

Organic Solvent Stability and Long-term Storage of Myoglobin-based Carbene Transfer Biocatalysts

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Abstract: Recent years have witnessed a rapid increase in the application of enzymes for chemical synthesis and manufacturing, including the industrial-scale synthesis of pharmaceuticals using multi-enzyme processes. From an operational standpoint, these bioprocesses often require robust biocatalysts capable of tolerating high concentrations of organic solvents and possessing long shelf-life stability. In this work, we investigated the activity and stability of myoglobin-based carbene transfer biocatalysts in the presence of organic solvents and after lyophilization. Our studies demonstrate that Mb-based cyclopropanases possess remarkable organic solvent-stability, maintaining high levels of activity and stereoselectivity in the presence of up to 30-50% (v/v) concentrations of various organic solvents, including ethanol, methanol, DMF, acetonitrile, and DMSO. Furthermore, they tolerate long-term storage in lyophilized form, both as purified protein and as whole cells, without significant loss in activity and stereoselectivity. These stability properties are shared by myoglobin-based carbene transferases useful for other type of asymmetric carbene transfer reactions. Finally, we report on simple protocols for catalyst recycling as whole-cell system and for obviating the need for strictly anaerobic conditions to perform these transformations. These findings demonstrate the robustness of myoglobin-based carbene transferases under operationally relevant conditions and should help guide the application of these biocatalysts for synthetic applications.

Introduction

Over the past two decades, biocatalysis has covered an increasingly important role in organic synthesis and in the chemical manufacturing of drugs, advanced pharmaceutical intermediates (APIs), food additives, biofuels, and other important chemical products.[1-6] Attractive features of enzymes in this regard include their potentially high degrees of chemo-, regio- and stereoselectivity,[1-3] which can be altered and tuned by protein engineering and directed evolution.[7-9] As a prominent example, a multi-enzyme cascade process involving a total of nine enzymes, five which were modified via protein engineering, was recently implemented by Merck for the large-scale synthesis of the anti-HIV drug islatravir, providing a much shorter and more efficient route for the production of this molecule compared to previously reported synthetic routes.[10] In addition to the desired activity and selectivity, an important requirement for the application of enzymes in industrial and large-scale processes is an inherent (or acquired) stability in the presence of organic solvents, since high concentration of organic co-solvents are often required to solubilize lipophilic substrates and/or achieve high substrates loadings.[2, 4, 11-14] For example, a recently implemented industrial-scale biocatalytic process for the production of the anti-diabetic drug sitagliptin involved an engineered transaminase capable of operating in the presence of 50% (v/v) DMSO, which was necessary to permit the use of high substrate loadings (100 g/L).[15] Since most proteins and enzymes are readily denatured by organic solvents, significant protein engineering/directed evolution efforts are typically required to equip biocatalysts with sufficient activity and stability properties to fit the target manufacturing processes.[2, 16] For example, the aforementioned engineered transaminase was obtained after 11 rounds of directed evolution[15] and similar efforts (18 rounds of engineering/screening) were required for engineering a halohydrin dehalogenase biocatalyst for the manufacturing of the

cholesterol-lowering drug atorvastatin (Lipitor).[17] Along with organic solvent stability, shelf-life stability constitutes another highly desirable feature for enzymes to be employed for organic synthesis.[2, 4, 11]

While a number of natural enzyme classes represent key components of the biocatalytic toolbox for organic synthesis (e.g., hydrolases, ketoreductases, transaminases),[1, 4, 18, 19] we and others have reported that heme-containing enzymes and proteins are functional biocatalysts for ‘abiological’ carbene transfer reactions, including olefin cyclopropanation,[20-29] Y–H carbene insertion (where Y= N, S, B, or Si),[30-36] C–H functionalization,[37-41] aldehyde olefination,[42, 43] and others.[44, 45] In particular, engineered myoglobins (Mb) have emerged as particularly selective and versatile scaffolds for promoting a variety of carbene-mediated transformations useful for the selective construction of new carbon-carbon and carbon-heteroatom bonds.[23-29, 31, 32, 35, 36, 38, 39, 42, 44, 46] In the interest of further exploring the utility of these Mb-based carbene transferases for practical synthesis, we investigated here the activity and stability of these biocatalysts in the presence of organic solvents, after lyophilization, and under other operationally relevant conditions.

Materials and Methods

Experimental Procedures:

General Information. All the chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich, Alfa Aesar, ACS Scientific, Acros) and used without any further purification. All dry reactions were carried out under argon in flame-dried glassware with magnetic stirring using standard gas-tight syringes, cannula, and septa. ^1H and ^{13}C NMR spectra were measured on Bruker DPX-500 (operating at 500 MHz for ^1H and 125 MHz for ^{13}C) or Bruker DPX-400 (operating at

400 MHz for ^1H and 100 MHz for ^{13}C). Tetramethylsilane (TMS) served as the internal standard (0 ppm) for ^1H NMR and CDCl_3 was used as the internal standard (77.0 ppm) for ^{13}C NMR. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230-400 mesh. Thin Layer Chromatography (TLC) was carried out using Merck Millipore TLC silica gel 60 F254 glass plates.

Product Analysis. The reactions were analyzed by adding 20 μL of internal standard (1,3-benzodioxole, 50 mM in ethanol) to each reaction mixture, followed by extraction with 400 μL of dichloromethane (CH_2Cl_2) and analyzed by GC-FID using a Shimadzu GC-2010 gas chromatograph equipped with an FID detector and a Cyclosil-B column (30 m x 0.25 mm x 0.25 μm film). Calibration curves for quantification of all reactions were constructed using authentic standards produced synthetically as described in **Synthetic Procedures** and analyzed by GC-FID. Separation method: 1 μL injection, injector temperature: 250 $^\circ\text{C}$, detector temperature: 300 $^\circ\text{C}$, column temperature set at 120 $^\circ\text{C}$ for 3 min, then to 140 $^\circ\text{C}$ at 0.85 $^\circ\text{C}/\text{min}$, then to 245 $^\circ\text{C}$ at 30 $^\circ\text{C}/\text{min}$. Total run time was 31.03 min. Enantiomeric excess of cyclopropane **3** was determined by chiral GC-FID using the previously describe method. Enantiomeric excess of rearrangement product **10** and cyclopropane **11** was determined by Supercritical Fluid Chromatography (SFC) analysis, using a JASCO Analytical and SemiPreparative SFC instrument equipped with a column oven (35 $^\circ\text{C}$), photodiode array detector, a backpressure regulator (12.0 MPa), a carbon dioxide pump, and a sample injection volume of 3 μL . Daicel Chiralpak IB or IC (0.46 cm ID $\times$ 25 cm L) was used for separation of enantiomers. All samples were eluted using an isocratic solvent system with isopropyl alcohol (IPA) as the modifier solvent in liquid CO_2 at an elution rate of 4 mL/min and detected at $\lambda = 220$ nm. Total run time was 10.2 min.

Synthetic Procedures. Procedures for the synthesis of reagents and authentic standards are provided as Supporting Information.

Protein Expression and Purification. Engineered Mb variants were expressed in *E. coli* BL21(DE3) cells as described previously.[23] Briefly, cells were grown in terrific broth (TB) medium (ampicillin, 100 mg L⁻¹) at 37 °C (150 rpm) until OD₆₀₀ reached 0.9-1.2. Cells were then induced with 0.25 mM β-D-1-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid (δ ALA). After induction, cultures were shaken at 180 rpm and 27 °C and harvested after 18-20 hours by centrifugation at 4 °C. After cell lysis by sonication, the proteins were purified by Ni-affinity chromatography. The lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA Lysis Buffer. The resin was washed with 50 mL of Ni-NTA Lysis Buffer and then 50 mL of Ni-NTA Wash Buffer (50 mM KPi, 250 mM, NaCl, 20 mM imidazole, pH 8.0). Proteins were eluted with Ni-NTA Elution Buffer (50 mM KPi, 250 mM, NaCl, 250 mM histidine, pH 7.0). After elution, the proteins were buffer exchanged against 50 mM potassium phosphate (KPi) buffer (pH 7.0 or 8.0) using 10 kDa Centricon filters. Myoglobin concentration was determined using an extinction coefficient $\epsilon_{410} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$.

Catalyst Recovery after Lyophilization. Samples of purified Mb(H64V,V68A) or whole cells expressing Mb(H64V,V68A) in KPi buffer solution (50 mM, pH 7.0) were flash-frozen in dry ice and lyophilized using a LabConco Freeze-dryer Freezone for 24 hours. The lyophilized sample was resuspended in the original volume of Milli-Q water and analyzed via UV-Vis spectroscopy. For the purified protein, the % of protein recovery (Table S2) was determined by comparison of the protein concentration prior and after lyophilization based on the Soret bands at 408, 436, and 425 nm for the ferric, ferrous, and CO-bound form of the hemoprotein, respectively. For the whole cell catalyst, optical density at 600 nm (OD₆₀₀) was measured prior and after lyophilization to

measure percent cell recovery. To determine protein recovery, the whole cells were lysed by sonication and the concentration of Mb was measured from the cell lysate via UV-Vis spectroscopy.

