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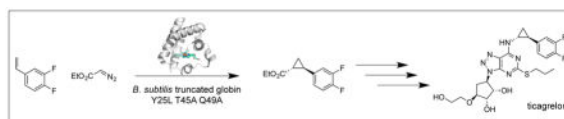
Highly Stereoselective Biocatalytic Synthesis of Key Cyclopropane Intermediate to Ticagrelor

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Abstract

Extending the scope of biocatalysis to important non-natural reactions such as olefin cyclopropanation will open new opportunities for replacing multi-step chemical syntheses of pharmaceutical intermediates with efficient, clean, and highly selective enzyme-catalyzed processes. In this work, we engineered the truncated globin of *Bacillus subtilis* for the synthesis of a cyclopropane precursor to the antithrombotic agent ticagrelor. The engineered enzyme catalyzes the cyclopropanation of 3,4-difluorostyrene with ethyl diazoacetate on a preparative scale to give ethyl-(1*R*, 2*R*)-2-(3,4-difluorophenyl)-cyclopropanecarboxylate in 79% yield, with very high diastereoselectivity (>99% dr) and enantioselectivity (98% *ee*), enabling a single-step biocatalytic route to this pharmaceutical intermediate.

Graphical Abstract



Keywords

biocatalysis; directed evolution; ticagrelor; cyclopropanation; *Bacillus subtilis*; truncated globin^{*}Corresponding author: frances@caltech.cheme.edu (Frances H. Arnold).[§]Current address: Department of Chemistry, Scripps Research Institute, Jupiter, FL, 33458, United States[#]Current address: Merck, Kenilworth, NJ, 07033, United States

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Supporting information available: experimental procedures, standard curves, and characterization of ticagrelor cyclopropyl ester (*R,R*)-5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Ticagrelor (**1**) is a P2Y₁₂ antagonist developed by AstraZeneca under the trade name Brilinta[®] (Figure 1). It was approved by the FDA in 2011 for the prevention of platelet aggregation after the occurrence of a thrombotic event.¹ Sales in 2015 were \$619 million and are projected to go as high as \$3.5 billion by 2023.^{2–3} Most of the patented syntheses of ticagrelor use a key chiral cyclopropane intermediate, (1*R*,2*S*)-2-(3,4-difluorophenyl)cyclopropan-1-amine (**2**). The various methods reported for the preparation of this building block are lengthy, requiring resolutions, chiral auxiliaries or expensive catalysts to obtain the desired enantiomer.^{4–18} Recently, there has been a push to use biocatalysts in order to decrease toxic wastes and make pharmaceutical production ‘greener’.^{19–20} A notable example of success in the development of biocatalytic alternatives can be found in Merck’s production of sitagliptin, used to treat Type 2 diabetes. Directed evolution was used to engineer a transaminase to synthesize the drug under industrial process conditions; the result was a biocatalytic process with 10–13% higher product yield, a 19% reduction in overall waste, and elimination of all heavy metals.²¹ Similarly, biocatalysis can potentially offer very high selectivity and environmentally friendly process conditions for the synthesis of ticagrelor. Turner and coworkers recently examined three complementary biocatalytic routes for the preparation of cyclopropylamine **2**.²² They demonstrated that a ketoreductase could convert 1-(3,4-difluorophenyl)-3-nitropropan-1-one to the corresponding enantiopure alcohol, a building block that could lead to cyclopropylamine **2** in two to three steps; alternatively, a lipase or amidase could be used for kinetic resolution of a racemic mixture of cyclopropyl ester or amide to yield chiral precursors suitable for the preparation of **2**.

