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# Stereoselective Olefin Cyclopropanation under Aerobic Conditions with an Artificial Enzyme Incorporating an Iron-Chlorin e6 Cofactor

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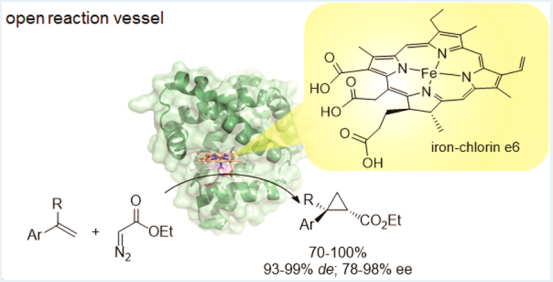
Supporting Information

**ABSTRACT:** Myoglobin has recently emerged as a promising biocatalyst for catalyzing carbene-mediated cyclopropanation, a synthetically valuable transformation not found in nature. Having naturally evolved for binding dioxygen, the carbene transferase activity of this metalloprotein is severely inhibited by it, imposing the need for strictly anaerobic conditions to conduct these reactions. In this report, we describe how substitution of the native heme cofactor with an iron-chlorin e6 complex enabled the development of a biocatalyst capable of promoting the cyclopropanation of vinylarenes with high catalytic efficiency (up to 6970 TON), turnover rate (>2000 turnovers/min), and stereoselectivity (up to 99% de and ee) in the presence of oxygen. The artificial metalloenzyme can be recombinantly expressed in bacterial cells, enabling its application also in the context of whole-cell biotransformations. This work makes available a robust and easy-to-use oxygen-tolerant biocatalyst for asymmetric cyclopropanations and demonstrates the value of porphyrin ligand substitution as a strategy for tuning and enhancing the catalytic properties of hemoproteins in the context of abiological reactions.




**KEYWORDS:** *cyclopropanation, myoglobin, artificial metalloenzyme, chlorin e6, biocatalysis*

Chiral cyclopropanes constitute highly valuable building blocks in medicinal chemistry, providing key pharmacophores in several drug molecules such as the antidepressant Tranylcypromine[1] and the platelet aggregation inhibitor Ticagrelor,[2] among others.[3] Major progress has been made in the development of synthetic methods to access these important structural motifs, including those relying on the transition-metal catalyzed addition of a carbenoid species to the carbon−carbon bond of an olefin.[4] Over the past few years, biocatalytic strategies for promoting olefin cyclopropanation reactions have also emerged, involving engineered variants of heme-containing proteins such as cytochrome P450s[5] and myoglobins (Mb)[6] or other proteins scaffolds.[7] In particular, our group has previously shown how engineered myoglobins can provide promising catalysts for asymmetric cyclopropanation reactions[6] and other carbene-mediated transformations.[8]

Myoglobin-catalyzed olefin cyclopropanation in the presence of diazo compounds is assumed to be mediated by a heme-carbenoid complex generated upon reaction of the carbene donor reagent with the protein-bound heme cofactor.[6a] Previous studies established that reduction of the hemoprotein to its ferrous state is critical for supporting cyclopropanation activity.[6a] Since ferrous myoglobin has high affinity for oxygen, this non-native reactivity is severely suppressed in the presence of air, imposing the need for strictly anaerobic conditions to realize these transformations. While this inhibitory effect is somewhat alleviated by mutations at the level of the distal histidine residue,[9] which is directly involved in stabilizing the oxy-form of myoglobin,[10] the catalytic activity of Mb-based cyclopropanation catalysts is drastically reduced in the presence of oxygen. A similar drawback concerns iron-porphyrins and other synthetic iron-based catalysts previously investigated for cyclopropanation reactions.[11] These limitations have prompted us to investigate strategies for overcoming the oxygen intolerance of these carbene transfer biocatalysts, a goal that would greatly simplify the application of these systems in organic synthesis. Herein, we report the development of a highly efficient and stereoselective myoglobin-based catalyst for promoting asymmetric olefin cyclopropanations under aerobic conditions.