Anaerobic Protocol for Protein Reactions via Cannulation. In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) was purged by bubbling argon into the mixture for 3 min in a sealed vial. A buffered solution containing myoglobin (20 μ M final concentration) was carefully degassed via its headspace in a similar manner in a separate sealed vial equipped with a stir bar. The two solutions were then mixed together via cannulation. Reactions were initiated by addition of organic solvent (if applicable), olefin (10 mM final concentration), and α -ethyl diazoacetate (EDA, 20 mM final concentration) bringing the mixture to a final volume of 400 μ L. The reaction mixture was stirred for 16 hours at room temperature.

Anaerobic Protocol for Protein Reactions via Anaerobic Chamber. In a typical procedure inside an anaerobic chamber, a solution of sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), organic solvent (if applicable), olefin (10 mM final concentration), and EDA (20 mM final concentration) were added to a reaction vessel equipped with a stir bar. Reactions were initiated by addition of myoglobin (20 μ M final concentration) to the vessel bringing the mixture to a final volume of 400 μ L. The reaction vessel was capped and stirred inside the chamber for 16 hours at room temperature. Reaction work up was performed outside the anaerobic chamber. All buffered solutions and organic solvents were purged with Ar prior to entering the anaerobic chamber.

Micro-aerobic Reaction Setup. In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) was added to a

reaction vessel equipped with a stir bar. The vessel was sealed and the solution purged by bubbling argon to the solution for 3 minutes. Reactions were initiated by the addition of myoglobin (20 μ M final concentration), olefin (10 mM final concentration), and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μ L. The reaction mixture was stirred for 16 hours at room temperature.

Semi-aerobic Reaction Setup. In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0) and myoglobin (20 μ M final concentration) were added to a reaction vessel equipped with a stir bar. The vessel was sealed, and the headspace was purged with argon for 3 minutes. Reactions were initiated by the addition of olefin (10 mM final concentration) and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μ L. The reaction mixture was stirred for 16 hours at room temperature.

Aerobic Protocol for Protein Reactions. In a typical procedure, a solution containing sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), olefin (10 mM final concentration), and EDA (10 or 20 mM final concentration) was added to a reaction vessel equipped with a stir bar. Reactions were initiated by addition of myoglobin (20 μ M final concentration) to the vessel bringing the solution to a final volume of 400 μ L in open air. The reaction mixture was capped and stirred for 16 hours at room temperature.

Initial Rates Measurements. In a typical procedure inside an anaerobic chamber, a solution of sodium dithionite (10 mM final concentration) in potassium phosphate buffer (50 mM, pH 7.0), organic solvent, olefin (10 mM final concentration), and EDA (20 mM final concentration) was added to a vessel equipped with a stir bar. The reaction vessel was sealed and brought out of the anaerobic chamber. Reactions were immediately initiated by addition of myoglobin (5 μ M final concentration, 2 mL final volume) and the resulting mixture was left stirring for 1 minute. An

aliquot (400 μL) was extracted via syringe and quenched by placing it in an Eppendorf tube containing 5 M HCl (100 μL), CH_2Cl_2 (400 μL), and internal standard (20 μL , 2.5 mM final concentration). The tube was immediately vortexed for 30 seconds, centrifuged at 13,000 x g for 5 minutes, and the CH_2Cl_2 layer was extracted for GC analysis.

Anaerobic Protocol for Whole Cells Reactions. In a typical procedure, a suspension of Mb(H64V,V68A)-expressing cells was added to a reaction vessel equipped with a stir bar containing potassium phosphate buffer (50 mM, pH 7.0). The vessel was sealed, and the headspace purged with Ar for 3 min. Reactions were initiated by addition of olefin (10 mM final concentration) and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μL . The reaction mixture was stirred for 16 hours at room temperature under positive argon pressure. The TON for whole-cell reactions were calculated based on Mb concentration in the reaction mixture as measured via UV-Vis spectroscopy ($\epsilon_{410} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$) after cell lysis.

Anaerobic Protocol for Whole Cells Reactions via Anaerobic Chamber. In a typical procedure inside an anaerobic chamber, a suspension of Mb(H64V,V68A)-expressing cells were added to a reaction vessel equipped with a stir bar containing potassium phosphate buffer (50 mM, pH 7.0). Reactions were initiated by addition of olefin (10 mM final concentration), and EDA (20 mM final concentration) bringing the solution to a final volume of 400 μL . The vessel was capped and stirred inside the chamber for 16 h at room temperature. Reaction work up was performed outside the anaerobic chamber. The TON for whole-cell reactions were calculated based on Mb concentration in the reaction mixture as measured via UV-Vis spectroscopy ($\epsilon_{410} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$) after cell lysis. All buffered solutions and organic solvents were purged with Ar prior to entering the anaerobic chamber.

Whole-cell Biocatalyst Recycling Experiments. All reactions were carried out using the anaerobic protocol for whole-cells reactions, except that each set of reactions were left stirring at room temperature under positive argon pressure for 30 min. After a reaction cycle was completed, 20 μL of internal standard (50 mM stock solution, 2.5 mM final conc.) was added to the vial. The reaction mixture was transferred to an Eppendorf tube and centrifuged at 13,000 x g for 5 minutes. The supernatant was removed and transferred to another Eppendorf tube. After centrifugation, the cell pellet was resuspended in 400 μL of a 1.5% (v/v) Tween 20 solution in potassium phosphate buffer (50 mM, pH 7.0). The centrifugation and supernant transfer step were repeated two additional times and all the supernatants were combined. An aliquot of 400 μL of the combined supernatant solution was extracted with DCM for GC analysis. After the last extraction of supernatant, the cell pellet was resuspended in 400 μL of potassium phosphate buffer (50 mM, pH 7.0) and transferred to a reaction vial to begin the next cycle of catalysis.

Results and Discussion

Effect of organic solvents on activity and stability of Mb-based cyclopropanase

To assess the impact of organic solvents on the function of Mb-based carbene transferases, we analyzed the catalytic activity and selectivity of sperm whale myoglobin variant Mb(H64V,V68A) in the presence of various concentrations of five water-miscible organic solvents which are commonly used in biocatalysis, namely ethanol (EtOH), methanol (MeOH), *N,N*-dimethylformamide (DMF), acetonitrile (ACN), and dimethyl sulfoxide (DMSO). Mb(H64V,V68A) was previously developed to catalyze the highly stereoselective cyclopropanation of styrene and other vinylarenes in the presence of ethyl α -diazoacetate (EDA, **2**) as the carbene donor with a high degree of diastereo- and enantioselectivity.[23, 24] Consistent

with previous reports,[23, 24] this Mb variant catalyzes the cyclopropanation of styrene (**1**) with α -ethyl diazoacetate (EDA, **2**) to give the *trans*-(1*S*,2*S*) cyclopropane **3** in 99% *de* and 99% *ee* (**Figure 1**). In addition to the solvent effect on activity, we envisioned the stereoselectivity of this enzyme could be used to gauge the effect of the applied reaction conditions on the catalytic properties of the biocatalyst.

Initially, we examined the activity and selectivity of Mb(H64V,V68A) in the styrene cyclopropanation reaction in the presence of EtOH from 6% to 60% vol/vol (v/v) in potassium phosphate buffer (KPi) under standard reaction conditions (10 mM substrate, 0.2 mol% catalyst). As shown in **Figure 1A** and **Table S1**, the Mb catalyst was found to maintain high levels of activity (>99% yield) and stereoselectivity (>99% *de* and *ee*) up to 30% v/v of EtOH. Upon increasing the concentration of organic co-solvent beyond this point, both the activity and stereoselectivity decrease resulting in 45% yield, 95% *de*, and 67% *ee* at 40% v/v EtOH and 22% yield and 88% *de*, and 32% *ee* at 60% v/v EtOH. Styrene cyclopropanation with EDA catalyzed by free hemin in buffer produces **3** in 79% *de* (*trans*) as a racemate (0% *ee*).[23] A reduction in % *ee* can thus derived from solvent-induced alteration of the enzyme active site and/or contribution from background reaction from hemin released upon denaturation of the hemoprotein. The experiments above thus showed that the Mb biocatalyst tolerates an EtOH concentration as high as 30% v/v without any deleterious effect on its catalytic function and stereoinduction properties.

Similar experiments were then extended to the other organic solvents (**Figure 1B-E**). In the presence of methanol, the Mb(H64V,V68A) catalyst was found to show no noticeable decrease in both activity and stereoselectivity (>99% *de* and *ee*) from 6% up to 40% (v/v) (**Figure 1B**). At 50% (v/v), only a slight reduction in stereoselectivity was observed (99% *de*, 96% *ee*), while maintaining high activity (77% yield) and no decrease in relative activity compared to the ‘no-

solvent' reference conditions (= 6% (v/v)). At 60% (v/v), although the relative activity of the enzyme decreases to about 30%, stereoselectivity remains high (96% *de*, 89% *ee*). These results thus revealed a remarkable robustness of the Mb biocatalyst against denaturation in the presence of methanol (i.e., up to 50-60% (v/v)) as well as its superior stability in this solvent compared to ethanol (**Figure 1A vs. 1B**).