Recent reports from our laboratory^{23–24} and others^{25–26} have shown that heme proteins can catalyze the cyclopropanation of various olefins using diazo compounds. Cyclopropyl motifs are present in many bioactive and synthetic compounds and serve as versatile intermediates in organic synthesis.^{27–28} The proteins reported to catalyze olefin cyclopropanation have proven to be amenable to directed evolution for increased activity and to obtain the desired product with high diastereo- and enantioselectivity in a single step: for example, Wang *et al.* engineered a cytochrome P450 to produce the chiral cyclopropane core of levomilnacipran.²⁴

Here we report an enantioselective synthesis of the chiral cyclopropane core of ticagrelor (Figure 1C) using a whole-cell biocatalyst expressing an engineered heme protein derived from *Bacillus subtilis* group II truncated hemoglobin (UniProt ID: O31607).²⁹ This engineered enzyme catalyzes the cyclopropanation of commercially available 3,4-difluorostyrene (DFS, **3**) using ethyl diazoacetate (EDA, **4**) to provide one-step access to ethyl-(1*R*,2*R*)-2-(3,4-difluorophenyl)-cyclopropanecarboxylate (*R,R*)-**5**, an ester precursor of the ticagrelor cyclopropylamine **2**, with excellent selectivity for the desired stereoisomer.

We began by screening various heme proteins for their ability to catalyze the reaction of DFS (**3**) with EDA (**4**) to produce the ticagrelor cyclopropyl ester (*R,R*)-**5**. Most of the proteins tested, such as the H64V V68A variant of sperm whale myoglobin,²⁵ gave primarily the opposite enantiomer of the desired precursor. Three proteins gave an enantio-enriched product with the desired (*R,R*)-configuration:

B. subtilis group II truncated globin, *Hydrogenobacter thermophilus* cytochrome *c* M59A Q62A, and *Bacillus megaterium* P450-BM3 T268A C400H, which gave product in 34, 44, and 8 % *ee*, respectively. Mutagenesis was performed on all three proteins, but substantial improvement in enantioselectivity was only observed with variants of the *B. subtilis* group II truncated globin. The directed evolution strategy we used to optimize the performance of this protein is outlined below. *B. subtilis* group II truncated globin is a small (132 residues, 15 kDa) monomeric protein with very high oxygen affinity and a published crystal structure (PDB ID: 1UX8).²⁹ We hypothesized that iron-carbenoid formation occurred in the distal region of the heme and that mutation of amino acid residues in this nascent “active site” could improve its catalytic performance. Bordeaux *et al.* showed that mutations at H64 and V68 in sperm whale myoglobin could tune the stereoselectivity of the cyclopropanation reaction between ethyl diazoacetate and styrene.²⁵ We chose the analogous residues in *B. subtilis* truncated globin (T45 and Q49, based on a sequence alignment, see Supplemental Information) and targeted those two sites for mutagenesis (Figure 2). The two residues were mutated simultaneously to one of three residues (leucine, phenylalanine, and alanine) in an attempt to find variants that would improve the yield and enantioselectivity of the reaction. The T45A Q49A double mutant stood out as highly selective, yielding the desired product in 95% *ee* with a yield of 57%. Modeling with a Dunbrack rotamer library³⁰ suggests that the amount of space in the nascent active site is increased when the polar and larger threonine and glutamine are mutated to smaller, non-polar alanine.

Truncated globins natively bind oxygen and do not have an active site for catalysis; we attribute the observed improvement in selectivity to the formation of a binding pocket on the distal side of the heme, which presumably binds the substrates in an orientation that favors formation of the desired product. To further improve enantioselectivity, site-saturation mutagenesis was performed at position A45, and the distal heme ligand Y25. While mutations at A45 failed to improve enantioselectivity, screening of the Y25 library led to the discovery of variant Y25L T45A Q49A, which maintained the yield of the parent protein while affording >99% *dr* and 97% *ee* at 20 mM DFS and 40 mM EDA.

To further increase product yield, the Y25L T45A Q49A variant was expressed in two *E. coli* BL21 derivative cell lines C41(DE3) and C43(DE3), which have been optimized to overexpress mildly toxic proteins.³¹ Both C41(DE3) and C43(DE3) cells expressing the Y25L T45A Q49A variant showed increases in enantio- and diastereoselectivity through improved expression of the recombinant protein. The engineered protein now effectively outcompetes the small amount of background reaction from the cells (which produces racemic product).³² The C43(DE3) line displayed the highest diastereo- and enantioselectivity (>99% *dr* and *ee*) at 20 mM DFS/40 mM EDA and was selected for use in preparative-scale reactions.

