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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, CO_2) 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 types of carbenes involved in enzymatic catalysis to include iron carbenes by the engineering of hemin-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.

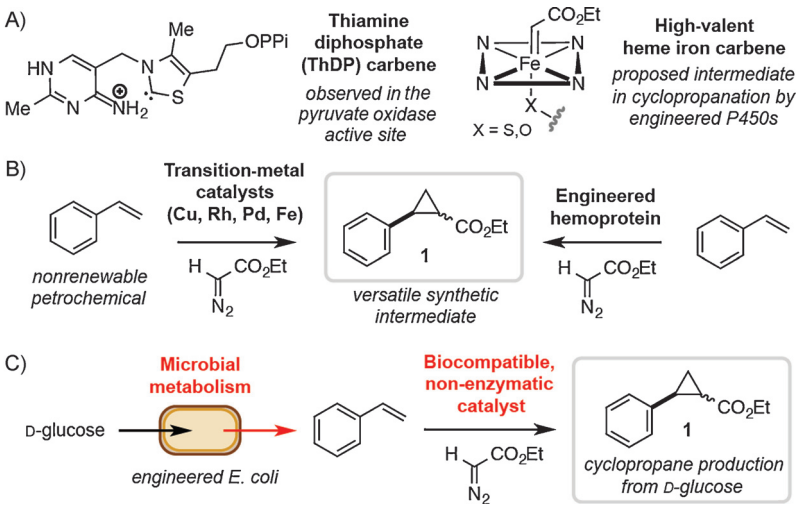

Figure 1. Design of a biocompatible cyclopropanation reaction. A) Carbene intermediates in biological catalysis. B) Current approaches for accessing ethyl 2-phenylcyclopropane-1-carboxylate (**1**) from styrene by transition-metal-mediated carbene chemistry. C) Phenyl cyclopropane production from D-glucose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

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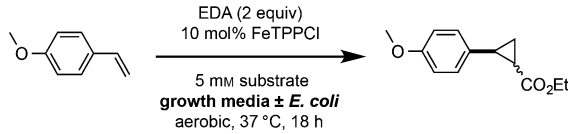
styrene generated by engineered microbial metabolism. This reaction is both a new biocompatible iron-mediated transformation and, to the best of our knowledge, the first example of metallocarbene chemistry interfaced with cellular metabolism for small-molecule production.

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), $OD_{600} = 0.5$) 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).

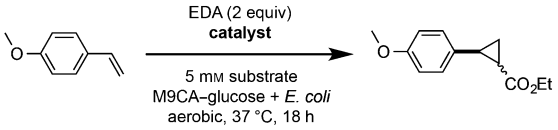
Table 1: FeTPPCl-catalyzed cyclopropanation reactions in aqueous media and in the presence of *E. coli*.[a]



Entry	Growth medium	*E. coli* cells added?	Yield [%]	*trans*/*cis*
1	H_2O	no	46	3:1
2	0.1 M K_2HPO_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

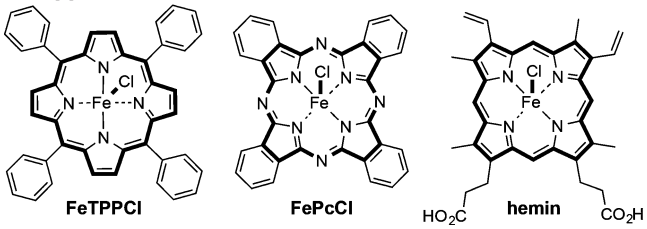
[a] Reactions were performed with 4-vinylanisole (5 mM), EDA (10 mM), and FeTPPCl (0.5 mM) in sealed Hungate tubes under an atmosphere of air. All cultures were grown in the presence of kanamycin (50 mg L^{-1}) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.2 mM). *E. coli* BL21 cells transformed with an empty *p*ET-29b(+) expression plasmid ($OD_{600} = 0.5$) were used. Product concentrations in crude culture extracts were determined by ^{1}H 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 [%]	*trans*/*cis*
1	FeTPPCl (10)	75	3.9:1
2	$Fe(F_{20}TPP)Cl$ (10)	55	5.1:1
3	$Fe(OMe)_4TPPCl$ (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 $FeCl_3$ 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