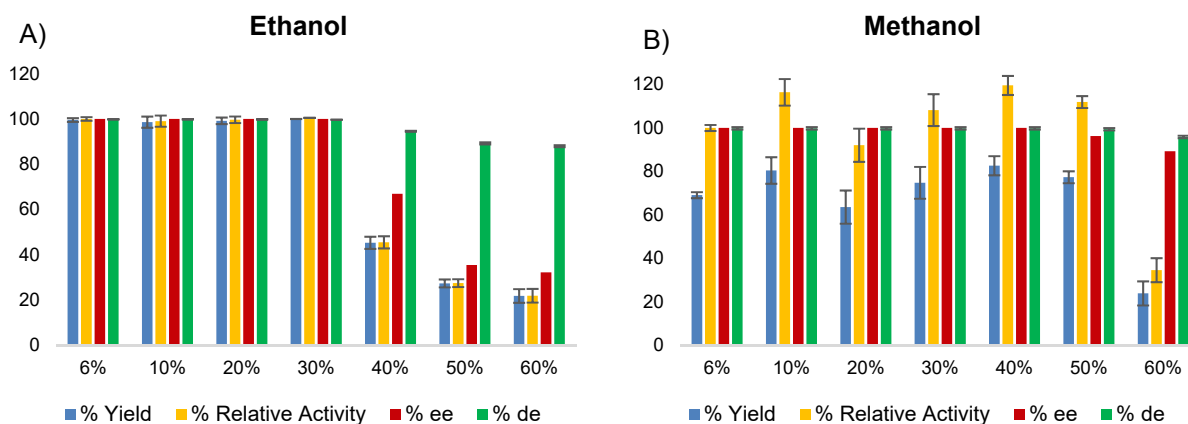
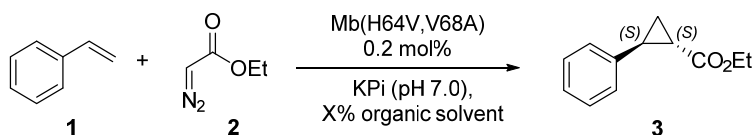
The experiments with *N,N*-dimethylformamide (DMF) and acetonitrile (ACN) show an overall similar behavior of the biocatalyst in the presence of these organic solvents (**Figure 1C-D**). In both cases, no deleterious effect on the diastereo- and enantioselectivity of the catalyst was observed up to 40% (v/v) of the organic co-solvent, under which conditions the Mb(H64V,V68A) produced **3** with excellent stereoselectivity (>99% *de* and *ee*). With ACN, only a small reduction in diastereoselectivity was observed at 40% (v/v) (96% *de*, >99% *ee*). Within this concentration range, the catalytic activity of Mb(H64V,V68A) also remained largely unaffected in the presence of DMF, whereas a two-fold reduction in relative activity was observed upon transitioning from 30% to 40% acetonitrile, thus indicating a slightly higher robustness of the catalyst toward DMF over acetonitrile. Interestingly, a small but significant increase in relative activity (+20%) was consistently observed between 10% and 30% (v/v) of acetonitrile, indicating a beneficial effect of this solvent on catalytic activity. With both solvents, a noticeable reduction in activity and stereoselectivity occurred by increasing the organic solvent concentration to 50% and 60% v/v, with the biocatalyst providing 8-30% yields with 37-81% *ee* (**Figure 1C-D**). This notwithstanding, the Mb(H64V,V68A)-catalyzed reaction was found to maintain a significant degree of stereoselectivity in the presence of a 1:1 mixture of buffer : DMF (98% *de*, 81% *ee*).

In DMSO, the cyclopropanation biocatalyst showed no to little reduction in relative activity (74-100% rel. act., corresponding to 67-90% yield) observed up to 30% (v/v) of the organic co-

solvent, after which point a progressive decrease in activity accompanied an increase in the co-solvent concentration (**Figure 1E**). Under the applied conditions, the maximal co-solvent concentration for detectable activity was 50% (v/v) DMSO, corresponding to a 13% yield in the cyclopropanation reaction. Interestingly, in the presence of this solvent only excellent stereoselectivity (99% *de* and 98-99% *ee*) was displayed up to 40% (v/v) and high stereoselectivity (99% *de* and 88-94% *ee*) was maintained at the high organic solvent concentrations of 50% and 60% (v/v) (**Figure 1E**). Thus, while the concentration-dependent effect of DMSO on enzymatic activity is similar to that of ethanol and acetonitrile, the enzyme's robustness toward this solvent in terms of stereoselectivity is similar to its robustness in methanol and, to a lesser extent, in DMF.

Altogether, these studies show that the Mb-based cyclopropanation biocatalyst retain high levels of activity and stereoselectivity in up to 40-50% (v/v) of various organic co-solvents. Such organic solvent stability is remarkable considering that most enzymes, and in particular cofactor-dependent enzymes,[15] are readily denatured under these conditions.[14] For example, cytochromes P450, which also contain a heme cofactor, are readily inactivated by low concentrations (<5–10% vol/vol) of organic solvents such as ethanol, DMF, or acetonitrile.[47-50] On balance of both activity and stereoselectivity, the Mb(H64V,V68A) biocatalyst was found to display the following order of organic solvent tolerance: MeOH > DMSO > DMF ~ ACN > EtOH. This order shows only a modest correlation with the dielectric constant of these solvents ($\epsilon = 32$, 47, 36, 37, and 24, respectively), indicating that multiple factors are at play in influencing the solvent effect on activity and stereoselectivity. With the least polar solvent, ethanol, a decrease in activity is accompanied by a decrease in stereoselectivity, suggesting that protein denaturation could be occurring at high solvent concentrations. For DMSO and DMF, high stereoselectivity is maintained at high solvent concentrations (50% and 40% v/v, respectively), despite a decrease in

the product yield and thus in catalytic activity. This suggests an inhibitory effect of the solvent on the enzyme activity without involving denaturation of the hemoprotein. Coordination of DMSO to the heme iron has been proposed to represent a key mechanism of inactivation of P450s in this solvent.[50, 51] and a similar phenomenon could be at the basis of the reduced cyclopropanation activity of the Mb catalyst at high concentrations of DMSO. A similar phenomenon could be invoked for DMF, which can also bind metals. As noted earlier, the enzyme shows highest stability toward methanol, in which it maintains high activity and stereoselectivity up to 50% v/v, this indicating both a lack of solvent-induced protein denaturation and activity inhibition.



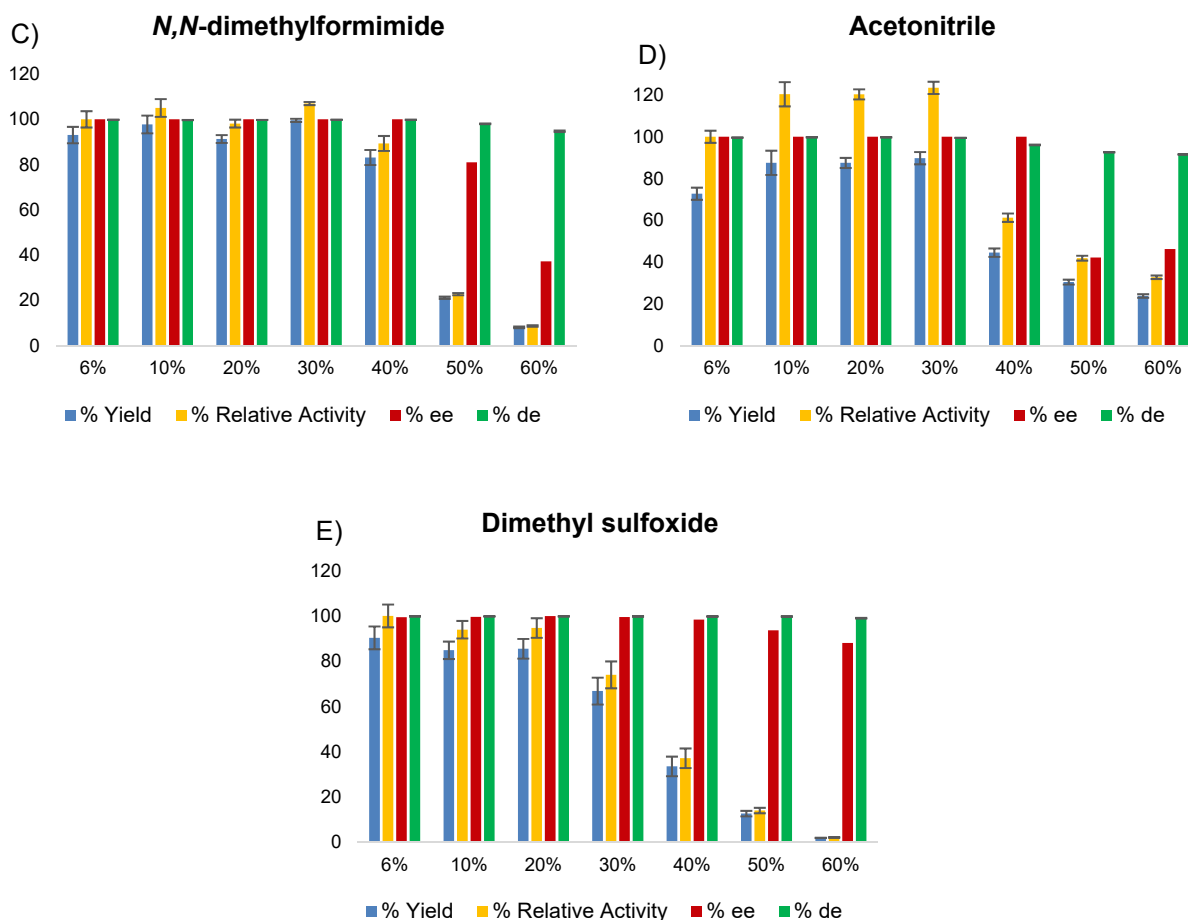


Figure 1. Product yield, relative activity and stereoselectivity of Mb(H64V,V68A) for the conversion of styrene and EDA into **3** in the presence of the indicated organic solvent at varying concentrations. Relative activity correspond to the yields normalized to the yield at the minimal solvent concentration (=6% (v/v)), which corresponds to 100% for EtOH, 69% for MeOH, 93% for DMF, 73% for ACN, and 90% for DMSO. Reactions conditions: 10 mM styrene, 20 mM EDA, 10 mM Na₂S₂O₄, 20 μ M Mb(H64V,V68A) in 50 mM potassium phosphate buffer (pH 7.0), anaerobic, R.T., 16 h. Product yield, diastereomeric excess (% *de*), and enantiomeric excess (% *ee*) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

