

Interfacing Microbial Styrene Production with a Biocompatible Cyclopropanation Reaction**

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Abstract: The introduction of new reactivity into living organisms is a major challenge in synthetic biology. Despite an increasing interest in both the development of small-molecule catalysts that are compatible with aqueous media and the engineering of enzymes to perform new chemistry *in vitro*, the integration of non-native reactivity into metabolic pathways for small-molecule production has been underexplored. Herein we report a biocompatible iron(III) phthalocyanine catalyst capable of efficient olefin cyclopropanation in the presence of a living microorganism. By interfacing this catalyst with *E. coli* engineered to produce styrene, we synthesized non-natural phenyl cyclopropanes directly from D-glucose in single-vessel fermentations. This process is the first example of the combination of nonbiological carbene-transfer reactivity with cellular metabolism for small-molecule production.

The field of synthetic biology is changing how important small molecules are manufactured.^[1] By using renewable starting materials (e.g. sugar, plant biomass, CO₂) metabolic engineers overproduce small molecules in single-vessel fermentations by optimizing both known and *de novo* biosynthetic pathways in host microorganisms. Despite significant progress in this discipline, a major remaining challenge is the engineering of organisms to access compounds of non-natural origin. This objective is important because many small molecules of commercial interest are not currently accessible by the use of known enzymatic chemistry. Two strategies toward this goal have emerged: the engineering of nonbiological reactivity into enzymes and the development of nonenzymatic catalysts that can be interfaced with cellular metabolism.^[2,3]

Exploration of the reactivity of carbenes has been particularly fruitful for enzyme engineering efforts. Carbene intermediates are accessed and utilized in cells by thiamine diphosphate dependent enzymes (Figure 1 A).^[4] In previous studies, ruthenium carbene complexes have been introduced within artificial metalloenzymes for olefin ring-closing metathesis in water and phosphate buffer.^[5] More recent studies by the Arnold and Fasan research groups have extended the

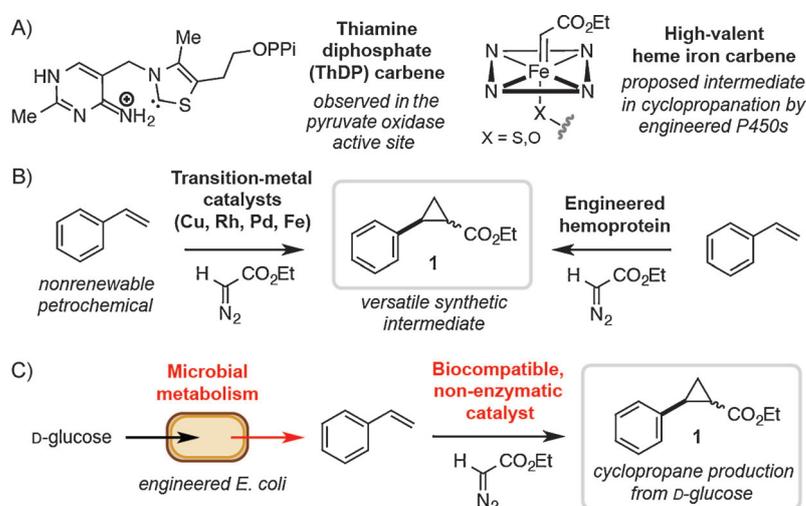


Figure 1. Design of a biocompatible cyclopropanation reaction. A) Carbene intermediates in biological catalysis. B) Current approaches for accessing ethyl 2-phenylcyclopropane-1-carboxylate (1) from styrene by transition-metal-mediated carbene chemistry. C) Phenyl cyclopropane production from D-glucose by combining *in vivo* styrene production with biocompatible chemistry.

types of carbenes involved in enzymatic catalysis to include iron carbenes by the engineering of heme-binding proteins to catalyze enantioselective olefin cyclopropanation with ethyl diazoacetate (EDA) both *in vitro* and in a whole-cell format (Figure 1 B).^[2b,f,g,6] These studies were inspired by the mechanistic similarities between cytochrome P450 oxene-transfer catalysis and transition-metal-mediated carbene-transfer reactions. This research provides a biocatalytic route to cyclopropanes, which are found in many bioactive synthetic molecules.^[7] Despite the success of these engineering efforts, enzymes that utilize metallocarbene intermediates have not yet been integrated into engineered metabolic pathways.