Preparative scale (0.4 mmol, 20 mL) reactions were performed anaerobically^{23–24} using whole-cell catalyst (OD₆₀₀=80) and 20 mM DFS/40 mM EDA (Figure 3). Initially these reactions were done with simultaneous addition of the bacterial catalyst and reagents, which gave high selectivity for the desired product, but only ~50% yield. After testing a range of reaction conditions, we found that slow addition of the whole-cell catalyst and EDA (**4**) solutions to DFS (**3**) over the course of 3 hours gave the ticagrelor cyclopropyl ester (*R,R*)-**5**

as virtually a single isomer (>99% dr, 98% ee) in 79% yield (see Supporting Information). It is likely that slow addition slows down the formation of EDA dimer and reduces catalyst inactivation by carbene transfer to the protein (heme cofactor and nucleophilic side chains) rather than the DFS, an inactivation mechanism we recently analyzed in detail in another cyclopropanation enzyme.³³

This work has generated an efficient, stereoselective, and potentially low-cost bacterial biocatalyst for producing the cyclopropane precursor to the antithrombotic agent ticagrelor. The ester product of the biocatalytic reaction (*R,R*)-**5**, can be converted easily to the amine ticagrelor precursor **2** via ammonolysis followed by Hofmann rearrangement¹⁶ of the corresponding amide or a Curtius rearrangement⁹ on the corresponding acyl azide. This work has also demonstrated how directed evolution can rapidly optimize a newly discovered enzyme activity, olefin cyclopropanation, to achieve desired product selectivity and yield. With careful process optimization, processes based on biological catalysts such as reported here can continue to replace far less environmentally friendly methods for producing pharmaceutical intermediates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

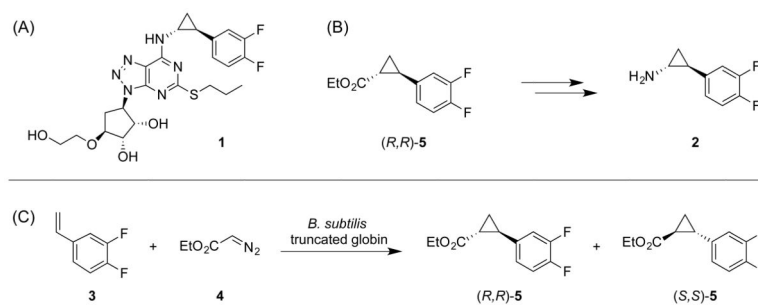
EDA	ethyl diazoacetate
DFS	3,4-difluorostyrene

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**Figure 1.**

(A) Structure of ticagrelor **1**. (B) The cyclopropyl ester precursor **5** can be converted to the cyclopropylamine precursor **2** by making the acyl azide and performing a Curtius rearrangement⁹ or via ammonolysis followed by Hofmann rearrangement¹⁶ of the corresponding amide. (C) Cyclopropanation of 3,4-difluorostyrene (**3**) with ethyl diazoacetate (**4**) catalyzed by engineered heme protein makes the cyclopropyl ester **5**.

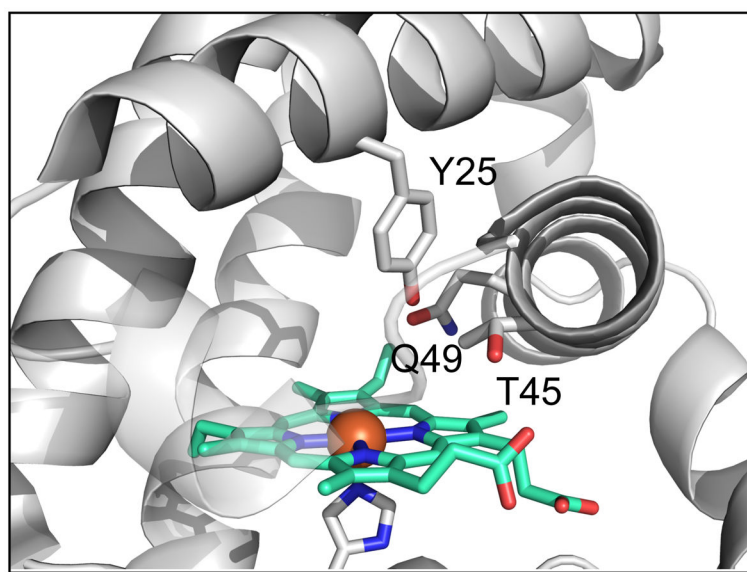


Figure 2. Positions of the Y25, T45, and Q49 residues near the heme iron in the *B. subtilis* wild-type protein (PDB ID: 1UX8).