Effect of organic solvents on cyclopropanation rate

To further assess the effect of the different solvents on the catalytic properties of the Mb-based carbene transferase, we measured initial rates of product formation in the presence of 30% (v/v) organic co-solvent using 0.05 mol% catalyst. As summarized in **Table 1**, these experiments showed that the Mb-catalyzed cyclopropanation reaction proceeds with faster rates in the presence of ethanol and methanol compared to the reactions carried out in the presence of acetonitrile, DMF and DMSO. The measured cyclopropanation rates in the presence of the alcohols correspond to

195-227 turnovers min⁻¹, which are about five-fold lower than that measured in the absence of organic solvent (1,000 turnovers min⁻¹).^[23] Compared to the alcohol-based solvents, two- to five-fold slower cyclopropanation rates were observed in the presence of acetonitrile (148 TON/min), DMF (105 TON/min) and DMSO (44 TON/min). The more pronounced effects of the latter solvents on the catalytic rates can be explained on the basis of their Lewis basicity and thus their potential ability to coordinate the heme-Fe when present at high concentrations (30% v/v corresponds to 5.7 M acetonitrile, 3.9 M DMF, and 4.2 M DMSO). Reasonably, this process would compete for the interaction of the heme cofactor with EDA, as required for carbene transfer catalysis,^[52] resulting in a slowing down of the cyclopropanation rate.

Table 1. Initial rates of Mb(H64V,V68A) for the cyclopropanation of styrene and EDA in various organic solvents at 30% (v/v).^[a]

	Solvent				
	Ethanol	Methanol	<i>N,N</i> -dimethylformamide	Acetonitrile	Dimethylsulfoxide
Rate (TON min ⁻¹)	195 ± 37	227 ± 41	105 ± 16	148 ± 4.0	44 ± 18

^[a] Reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM Na₂S₂O₄, 30% organic solvent (v/v), 5 μM Mb(H64V,V68A), potassium phosphate buffer (50 mM, pH 7.0), anaerobic, R.T., 1 min. Product yield and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

Biocatalytic activity under micro- and semi-aerobic conditions

Hemoprotein-catalyzed carbene transfer reactions show optimal performance under anaerobic conditions due to the inhibitory effect of O₂ on carbene transferase activity resulting from O₂ binding to the heme cofactor.^[20, 23, 53] For our Mb-catalyzed carbene transfer reactions, anaerobic conditions are typically achieved by performing these reactions in an anaerobic chamber or through cannula transfer methods. Since these equipment and techniques may not be available in all laboratories, we wished to develop operationally simpler protocols to conduct these biocatalytic reactions. To this end, we tested an alternative protocol, referred here as ‘micro-

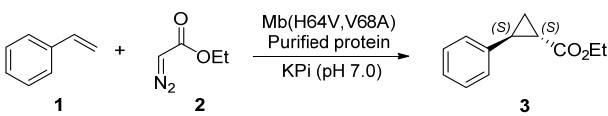
aerobic', which requires simple purging of the vial headspace and buffer solution containing the reductant with argon (using a balloon) followed by addition of the protein, substrate, and diazo reagent via a syringe. This protocol removes the need for the use of cannula transfer techniques. As shown in **Table 2**, under these micro-aerobic conditions, the Mb(H64V,V68A) biocatalyst performed with the same level of activity and stereoselectivity as under strictly anaerobic conditions (Entry 3 vs 1), providing full conversion of styrene to the *trans*-1*S*,2*S* cyclopropane **3** with >99% *de* and *ee*. This is in contrast to the reaction carried out under aerobic conditions which showed a significantly reduced yield as expected (**Table 2**, Entry 2). Encouraged by these results, we tested an even simpler 'semi-aerobic' protocol, in which the protein is simply added to a sealed vial containing a non-degassed buffered solution with the protein and reductant, followed by filling of the head space with argon, and addition of the substrate and diazo reagent. Under these conditions, quantitative yield of **3** was obtained with excellent diastereo- and enantioselectivity (>99% *de* and *ee*), thus demonstrating the functionality of these simplified micro- and semi-aerobic protocols for performing Mb-catalyzed carbene transfer reactions.

Reactions with lyophilized proteins and whole cells

Long shelf-life stability is a highly desirable feature for enzymes to be applied in biocatalysis[2, 4, 11] but cofactor-dependent enzymes such as heme enzymes often tend to lose catalytic activity upon long-term storage in solution or after lyophilization due to cofactor loss and/or denaturation.[54, 55] To investigate this aspect, the Mb(H64V,V68A) variant was lyophilized via a freeze-dry cycle (**Figure 2A**) and reconstituted in buffer after storage as lyophilized powder at room temperature. UV-vis absorption spectroscopy experiments showed nearly quantitative recovery of the Mb variant (94-99%) after reconstitution in buffer, as determined based on the intensity of the Soret bands in its ferric, ferrous, and CO-bound form

(Table S2). To further assess the impact of lyophilization on the catalytic properties of the Mb variant, the re-solubilized hemoprotein was tested for activity in the model cyclopropanation reaction with styrene and EDA. Importantly, under standard anaerobic conditions, the lyophilized protein showed identical levels of high activity (>99% yield) and excellent stereoselectivity as the control (non-lyophilized) protein (Table 2, Entry 5 vs. 1). At lower catalyst loading of 0.05 mol%, the re-solubilized Mb variant was found to provide quantitative yields of the enantiopure cyclopropanation product, supporting 2,000 TON (Table 2, Entry 6). These results thus demonstrate that the Mb-based cyclopropanase is able to tolerate well the lyophilization process without exhibiting loss in catalytic activity or stereoselectivity. Upon testing the lyophilized protein under the micro- and semi-aerobic protocols described above, excellent levels of activity and stereoselectivity were found in both cases (Table 2, Entry 7-8), further demonstrating the functionality and catalytic efficiency of the resolubilized protein in combination with these operationally simpler reaction setups. Although various additives[54, 55] were also tested during the lyophilization process (e.g., sucrose, glucose, and PEG 4000), the results above demonstrated that additives were not required for full recovery of the protein and its activity.

Table 2. Catalytic activity and selectivity of freshly prepared and lyophilized Mb(H64V,V68A) toward styrene cyclopropanation with ethyl diazoacetate under various atmospheric conditions.^[a]

						
Entry	Catalyst	Condition	Yield	TON	de	ee
1	Solution	Anaerobic	>99%	500	>99%	>99%
2	Solution	Aerobic	4%	19	>99%	>99%
3	Solution	Microaerobic	>99%	500	>99%	>99%
4	Solution	Semi-aerobic	>99%	500	>99%	>99%
5	Lyophilized	Anaerobic	>99%	500	>99%	>99%
6	Lyophilized	Anaerobic ^[b]	>99%	2,000	>99%	>99%

7	Lyophilized	Microaerobic	>99%	500	>99%	>99%
8	Lyophilized	Semi-aerobic	>99%	500	>99%	>99%
9	Lyophilized	Aerobic	7%	37	>99%	>99%

^[a] Reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM Na₂S₂O₄, 8% EtOH (v/v), 20 μ M Mb(H64V,V68A), potassium phosphate buffer (50 mM, pH 7.0), varying atmospheric conditions, room temperature, 16 hours. ^[b] 5 μ M protein. Product yield, diastereomeric excess (% *de*), enantiomeric excess (% *ee*), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

Encouraged by the results with the lyophilized protein, we investigated the effect of lyophilization on *E. coli* whole-cells expressing Mb(H64V,V68A). Similarly to the control reactions with freshly prepared cells, cyclopropane **3** could be obtained in quantitative yields and with excellent stereoselectivity (99% *de* and *ee*) using re-solubilized Mb(H64V,V68A)-containing *E. coli* cells at a cell density (OD₆₀₀) of 40 (**Table 3**, Entry 2 vs. 1). The experiments were then repeated under catalyst-limited conditions (OD₆₀₀ = 20) to better assess the impact of lyophilization on the activity of the intracellular biocatalyst. Surprisingly, the re-solubilized Mb(H64V,V68A)-containing cells provided an even higher yield of the cyclopropanation product compared to fresh cells (62% vs. 44%), while maintaining excellent stereoselectivity (**Table 3**, Entries 4 vs. 3). Furthermore, the lyophilized cells could be utilized after storage on a shelf at room temperature for over six months (**Figure 2B**) without exhibiting any loss in catalytic activity and stereoselectivity (**Table 3**, Entry 5). Overall, these experiments demonstrate the robustness of the Mb catalyst to lyophilization both in the form of purified protein and in whole cells as well as its long shelf-life stability in lyophilized form. As noted above, the observation of the higher catalytic activity of the lyophilized cells compared to the fresh cells was a positive yet unexpected outcome of these experiments. While further studies are required to elucidate the basis of this phenomenon, a possible explanation is that the lyophilization process may increase the permeability of the cells

to the reagents and/or contribute to reduce cellular components that interfere with the reaction in the intracellular settings.