We envisioned approaching this challenge by using biocompatible chemistry: nonenzymatic reactions capable of modifying metabolites as they are made by living organisms (Figure 1 C).^[3] Herein we describe the identification of an iron phthalocyanine catalyst that cyclopropanates

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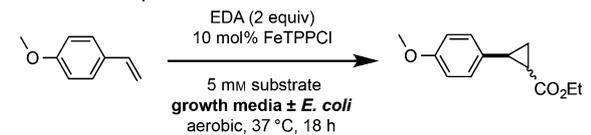
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styrene generated by engineered microbial metabolism. This reaction is both a new biocompatible iron-mediated transformation and, to the best of our knowledge, the first example of metallocarbene chemistry interfaced with cellular metabolism for small-molecule production.

We began our studies by investigating the efficiency of olefin cyclopropanation under conditions compatible with the growth of *Escherichia coli*. We initially examined the tetraphenylporphyrin iron(III) chloride (FeTPPCL) catalyzed cyclopropanation of 4-vinylanisole and EDA. This reaction has been previously reported to occur under aqueous alkaline conditions with diazomethane, and also in aqueous phosphate buffer (pH 7.0) under anaerobic conditions with the hemin cofactor as a catalyst (29% conversion).^[2g,8] We performed the FeTPPCL-mediated reaction in water, phosphate buffer (pH 7.0), and growth media of increasing complexity. We found that although the reaction was moderately efficient in water, phosphate buffer and growth media provided significantly higher conversions and diastereoselectivities (Table 1). Unlike other biocompatible transformations, cyclopropanation proceeded efficiently in the complex medium Luria-Bertani broth (LB).^[3a] The reasons for the increased conversion in phosphate buffer and growth media relative to water are unclear; however, similar effects observed for other reactions carried out in highly ionic solvents are hypothesized to arise from rate acceleration due to an increased hydrophobic effect.^[9] Finally, the addition of bacterial cells (*E. coli* BL21(DE3), OD₆₀₀ = 0.5) had no detrimental effect on the reaction yield or selectivity (Table 1, entries 5 and 7).

We next conducted a catalyst screen in M9CA–glucose media containing *E. coli* (Table 2; see also Table S1 in the Supporting Information). The presence of electron-withdrawing and electron-donating substituents on the porphyrin ring system influenced product diastereoselectivity but diminished conversion relative to FeTPPCL (Table 2, entries 2 and 3).

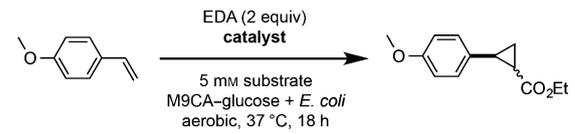
Table 1: FeTPPCL-catalyzed cyclopropanation reactions in aqueous media and in the presence of *E. coli*.^[a]



Entry	Growth medium	<i>E. coli</i> cells added?	Yield [%]	<i>trans/cis</i>
1	H ₂ O	no	46	3:1
2	0.1 M K ₂ HPO ₄	no	77	3.7:1
3	M9–glucose	no	71	3.7:1
4	M9CA–glucose	no	72	4:1
5	M9CA–glucose	yes	75	3.9:1
6	LB	no	71	4:1
7	LB	yes	70	4:1

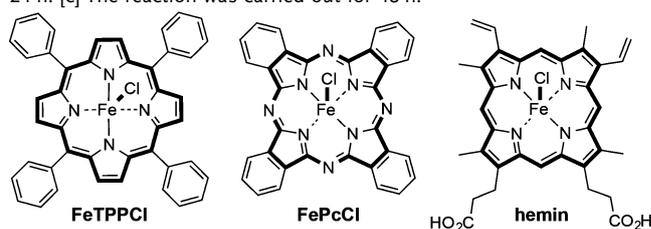
[a] Reactions were performed with 4-vinylanisole (5 mM), EDA (10 mM), and FeTPPCL (0.5 mM) in sealed Hungate tubes under an atmosphere of air. All cultures were grown in the presence of kanamycin (50 mg L⁻¹) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.2 mM). *E. coli* BL21 cells transformed with an empty pET-29b(+) expression plasmid (OD₆₀₀ = 0.5) were used. Product concentrations in crude culture extracts were determined by ¹H NMR spectroscopy relative to an internal standard of 1,3,5-trimethoxybenzene. All data are shown as an average of three experiments to one standard deviation.

