synthesis. Prominent examples include the production of sitagliptin² and islatravir³. Usually, the enzymes used in these processes are highly engineered and show exceptional activity and selectivity for a given task, features which often result in a narrow substrate scope. While this specialization is useful for biocatalysts in the production stage, it limits the utility of enzymes in the initial phases of drug discovery, where broad substrate scopes are key to rapidly generating diverse molecules.⁴

An emerging strategy for the incorporation of biocatalysis into earlier stages of drug development is the enzymatic synthesis of a core motif that can be further derivatized.^{5–8} An enantiopure core motif can be generated with high selectivity, followed by the use of reactions commonly applied in diversityoriented synthesis to derivatize the molecule. One chiral motif found in several pharmaceutical and agrochemical compounds is the substituted cyclopropane.^{9–11} The Arnold lab and others have shown that heme proteins can catalyze cyclopropanation via carbene transfer¹², and engineered heme proteins have been used to generate the cyclopropane-containing pharmaceuticals levomilnacipran,¹³ ticagrelor,^{14,15} and grazoprevir¹⁶.

To bring the benefits of biocatalytic cyclopropanation to early drug discovery, we envisioned a chemoenzymatic approach in which a tandem enzymatic cyclopropanation-chemical derivatization sequence would enable preparation of diverse enantiopure cyclopropanes. To this end, we engineered heme proteins which catalyze carbene transfer to vinyl boronic acid pinacol ester (1) from ethyl diazoacetate (EDA, 2) to produce *cis*- and *trans*-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-cyclopropanecarboxylic acid ethyl ester (3) with high diastereoand enantioselectivity (Figure 1). Unlike small-molecule approaches for the stereoselective construction of cyclopropylboronates,^{17–21} this biocatalytic approach avoids the need for chiral ligands, expensive transition-metal catalysts, or highly reactive substrates. It also reduces waste in the form of organic solvents and undesired regio- and stereoisomers. The boronate functional group in 3 allows for the rapid generation of enantiopure cyclopropanes using Suzuki-Miyaura coupling.^{22–24} This method provides a new avenue for using biocatalysts in medicinal chemistry and other processes where the rapid generation of molecular diversity is desired.

To find a starting enzyme having some level of the desired cyclopropanation activity, we screened a panel of heme proteins (including variants of cytochromes P411 (serine-ligated P450s), cytochromes c, and globins) for the ability to produce **3** using EDA as a carbene precursor for the cyclopropanation of **1** (Supporting Information Table S1). We found that the *Rhodothermus marinus* nitric oxide dioxygenase (*Rma*NOD) variant *Rma*NOD Q52A catalyzed the desired reaction with low activity (17 total turnovers (TTN)) to preferentially produce *trans*-**3** (20:80 *cis:trans* diastereomeric ratio (dr)). The Q52A mutation was found during engineering for unactivated alkene cyclopropanation²⁵ and is analogous to mutations found to enhance myoglobin-catalyzed cyclopropanation reactions.²⁶

With *Rma*NOD Q52A as a starting point and using a crystal structure we obtained of *Rma*NOD Q52V (PDB ID: 6WK3) to guide selection of amino-acid residues in the distal heme pocket



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Figure 1. Directed evolution of *Rma*NOD for production of cyclopropylboronate **3**. All reactions were performed with whole *E. coli* resuspended in 20 mM MOPS buffer (pH = 7) to OD₆₀₀=30, 20 mM **1**, 40 mM **2**, anaerobic conditions, 5% EtOH cosolvent. Experimental details are provided in the Supporting Information, Materials and Methods section. (a) Diastereomeric ratio (%*cis*) and total turnover number (TTN) of the evolutionary lineage toward production of *cis*-**3**. (b) Diastereomeric ratio (%*trans*) and TTN of the evolutionary lineage of *trans*-**3** production.

for mutagenesis, we began iterative rounds of single-site-saturation mutagenesis and screening and recombined the beneficial mutations (Supporting Information). In an initial round of sitesaturation mutagenesis, we discovered that mutation Y32T caused an inversion of diastereoselectivity to favor production of *cis*-**3** and boosted overall enzymatic activity 24-fold (420 TTN, 90:10 dr, >99% enantiomeric excess (ee)). One further round of single- site-saturation mutagenesis and recombination yielded the variant *Rma*NOD Y32T Y39H L48R Q52A R79W (*Rma*NOD THRAW), which exhibited both higher activity and diastereoselectivity than *Rma*NOD Y32T Q52A while maintaining high enantioselectivity (Figure 1a). At analytical scale, *Rma*NOD THRAW produces *cis-***3** with a dr of 94:6, ee of >99%, and TTN of 1300.

In parallel to the evolution of the *cis*-lineage, we also focused on engineering an enzyme that produces *trans*-**3**. Starting from *Rma*NOD Q52A, we performed three sequential rounds of single-site-saturation mutagenesis and recombination to generate *Rma*NOD L20W Q52A L56I L60H L101N I105M (*Rma*NOD WAIHNM), which produces *trans*-**3** with a dr of <1:99, ee of >99%, and TTN of 2000 (Figure 1b). As we would expect, the most impactful mutations identified in both the *cis*- and *trans*lineages are found at first-shell residue positions (Supporting Information Figures S1 and S3). Mutations to first-shell residues can be expected to affect substrate and reactive intermediate binding, leading to changes in diastereoselectivity and activity. Future mechanistic studies might elucidate why these mutations exert the observed effects.