Figure 2. Lyophilized Mb(H64V,V68A) variant (A) and lyophilized *E. coli* cells containing Mb(H64V,V68A) (B).

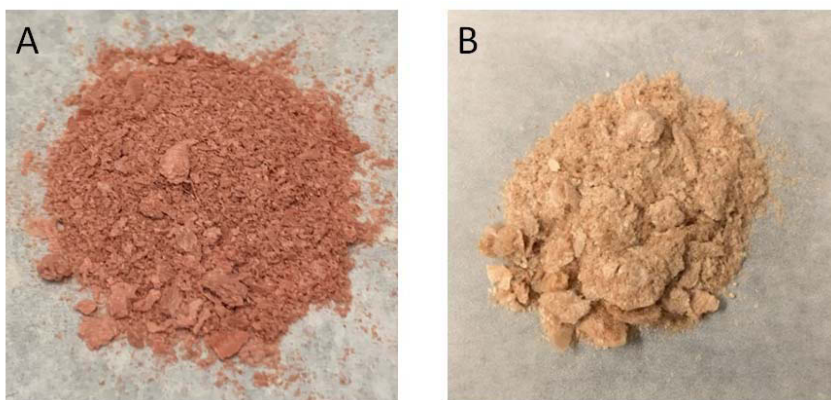
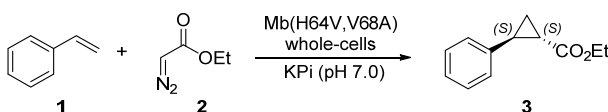


Table 3. Catalytic activity of freshly prepared and lyophilized whole cells expressing Mb(H64V,V68A) for styrene cyclopropanation with ethyl diazoacetate.^[a]



Entry	Catalyst	Yield	TON	de	ee
1	Fresh	>99%	363	>99%	>99%
2	Lyophilized	>99%	n.d.	>99%	>99%
3	Fresh ^[b]	44%	402	>99%	99%
4	Lyophilized ^[b]	62%	560	>99%	>99%
5	Lyophilized ^[c]	71%	n.d.	>99%	98%

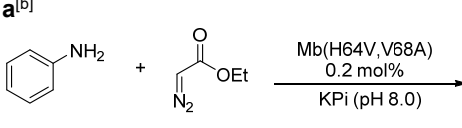
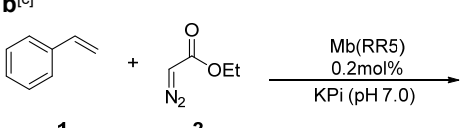
^[a] Reaction conditions: 10 mM styrene, 20 mM EDA, 8% EtOH (v/v), OD₆₀₀=40, potassium phosphate buffer (50 mM, pH 7.0), anaerobic, R.T., 16 h. ^[b] OD₆₀₀ = 20. ^[c] OD₆₀₀ = 20 using lyophilized cells stored at room temperature for six months. Product yield, diastereomeric excess (% de), enantiomeric excess (% ee), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards n.d. = not determined.

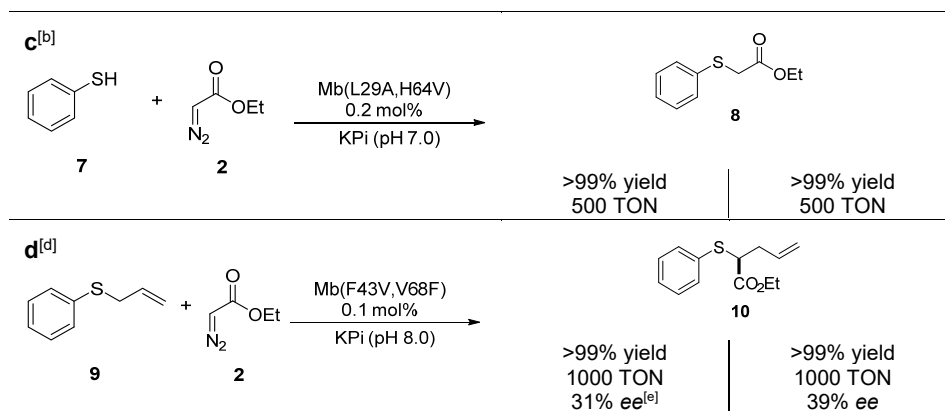
Other Mb-based carbene transferases

Having established the ability of Mb(H64V,V68A) cyclopropanase to maintain activity and stereoselectivity after lyophilization, further experiments were carried out to determine the generality of this property across other Mb(H64V,V68A)-catalyzed reactions (**Table 4a**) as well

as across other Mb-based carbene transferases (**Table 4b-d**). Mb(H64V,V68A) was previously reported to catalyze the N-H insertion of amines in the presence of diazo compounds.[31] In a model reaction with aniline (**4**) and EDA (**2**), lyophilized Mb(H64V,V68A) was found to produce the N-H insertion product **5** in quantitative yield and support 500 TON (**Table 4a**), thus displaying comparable activity to the non-lyophilized control. Next, we tested the catalytic activity of lyophilized Mb(L29T,F43W,H64V,V68F) (a.k.a. Mb(RR5)), which was previously developed to catalyze the cyclopropanation of vinylarenes with EDA with high *trans*-1*R*,2*R* selectivity, i.e. with opposite enantioselectivity compared to Mb(H64V,V68A) (**Table 4b**).[24] After lyophilization, this variant produced cyclopropane **6** in quantitative yield and high stereoselectivity (99% *de*, 95% *ee*), thus exhibiting no reduction in activity and selectivity compared to the non-lyophilized protein. Similar experiments were carried with Mb(L29A,H64V), which was previously optimized to catalyze S-H insertion reactions with thiols and diazoacetates.[32] Also in this case, the Mb variant was found to maintain its catalytic activity after lyophilization, resulting in the quantitative yield of the desired S-H insertion product **8** from the reaction with thiophenol (**7**) and EDA (**2**) (**Table 4c**).

Table 4. Catalytic activity and selectivity of engineered Mb catalysts for different carbene transfer reactions before and after lyophilization.^[a]

	Solution	Lyophilized
a ^[b] 	>99% yield 500 TON	>99% yield 500 TON
b ^[c] 	>99% yield 500 TON 99% <i>de</i> and 95% <i>ee</i>	>99% yield 500 TON 99% <i>de</i> and 95% <i>ee</i>



^[a] Reaction conditions: 10 mM substrate, 10 or 20 mM EDA, 10 mM Na₂S₂O₄, 10% EtOH, potassium phosphate buffer (50 mM, pH 7.0 or 8.0), anaerobic, room temperature, 16 hours. ^[b] 20 mM EDA and pH 7.0. ^[c] 10 mM EDA and pH 8.0. ^[d] 20 mM EDA and pH 8.0. ^[e] According to reference 44. Product yield, diastereomeric excess (% *de*), enantiomeric excess (% *ee*), and turnover number (TON) were determined by chiral GC-FID analysis using calibration curves with authentic (racemic) standards.

Lastly, we tested Mb(F43V,V68F), a Mb-based catalyst previously optimized for catalyzing asymmetric Doyle-Kirmse reactions with allenyl sulfides and EDA.[44] In a model reaction with allyl phenyl sulfide (**9**) and EDA (**2**), the lyophilized Mb variant was able to support 1,000 TON and produce the [2,3]-sigmatropic rearrangement product **10** in quantitative yield and 39% *ee*, thus mirroring the results obtained with the same protein prior to lyophilization. Altogether, these results combined demonstrate that robustness to lyophilization is shared by different Mb variants and across different types of Mb-catalyzed carbene transfer reactions.

Recyclability of Mb-containing whole-cell biocatalyst

Recyclability is another attractive feature of biocatalysts used for organic synthesis,[56] which is often pursued through immobilization of enzyme to solid supports.[57-60] Given the demonstrated functionality of our Mb-based catalysts in whole-cell systems, we were interested in investigating the possibility of recycling Mb-containing whole cells in multiple rounds of catalysis. In particular, we envisioned that entrapment of the biocatalyst in the cell would facilitate re-

isolation of the biocatalyst after the reaction, while protecting it from denaturation during the product extraction process (typically performed with halogenated solvents such as dichloromethane). For these experiments, *E. coli* cells expressing Mb(H64V,V68A) were applied to catalyze the cyclopropanation of *p*-methoxy-styrene (**11**) with EDA over 3 three consecutive rounds of catalysis. After each round, the cells were pelleted, washed with buffer containing 1.5% (v/v) Tween 20 surfactant to remove residual product (and unreacted reagents), and then resuspended in buffer for use in the next round of catalysis. At each cycle, the product was isolated from the supernatant of the cell suspension and wash solutions. These experiments showed a relative activity of 80-85% for both the second and third round of catalysis compared to the first round. In addition, excellent levels of stereoselectivity (>99% *de* and *ee*) were observed after each rounds of catalysis, indicating that the Mb catalyst is responsible for the observed cyclopropanation activity and no erosion of stereoselectivity occurred during the biocatalyst recovery process. These results thus provide a proof-of-principle demonstration of the recyclability of the Mb carbene transferase in whole cells and its ability to maintain high levels of activity and stereoselectivity across multiple cycle of catalysis using these systems.