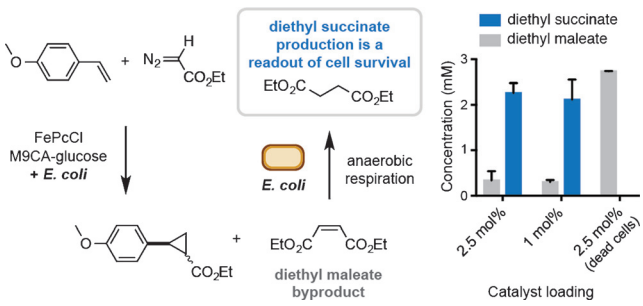


Figure 2. A reaction by-product reports on *E. coli* survival under the cyclopropanation reaction conditions. Reactions were performed as described in Table 1.

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 3A).[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 (*p*Trc99A_*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 ($OD_{600} = 0.5–0.6$, $t = 0$ h). After 48 h we observed cyclopropane **1** by GC (44% conversion, d.r. 3.0:1). ^{1}H 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 3B).

We performed a series of control experiments to confirm that cyclopropanation required the presence of the catalyst, EDA, and living *E. coli* (Figure 3C). To obtain information about the timing and rate of cyclopropanation relative to in vivo styrene production, we analyzed product distributions in fermentations with and without the reaction components (Figure 3D; see also Figure S4). In the presence of FePcCl and EDA, styrene reached a maximum concentration of 0.6 mM after 18 h and then steadily depleted as **1** accumulated. Ultimately, the concentration of **1** equaled the concentration of styrene produced in the absence of the reaction components. This observation confirms that the biocompatible cyclopropanation is interfaced with styrene output from *E. coli* and that after 18 h the rate of styrene consumption by cyclopropanation probably exceeds that of styrene generation by metabolism. This analysis also demonstrates that the reaction components have a minimal effect on the overall levels of styrene production. Interestingly, in the absence of FePcCl, accumulating EDA significantly inhibited styrene production after 12 h ($P < 0.05$), thus indicating that this reagent is toxic to *E. coli* and that the activity of the catalyst prevents this adverse effect in the full reaction (see Figure S5). A preliminary investigation of the cyclopropanation by using a three-phase test suggested that catalysis by FePcCl occurs at a solid–liquid interface, as no product was detected when a polymer-supported styrene was used (see Figure S6). This result indicates that FePcCl is probably functioning as a heterogeneous catalyst in this transformation.

We evaluated the scope of the in vivo cyclopropanation by examining other diazoacetate derivatives. Using additional electron-poor (acceptor) carbenes afforded cyclopropanes **2–4**,