We performed a gram-scale reaction using RmaNOD THRAW. Whole E. coli cells ($OD_{600}=30$ in 1x M9-N, 60 mM 1, 120 mM 2, anaerobic conditions, 5% EtOH cosolvent, details in Supporting Information. Compound Synthesis and Characterization) expressing RmaNOD THRAW were used to produce 3.7 g of cis-3 (>99% ee, 95:5 dr, 36% isolated yield, 41% isolated yield based on recovered starting material). Some of the chiral product was converted to the potassium trifluoroborate salt (4, Figure 2a), which was crystallized to determine that the absolute stereochemistry of the enzymatic product was (1R, 2S)(Supporting Information). During preparative-scale reactions, we found that 3 in aqueous buffer partially converted to the pinacol-deprotected form, cyclopropylboronic acid. We found that the deprotected boronic acid could either be re-protected with additional pinacol or converted into the trifluoroborate salt (Supporting Information, Compound Synthesis and Characterization).

We also sought to improve access to the final product by simplifying both the catalyst formulation and the product isolation procedure. Currently most biocatalytic carbene-transfer reactions are catalyzed using freshly prepared whole E. coli cells harboring engineered heme proteins.12 While reactions run using whole cells are straightforward to perform, the whole cells have a multi-day preparation prior to use, have a short shelf life, and require biological infrastructure not available to many chemists. In contrast, using lyophilized biocatalysts enables preparation of a large batch of catalyst (e.g. via fermentation) which can be shelf-stable²⁹ and used by researchers without cell culture experience. We found that both RmaNOD THRAW and *Rma*NOD WAIHNM function as lyophilized enzyme in whole E. coli cells resuspended in aqueous buffer, and that they could be used in preparative-scale reactions (Supporting Information section, Materials and Methods).

A challenging step in the isolation of **3** was efficient separation of **3** from the two EDA dimers, diethyl maleate and diethyl fumarate, via silica column chromatography. We therefore modified the procedure to isolate the product from starting material and EDA dimer byproducts without the use of chromatography. After aqueous work-up and extraction of the crude reaction mix into organic solvents, unreacted **2** can be removed under reduced pressure. Then, to efficiently separate the cyclopropane product from the maleate and fumarate byproducts, **3** can be derivatized in crude extract to form **4**.³⁰ The trifluoroborate salt is then easily separated from the remaining impurities

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Figure 2. Derivatization of *cis*-**3**. (a) Conversion of *cis*-**3** to the trifluoroborate salt (**4**). Products are drawn with absolute stereochemistry, which was determined as (1R,2S) from X-ray diffraction experiments on **4**. (b) Conversion of *cis*-**3** to a variety of aryl cyclopropanes using stereoretentive Suzuki-Miyaura coupling. Isolated yields are reported for the derivatization step.

(Supporting Information section, *Materials and Methods*). Through the combination of using lyophilized whole cells harboring *Rma*NOD THRAW and the improved product isolation procedure, we prepared (1R,2S)-4 on 1 mmol scale at 52% isolated yield.

Using diverse coupling partners, many compounds could be produced from cis- and trans-3 or their respective trifluoroborate derivatives. In addition to cross-coupling reactions, conversions of cyclopropylboronates to cyclopropylamines and cyclopropanols are known.^{20,22,23,27,28} As a proof of concept, chiral product from the gram-scale reaction was used in stereoretentive Suzuki-Miyaura coupling reactions to produce select aromatic cyclopropanes **5a–5d** (Figure 2b). These examples demonstrate that Suzuki-Miyaura cross-coupling with cis-3 tolerates different functional groups (5a, 5b, 5d), bulky aromatic groups (5c), and heterocycles (5d), with complete retention of stereochemistry. The full synthetic pathway to compounds 5a-5d demonstrates how combining small-molecule and biocatalytic systems takes advantage of the stereoselectivity of biocatalysts and the general activity of small-molecule catalysts. While we have focused on cross-coupling reactions on purified cyclopropylboronates, one could consider performing these

derivatizations immediately following the enzymatic reaction. Work by Lipshutz and coworkers has shown that cross-coupling reactions can be carried out in aqueous buffer using specialty surfactants.³¹ Further reaction optimization could provide a one-pot, two-step system utilizing these surfactants, in which the enzymatic cyclopropanation is followed by cross-coupling to provide the desired derivatized cyclopropane product.

By coupling the specificity of biocatalysts with the broad substrate scope of small-molecule catalysts, this study presents a chemoenzymatic approach that rapidly generates diverse enantiopure cyclopropane-containing compounds, a strategy that could be useful for preparing chiral small-molecule libraries at the early stages of drug discovery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and experimental methods, compound characterization data (PDF)

Full nucleotide and amino-acid sequences for all reported enzyme variants (XLSX)

X-ray crystallographic information file for (1*R*,2*S*)-4 (CIF).

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Author Contributions

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CONFLICT OF INTEREST

A provisional patent, on which A. M. K., B. J. W., and S. B. J. K. are inventors, has been filed through the California Institute of Technology based on the results presented here.

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ABBREVIATIONS

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Bpin, pinacolborane. EDA, ethyl diazoacetate. *E. coli, Escherichia coli. Rma*NOD, *Rhodothermus marinus* nitric oxide dioxygenase. TTN, Total turnover number. dr, diastereomeric ratio. ee, enantiomeric excess.

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Figure 1. Directed evolution of RmaNOD for production of cyclopropylboronate 3. All reactions were performed with whole E. coli resuspended in 20 mM MOPS buffer (pH = 7) to OD600=30, 20 mM 1, 40 mM 2, anaerobic conditions, 5% EtOH cosolvent. Experimental details are provided in the Supporting Information, Materials and Methods section. (a) Diastereomeric ratio (%cis) and total turnover number (TTN) of the evolutionary lineage toward production of cis-3. (b) Diastereomeric ratio (%trans) and TTN of the evolutionary lineage of trans-3 production.

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