Conclusions

In conclusion, our studies demonstrate that Mb-based carbene transferases show a remarkable organic solvent stability and tolerate the presence of various organic solvents such as methanol, ethanol, acetonitrile, DMF, and DMSO, in concentrations up to 30-50% (v/v) without significant reduction in activity and stereoselectivity. As these organic co-solvent concentrations are often required for biocatalysis at the large scale (e.g., 50% DMSO for sitagliptin manufacturing)[15], the inherent organic solvent stability of these Mb-based carbene transferases should facilitate their use in the context of these applications without requiring further protein engineering. This work

also shows that these biocatalysts are able to withstand lyophilization, both as a purified protein and in whole-cells, with excellent retention of catalytic activity and stereoselectivity. These lyophilized biocatalysts exhibit long shelf-life stability and could be stored at room temperature for over six months with any loss in activity or stereoselectivity. Finally, we demonstrate the recyclability of Mb-expressing whole cells across multiple rounds of carbene transfer catalysis and we implemented technically simple protocols for performing these biocatalytic transformations which bypasses the need for specialized instrumentation (anaerobic chamber) or techniques (cannulation) to ensure microaerobic conditions for optimal catalytic activity. Altogether, these findings demonstrate the robustness of myoglobin-based carbene transferases under operationally relevant conditions and should facilitate the practical exploitation of these biocatalysts for synthetic applications.

Acknowledgements

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Supporting Information for

Organic Solvent Stability and Long-term Storage of Myoglobin-based Carbene Transfer Biocatalysts

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Table S1. Product yield, catalytic turnovers (TON), and stereoselectivity selectivity of Mb(H64V,V68A)-catalyzed cyclopropanation of styrene and EDA at varying concentrations of the different organic solvents.

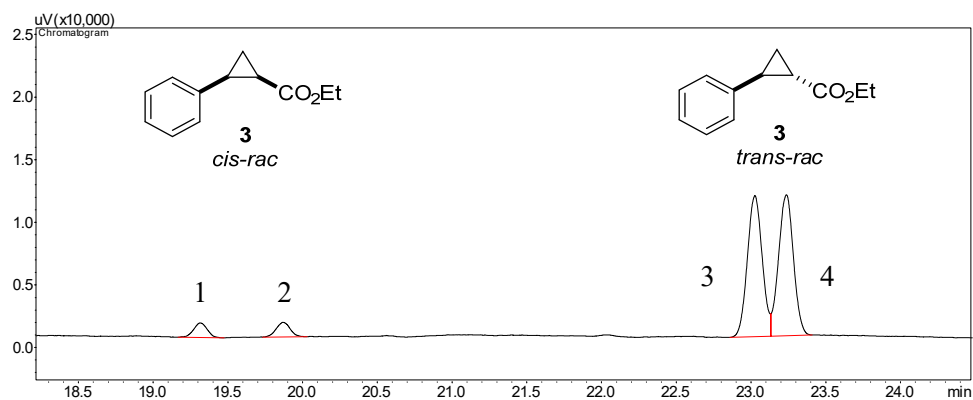
Solvent (v/v%)	Ethanol				Methanol				<i>N,N</i> -dimethylformamide			
	Yield (%)	TON	<i>de</i> (%)	<i>ee</i> (%)	Yield (%)	TON	<i>de</i> (%)	<i>ee</i> (%)	Yield (%)	TON	<i>de</i> (%)	<i>ee</i> (%)
6	>99 ± 0.8	498 ± 4	>99 ± 0.0	>99	69 ± 1.4	346 ± 7	>99 ± 0.0	>99	93 ± 3.6	465 ± 18	>99 ± 0.0	>99
10	>99 ± 2.5	493 ± 13	>99 ± 0.0	>99	80 ± 6.1	402 ± 30	>99 ± 0.0	>99	98 ± 3.9	489 ± 20	>99 ± 0.1	>99
20	>99 ± 1.4	496 ± 8	>99 ± 0.0	>99	64 ± 7.6	318 ± 38	>99 ± 0.0	>99	91 ± 1.7	457 ± 9	>99 ± 0.0	>99
30	>99 ± 0.0	500 ± 0	>99 ± 0.1	>99	75 ± 7.3	374 ± 37	>99 ± 0.0	>99	>99 ± 0.7	498 ± 3	>99 ± 0.0	>99
40	45 ± 2.7	226 ± 14	95 ± 0.2	67	83 ± 4.4	413 ± 22	>99 ± 0.0	>99	83 ± 3.3	416 ± 17	>99 ± 0.0	>99
50	27 ± 1.7	137 ± 9	89 ± 0.5	35	77 ± 2.8	387 ± 14	99 ± 0.1	96	21 ± 0.5	106 ± 2	98 ± 0.2	81
60	22 ± 3.0	109 ± 15	88 ± 0.4	32	24 ± 5.5	120 ± 28	96 ± 1.8	89	8.1 ± 0.3	41 ± 2	95 ± 0.4	37
Solvent (v/v%)	Acetonitrile				Dimethyl sulfoxide							
	Yield (%)	TON	<i>de</i> (%)	<i>ee</i> (%)	Yield (%)	TON	<i>de</i> (%)	<i>ee</i> (%)				
6	73 ± 2.9	364 ± 15	>99 ± 0.0	>99	90 ± 5.0	452 ± 25	>99 ± 0.0	>99				
10	88 ± 5.8	438 ± 29	>99 ± 0.0	>99	85 ± 3.9	424 ± 19	>99 ± 0.0	>99				
20	88 ± 2.4	437 ± 12	>99 ± 0.0	>99	86 ± 4.3	428 ± 22	>99 ± 0.0	>99				
30	90 ± 2.9	449 ± 15	99 ± 0.1	>99	67 ± 5.9	334 ± 30	>99 ± 0.0	>99				
40	45 ± 2.0	223 ± 10	96 ± 0.2	>99	34 ± 4.3	168 ± 22	>99 ± 0.0	98				
50	31 ± 1.2	152 ± 6	93 ± 0.0	42	13 ± 1.2	64 ± 6	>99 ± 0.0	94				
60	24 ± 0.8	119 ± 4	92 ± 0.1	46	2.0 ± 0.1	10 ± 0	99 ± 0.1	88				

Table S2. Recovery of Mb(H64V,V68A) catalyst after lyophilization as purified protein and whole cells. Percent recovery was determined by comparison between concentration (purified protein or whole cells) or optical density (whole cells) before and after lyophilization (experimental details can be found under **Synthetic Procedures**). Fe(III), Fe(II), and Fe(II)(CO) Soret bands calculated at 408, 436, and 425 nm, respectively. Myoglobin concentration was determined using an extinction coefficient $\epsilon_{410} = 157 \text{ mM}^{-1} \text{ cm}^{-1}$. Samples were lyophilized for 24 hours and resuspended in KPi buffer. ^[a] Concentrations were determined from lysate. ^[b] Optical density calculated at 600 nm. n.a. = not applicable.

Catalyst	% Recovery			
	Fe (III)	Fe (II)	Fe (II)(CO)	OD ₆₀₀
Purified Mb(H64V,V68A)	94% ± 2.3%	99% ± 6.2%	99% ± 11.0%	n.a.
Mb(H64V,V68A) whole cells	97% ^[a]	96% ^[a]	93% ^[a]	91% ^[b]

Figure S2. GC chromatograms for determination of enantiomeric excess in the styrene cyclopropanation reactions catalyzed by Mb(H64V,V68A) and Mb(RR5). Reference racemic sample was prepared using Rh₂(OAc)₄ catalyst as described in the **Synthetic Procedures** section.

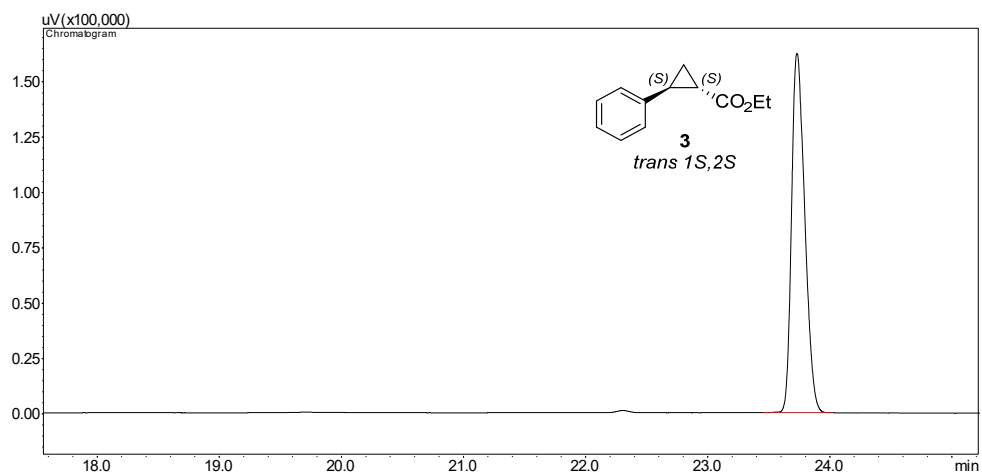
(a) Racemic (*cis* and *trans*) ethyl-2-phenylcyclopropane-1-carboxylate (**3**)



Peak	tR (min)
1 <i>cis</i> (1 <i>R</i> ,2 <i>S</i>)	19.316
2 <i>cis</i> (1 <i>S</i> ,2 <i>R</i>)	19.869
3 <i>trans</i> (1 <i>R</i> ,2 <i>R</i>)	23.026
4 <i>trans</i> (1 <i>S</i> ,2 <i>S</i>)	23.237