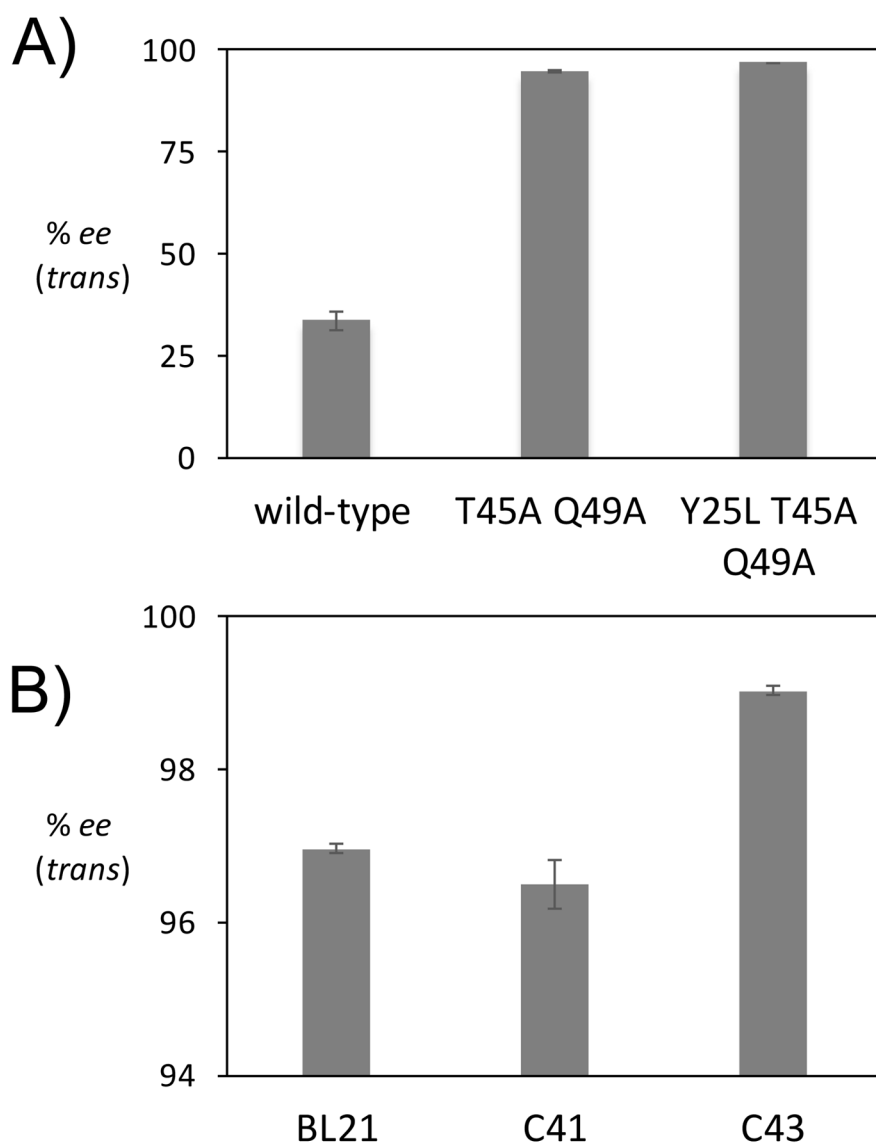


Figure 3. (A) Enantioselectivity of the cyclopropanation reaction catalyzed by various *B. subtilis* variants. Reaction conditions: $OD_{600}=60$, 20 mM DFS/40 mM EDA. (B) Enantioselectivity of the cyclopropanation reaction catalyzed by *B. subtilis* T45A Q49A Y25L expressed in various *E. coli* strains. Reaction conditions: $OD_{600}=60$, 20 mM DFS/40 mM EDA. The error bars represent standard deviation of % ee (*trans*) for the reactions run in triplicate.