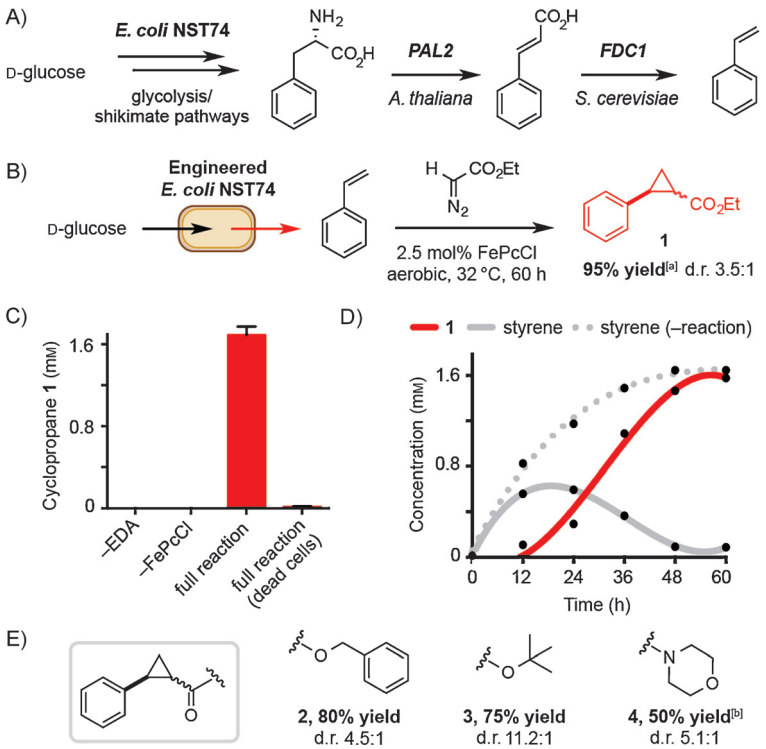


Figure 3. The biocompatible cyclopropanation reaction can be interfaced with microbial styrene production. A) Engineered pathway for styrene production in the L-phenylalanine overproducer *E. coli* NST74. B) Cyclopropanation of metabolically generated styrene. C) Graph showing that cyclopropane production 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.

which were isolated in good yields (Figure 3E). We also reexamined hemin as a catalyst to test the extent to which the results of our initial screening procedure predicted catalyst performance with metabolically generated styrene. Hemin remained a less efficient cyclopropanation catalyst (27% conversion after 60 h; see Table S4), thus confirming the utility of our catalyst-identification strategy. The low reactivity of hemin also resulted in EDA accumulation, which dramatically reduced overall styrene production levels to 0.23 mM. This finding shows that the identity of the nonenzymatic catalyst can influence multiple variables of in vivo reactions. Overall, these studies not only provide a new route to cyclopropanes but also a potential roadmap for the discovery of other biocompatible reactions.

The combined use of enzymatic and nonenzymatic catalysis for chemical synthesis can provide unique benefits over the use of chemo- or biocatalysis alone.[3c,11] In the case of this biocompatible cyclopropanation, the generation of styrene from D-glucose is attractive, as it avoids the use of nonrenewable petroleum streams. By intercepting biologically produced styrene in the fermentation vessel we also circumvent the challenges associated with its isolation, including volatility and reactivity (polymerization during gas stripping).[12] Additionally, the use of bench- and air-stable phthalocyanines circumvents the need for strictly anaerobic conditions during cyclopropanation (as are required by many engineered biocatalysts). This feature is critical for overall cyclopropane production, as an aerobic growth environment is needed for maximum styrene production by engineered *E. coli*.[10] Notably, this requirement has most likely made the incorporation of cyclopropanating enzymes into engineered metabolic pathways challenging.

The use of an inexpensive and highly abundant early-transition-metal catalyst adds to the appeal of this process. Indeed, interactions between microorganisms and iron are widespread in natural habitats, and evolutionary pressure to accommodate the reactivity of iron-containing minerals could account for the biocompatibility of our catalyst. The success of this cyclopropanation suggests that iron-mediated reactions are a promising source of new biocompatible reactions. Natural microbe–mineral interactions may also be good future starting points for the design of additional biocompatible nonenzymatic transformations.

In summary, we have shown that biocompatible metallocarbene-transfer catalysis and engineered metabolism can be combined for small-molecule production. The integration of metallocarbene chemistry with the metabolism of living organisms is a new approach to the construction of nonnatural molecules. Important future challenges include the development of biocompatible, chiral catalysts for enantioselective cyclopropanation and the engineering of pathways for the production of substituted styrene substrates. Finally, other nonenzymatic reactions that use styrene may also be good targets for biocompatible reaction development and allow access to further structural diversity from this single, engineered metabolic pathway.

Keywords: iron · metabolism · phthalocyanine · synthetic biology · synthetic methods
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