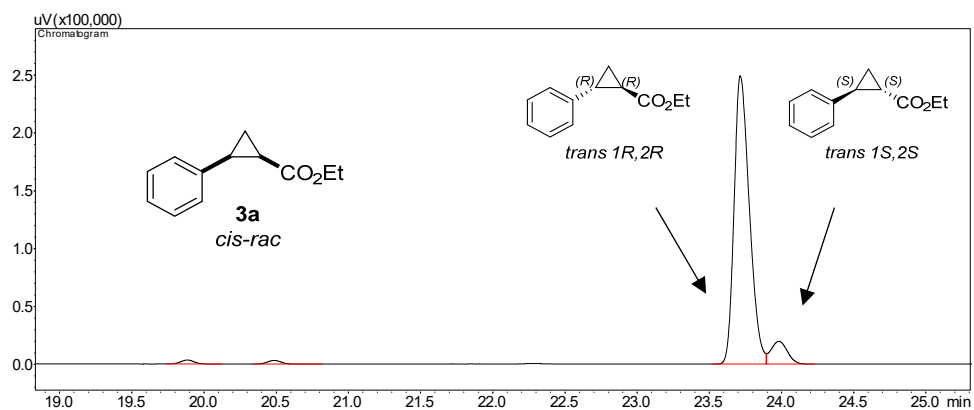
(b) Mb(H64V,V68A)-catalyzed ethyl-(1*S*,2*S*)-2-phenylcyclopropane-1-carboxylate (**3**).

n.a = not applicable



Peak	tR (min)
1 <i>cis</i> (1 <i>R</i> ,2 <i>S</i>)	n.a
2 <i>cis</i> (1 <i>S</i> ,2 <i>R</i>)	n.a
3 <i>trans</i> (1 <i>R</i> ,2 <i>R</i>)	n.a
4 <i>trans</i> (1 <i>S</i> ,2 <i>S</i>)	23.237

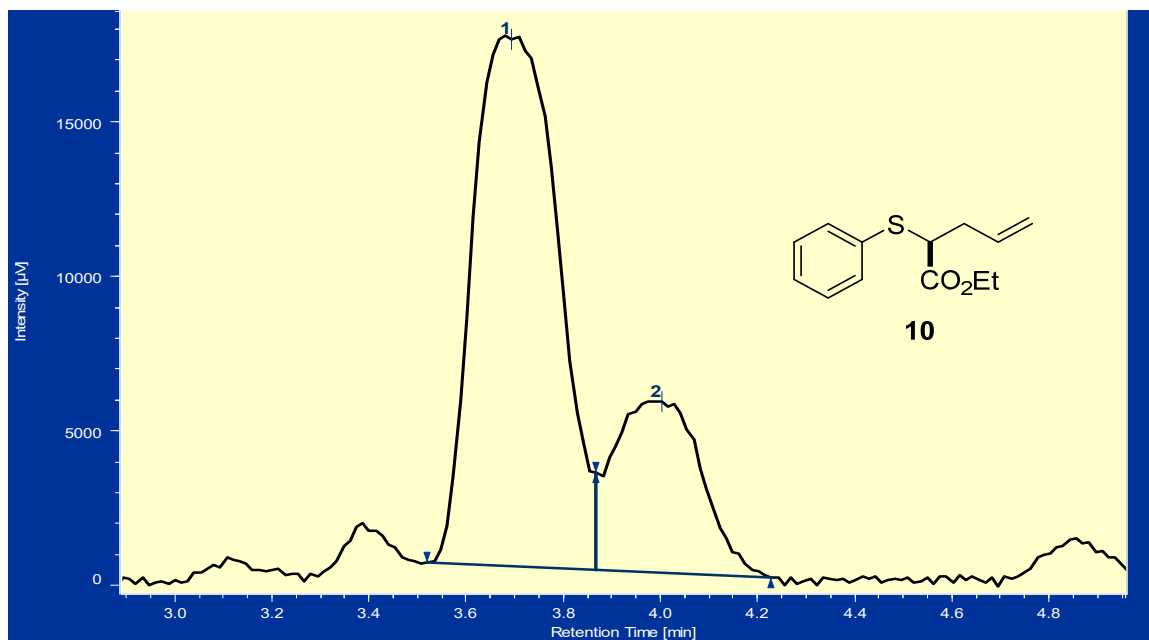
(c) Mb(RR5)-catalyzed ethyl-(1*R*,2*R*)-2-phenylcyclopropane-1-carboxylate (**6**)



Peak	tR (min)
1 <i>cis</i> (1 <i>R</i> ,2 <i>S</i>)	19.887
2 <i>cis</i> (1 <i>S</i> ,2 <i>R</i>)	20.484
3 <i>trans</i> (1 <i>R</i> ,2 <i>R</i>)	23.713
4 <i>trans</i> (1 <i>S</i> ,2 <i>S</i>)	23.982

Figure S3. SFC chromatogram for determination of enantiomeric excess in the allyl phenyl sulfide Doyle-Kirmse reactions catalyzed by Mb(H64V,V68A).

Separation method: Column IB, IPA:CO₂ (99.8:0.2).

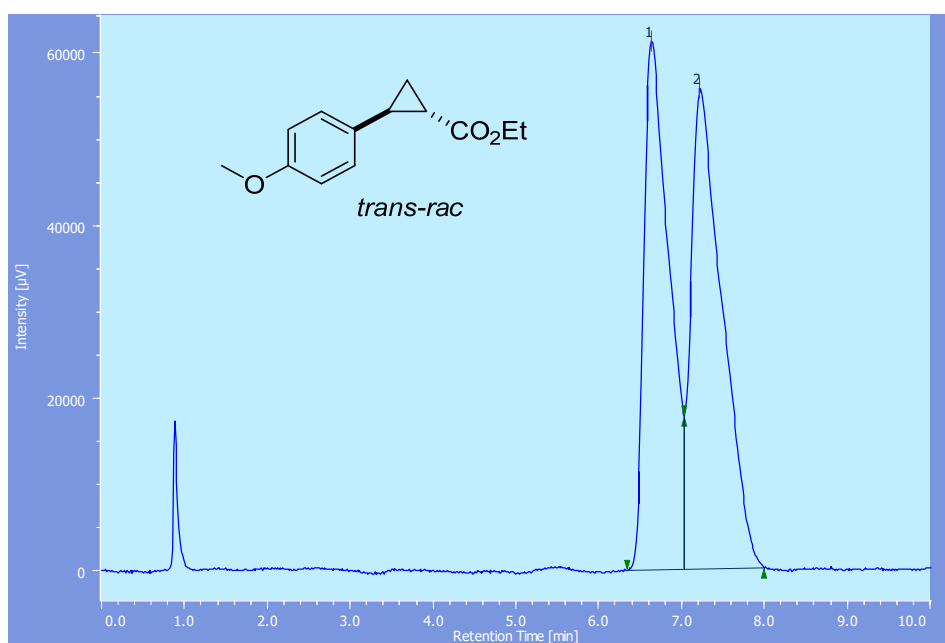


Peak	tR (min)
1	3.712
2	4.081

Figure S2. SFC chromatograms for determination of enantiomeric excess in the 4-vinylanisole cyclopropanation reactions catalyzed by Mb(H64V,V68A). Reference racemic sample was prepared using Rh₂(OAc)₄ catalyst as described in the **Synthetic Procedures** section.

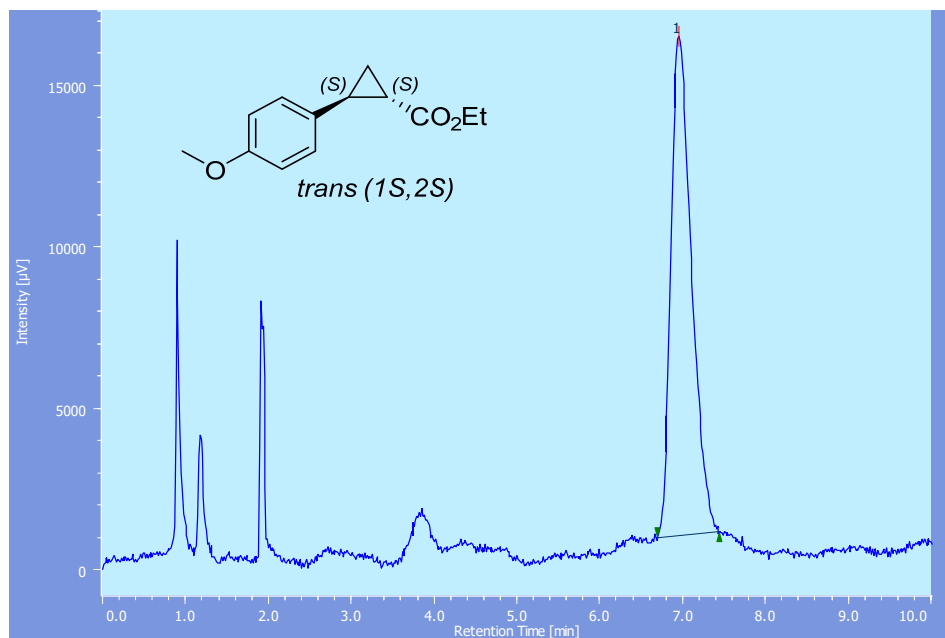
Separation method: Column IC, IPA:CO₂ (98:2).