Table 2: Catalyst screen in the presence of *E. coli*.^[a]



Entry	Catalyst (mol%)	Yield [%]	<i>trans/cis</i>
1	FeTPPCL (10)	75	3.9:1
2	Fe(F ₂₀ TPP)Cl (10)	55	5.1:1
3	Fe(OMe) ₄ TPPCL (10)	7	2.9:1
4	hemin (10)	0	–
5	FePcCl (10)	95	3.4:1
6	FePcCl (5)	93 ^[b]	3.4:1
7	FePcCl (2.5)	90 ^[c]	3.7:1
8	FePcCl (1)	80 ^[c]	4.0:1

[a] Reactions were performed as described in Table 1. All data are shown as an average of three experiments. [b] The reaction was carried out for 24 h. [c] The reaction was carried out for 48 h.



Replacement of the four aromatic rings of the TPP ring system with aliphatic substituents abolished catalytic activity (see Table S1). Hemin was unreactive under the reaction conditions (Table 2, entry 4), despite having previously been reported to catalyze this transformation *in vitro*.^[2g] Ferric phthalocyanine (FePcCl) proved to be an exceptional cyclopropanation catalyst and afforded the product in 95% yield (Table 2, entry 5). The catalyst loading of FePcCl could be reduced to as low as 1 mol% with only a moderate loss in conversion. No product was detected when FeCl₃ was used or in the absence of an added catalyst, thus ruling out the possibility that either endogenously biosynthesized metal-ion complexes or ferric ligands produced by *E. coli* contribute to catalysis *in vivo*.

The major by-product of the reaction at a catalyst loading of 10 mol% was diethyl maleate, which arises from EDA dimerization. Unexpectedly, at lower catalyst loadings we observed a new by-product, diethyl succinate (Figure 2; see

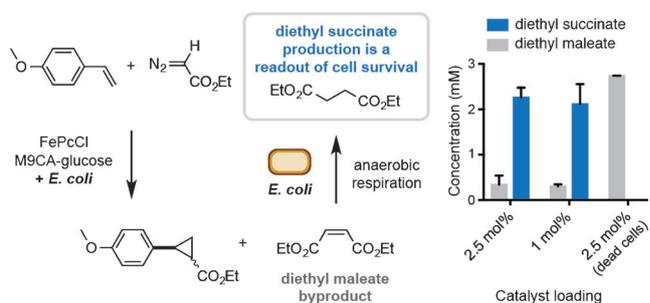


Figure 2. A reaction by-product reports on *E. coli* survival under the cyclopropanation reaction conditions. Reactions were performed as described in Table 1.

also Table S2). We hypothesized that this product arose from the use of diethyl maleate as a terminal electron acceptor by *E. coli* for anaerobic respiration under the progressively oxygen limiting conditions of the reaction setup. We also verified that living cells were required for diethyl succinate production. The formation of this by-product at a catalyst loading of 2.5 mol% therefore confirms that the *E. coli* bacteria are alive under our optimal reaction conditions.

Having shown that the FePcCl-catalyzed cyclopropanation was compatible with *E. coli*, we next attempted the reaction with styrene generated by bacterial metabolism. Styrene production from D-glucose was achieved in the L-phenylalanine-overproducing strain *E. coli* NST74 by introducing two enzymes: L-phenylalanine ammonia lyase from *Arabidopsis thaliana* (PAL2), which converts L-phenylalanine into *trans*-cinnamic acid, and a decarboxylase from *Saccharomyces cerevisiae* (FDC1) that generates styrene from *trans*-cinnamic acid (Figure 3 A).^[10]

We confirmed that FePcCl was effective under the conditions required for styrene production by performing the cyclopropanation reaction with 1.5 mM styrene in phosphate-limited minimal media (MM1) containing *E. coli* BL21(DE3) and D-glucose (82% yield, d.r. 1.7:1). Serial dilutions and plate counts directly from cultures with and