a) Racemic (*trans*) ethyl 2-(4-methoxyphenyl)cyclopropanecarboxylate:



Peak	tR (min)
1 (1 <i>R</i> ,2 <i>R</i>)	6.627
2 (1 <i>S</i> ,2 <i>S</i>)	7.213

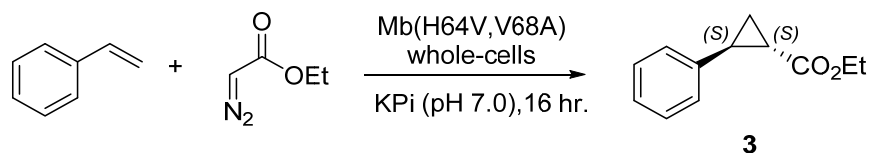
b) Mb(H64V,V68A)-catalyzed ethyl (1*S*,2*S*)-2-(4-methoxyphenyl)cyclopropanecarboxylate
(11):



Peak	tR (min)
1 (1 <i>R</i> ,2 <i>R</i>)	n.a
2 (1 <i>S</i> ,2 <i>S</i>)	6.960

Synthetic Procedures:

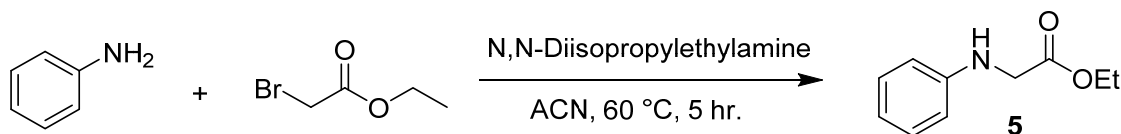
Preparative scale procedure for enzymatic synthesis of ethyl (1*S*,2*S*)-2-phenylcyclopropane-1-carboxylate (**3**):



To a 250 mL Erlenmeyer flask, equipped with a stir bar, was added *E. coli* (BL21(DE3)) whole-cells expressing Mb(H64V,V68A) at OD₆₀₀ = 40 in potassium phosphate buffer (KPi, 50 mM, pH = 7.0) and 10% glucose. Then, the head space of the flask was purged with Ar for 15 min. A solution of styrene in EtOH (1 equiv.) was added followed by the slow addition of EDA in EtOH (2 equiv.) using a syringe pump over 3 hours. The reaction was stirred overnight at room temperature under positive argon pressure. The crude product was extracted with CH₂Cl₂ (50mL x4) and dried over Na₂SO₄. After filtration, the crude was concentrated *in vacuo* and purified by silica gel column chromatography using 95:5 hexanes:ethyl acetate as elluent. Compound **3** was obtained as a yellow oil (90 mg, 90% yield). The collected data is in accordance with those reported in literature.¹

¹H NMR (400 MHz, CDCl₃): δ 7.28 (t, *J*=7.5 Hz, 2H), 7.20 (t, *J*=7.3 Hz, 1H), 7.10 (d, *J*=7.3 Hz, 2H), 4.18 (q, *J*=7.1 Hz, 2H), 2.52 (ddd, *J*=9.1, *J*=6.4, *J*=4.2 Hz, 1H), 1.91 (ddd, *J*=8.5, *J*=5.2, *J*=4.3 Hz, 1H), 1.60 (ddd, *J*=9.4, *J*=4.8 Hz, *J*=4.8 Hz, 1H), 1.33 (ddd, *J*=8.4, *J*=6.5 Hz, *J*=4.5 Hz, 1H), 1.28 ppm (t, *J*=7.1 Hz, 3H). **¹³C NMR** (100 MHz, CDCl₃): δ 173.2, 140.0, 128.3, 126.3, 126.0, 60.6, 26.1, 24.1, 17.0, 14.2 ppm.

Chemical Synthesis of ethyl phenylglycinate (**5**):



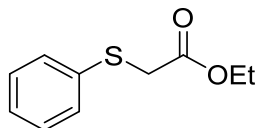
To a flame-dried round bottom flask, equipped with a stir bar, was added aniline (1 equiv.) and *N,N*-Diisopropylethylamine (2 equiv.) in acetonitrile (2-3 mL). The solution was heated to 60 °C and ethyl bromoacetate (1 equiv.) was added dropwise over 2 hours and left stirring for an additional 3 hours. The solvent was removed under reduced pressure and the leftover crude solid was thoroughly washed with distilled water and recrystallized in toluene. The product was further purified by flash column chromatography using a 9:1 hexanes:ethyl acetate solution affording 112.5 mg of a pale brown solid (17% yield). The collected data are in accordance with those reported in literature.²

¹H NMR (500 MHz, CDCl₃): δ 7.23 (t, *J*= 6.0 Hz, 2H), 6.79 (t, *J*= 6.5 Hz, 1H), 6.64 (d, *J*= 7.0 Hz, 2H), 4.34-4.24 (m, 3H), 3.91 (s, 2H), 1.33 (t, *J*= 6.5 Hz, 3H) ppm. **¹³C NMR** (125 MHz, CDCl₃): δ 171.2, 147.1, 129.3, 118.1, 113.0, 61.3, 45.8, 14.2 ppm.

General Procedure for Chemical Synthesis of S-H Insertion, Doyle-Kirmse Reaction, and Racemic Cyclopropanation Standards using Rh₂O(Ac)₄ (Procedure A): To a flame-dried round bottom flask, equipped with a stir bar, was added the desired nucleophile (styrene, thiophenol, or allyl phenyl sulfide, 1-5 equiv.) and Rh₂(OAc)₄ (5 mol%) in CH₂Cl₂ (2-5 mL) under argon. To this solution was added a solution of EDA (2 equiv.) in CH₂Cl₂ (5-10 mL) via slow addition (20 min to 3 hours) using a syringe pump. The resulting mixture was stirred for the indicated amount of time. The solvent was removed under reduced pressure and the crude mixture

was purified by flash chromatography using a 9:1 hexanes:ethyl acetate solution. The identities of the products were determined using ^1H and ^{13}C NMR.

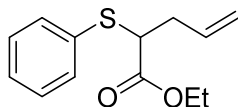
Ethyl(phenylthio)acetate (8):



Following the standard procedure A, compound **8** was obtained as a clear oil (0.5% yield). The collected data are in accordance with those reported in literature.³

^1H NMR (500 MHz, CDCl_3): δ 7.32 (d, $J=7.5$ Hz, 2H), 7.26-7.21 (m, 3H), 4.18 (q, $J=7.0$ Hz, 2H), 3.63 (s, 2H), 1.24 (t, $J=7.0$ Hz, 3H) ppm. **^{13}C NMR** (125 MHz, CDCl_3): δ 169.7, 135.0, 130.0, 129.0, 126.9, 61.5, 36.7, 14.1 ppm.

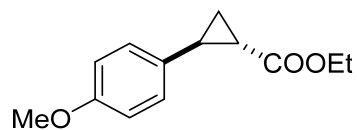
Ethyl 2-(phenylsulfanyl)-4-pentenoate (10):



Following the standard procedure A, compound **8** was obtained as a clear oil (6.0% yield). The collected data are in accordance with those reported in literature.⁴

^1H NMR (500 MHz, CDCl_3): δ 7.47 (d, $J= 5.5$ Hz, 2H), 7.30-7.26 (m, 3H), 5.85-5.76 (m, 1H), 5.15-5.08 (m, 2H), 4.18-4.09 (m, 2H), 3.71-3.67 (m, 1H), 2.66-2.60 (m, 1H), 2.54-2.49 (m, 1H), 1.18-1.14 (m, 3H) ppm. **^{13}C NMR** (125 MHz, CDCl_3): δ 171.6, 133.9, 133.1, 128.9, 128.0, 118.0, 61.1, 50.3, 35.8, 14.1 ppm.

Ethyl 2-(4-methoxyphenyl)cyclopropanecarboxylate (11):



Following the standard procedure **A**, *trans* isomer **12** was obtained as a clear oil (81% yield). The collected data are in accordance with those reported in literature.¹

¹H NMR (400 MHz, CDCl₃): δ 7.03 (d, *J*=8.4 Hz, 2H), 6.82 (d, *J*=8.4 Hz, 2H), 4.18 (q, *J*=7.2 Hz, 2H), 3.76 (s, 3H), 2.47 (ddd, *J*=9.2, *J*=6.4, *J*=4.2 Hz, 1H), 1.82 (ddd, *J*=8.4, *J*=5.0, *J*=4.2 Hz, 1H), 1.56-1.52 (m, 1H), 1.32 (t, *J*=7.2 Hz, 3H), 1.23 ppm (ddd, *J*=8.4, *J*=6.5, *J*=4.5 Hz, 1H). **¹³C NMR** (100 MHz, CDCl₃): δ 173.5, 158.3, 132.1, 127.5, 113.9, 60.6, 55.2, 25.6, 23.8, 16.7, 14.2 ppm.

References.

1. Bordeaux, M., Tyagi, V., Fasan, R. *Angew. Chem. Int. Ed.* **2015**, *54*, 1744-1748
2. Sreenilayam, G., Fasan, R. *Chem. Commun.* **2015**, *51* (8), 1532-1534.
3. Tyagi, V., Bonn, R. B., Fasan, R. *Chem. Sci.* **2015**, *6* (4), 2488-2494.
4. Tyagi, V., Sreenilayam, G., Bajaj, P., Tinoco, A., Fasan, R. *Angew. Chem. Int. Ed.* **2016**, *55* (43), 13562-13566.