without reaction components showed no difference in survival (see Figure S1 in the Supporting Information). Together, these results suggested that our reaction would be compatible with in vivo styrene production. Accordingly, the *PAL2* and *FDC1* genes were introduced into *E. coli* NST74 on a single expression plasmid (*pTrc99A_PAL2-FDC1*). Under optimized conditions, this strain accumulated 1.65 mM styrene in the culture medium over 48 h (see Figure S5). We next attempted the cyclopropanation reaction by adding FePcCl (2.5 mol%) and EDA (2 equiv) to cultures at the point of induction of the styrene-producing pathway ($OD_{600} = 0.5\text{--}0.6$, $t = 0$ h). After 48 h we observed cyclopropane **1** by GC (44% conversion, d.r. 3.0:1). ¹H NMR spectroscopic analysis of the reaction extract showed no unreacted EDA and significant levels of EDA by-products, thus indicating that competing carbene dimerization was probably limiting reaction conversion. This issue was circumvented by adding EDA portionwise over the course of the fermentation; in this way, the yield of **1** was increased to 81%. By adding an additional equivalent of EDA and extending the reaction time to 60 h we obtained **1** in 95% yield (as determined by GC; Figure 3 B).

We performed a series of control experiments to confirm that cyclopropanation required the presence of the catalyst, EDA, and living *E. coli* (Figure 3 C). To obtain information

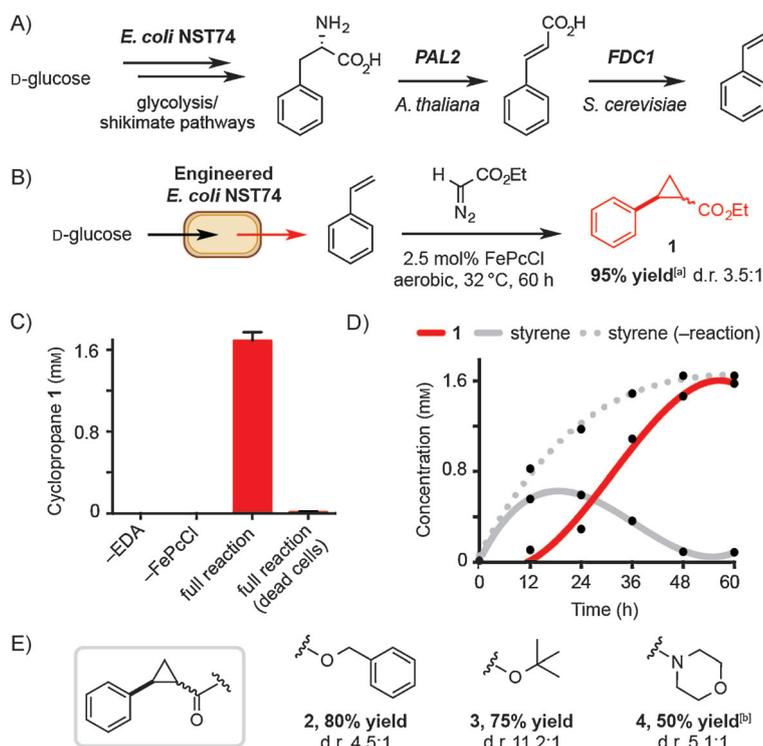


Figure 3. The biocompatible cyclopropanation reaction can be interfaced with microbial styrene production. A) Engineered pathway for styrene production in the L-phenylalanine overproducer *E. coli* NST74. B) Cyclopropanation of metabolically generated styrene. C) Graph showing that cyclopropane production requires all reaction components and living *E. coli*. D) Metabolite production during fermentations. E) Additional cyclopropanes accessed by this approach. Metabolite concentrations were determined by GC relative to an internal standard of 1,3,5-trimethoxybenzene. Yields in (E) are for isolated material from 800 mL cultures. All data are shown as an average of three independent experiments to one standard deviation. [a] The product was isolated in 93% yield. [b] The reaction was carried out for 72 h.

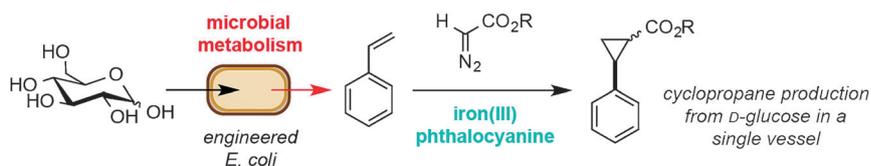
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Interfacing Microbial Styrene Production with a Biocompatible Cyclopropanation Reaction



The beauty of bugs: Iron(III) phthalocyanine was identified as a highly active and biocompatible carbene-transfer catalyst capable of cyclopropanating styrene generated by the metabolism of engineered *Escherichia coli*. The biocompatible reac-

tion is nontoxic to the producing organism, thus enabling the near-quantitative production of various phenyl cyclopropanes directly from D-glucose in single-vessel fermentations.