The native cofactor of myoglobin is iron-protoporphyrin IX (hemin), which is embedded in a hydrophobic pocket of the protein and bound via coordination of the iron atom by a conserved "proximal" histidine residue (His93 in sperm whale Mb).[10a] We and others recently showed how substitution of the metal center in this cofactor can provide a means to alter the reactivity of myoglobin in carbene and nitrene transfer reactions.[9,12] We reasoned that substitution of the porphyrin ligand could provide an alternative approach for tuning the electronic properties of the metal center and thus the reactivity of these biocatalysts, including their susceptibility to oxygen inhibition. With this idea in mind, we focused our attention on chlorin e6 (Ce6), a derivative of the tetrapyrrole chromophore of chlorophyll. Compared to protoporphyrin IX, Ce6 (**1**)

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contains a partially saturated pyrrole group (ring D, Figure 1a) and three carboxylic groups, one of which is directly linked to

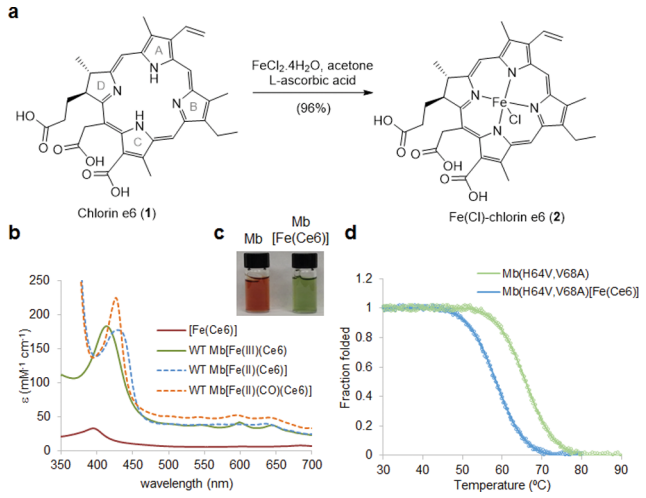


**Figure 1.** Iron-chlorin e6-substituted myoglobins. (a) Synthesis of Fe(Cl)-chorin e6 complex ([Fe(Ce6)]). (b) Overlay of the absorption spectra for [Fe(Ce6)], and Mb[Fe(Ce6)] in its ferric, ferrous, and CO-bound state. (c) Solutions of Mb and Mb[Fe(Ce6)]. (d) Thermal denaturation curves for Mb(H64V,V68A) and Mb(H64V,V68A)[Fe(Ce6)] as determined by circular dichroism ($\theta_{222}$). See also Figure S4.

the pyrrole ring C (instead of two propionic groups connected to rings C and D). These structural differences were expected to make Ce6 a more electron-deficient ligand than ppIX, possibly increasing the electrophilicity and thus the reactivity of the putative iron-carbene intermediate[6a,13] implicated in hemoprotein-catalyzed carbene transfer reactions. In addition, the introduction of electron-withdrawing groups in heme analogues was previously reported to reduce their relative affinity for oxygen,[14] suggesting that a similar effect could be obtained with the more readily available Ce6 ligand. Previous investigation of metalated chlorin e6 complexes have been limited to Mg(II)[15] and Zn(II) complexes, the latter being involved in electron-transfer processes as part of artificial photosynthetic systems.[16]

To prepare the desired iron-Ce6 complex (**2**), commercially available Ce6 was refluxed with excess Fe(II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) and L-ascorbic acid in acetone, resulting in the isolation of [Fe(Cl)(Ce6)] (**2**) as a dark green compound in 96% yield (Figure 1a). The iron complex shows a strong absorbance in the 390−400 nm range of the visible spectrum, corresponding to the Soret band (Figure 1b). Despite the structural differences between hemin and iron-chlorin e6 (**2**), inspection of the crystal structure of Mb suggested the steric feasibility of accommodating the non-native cofactor into the Mb scaffold (Figure S1). Indeed, the C and D rings of the complex **2** and the "extra" acetyl group at the C15 meso position, were expected to occupy the solvent-exposed side of the heme pocket. In addition, heme analogues carrying bulky groups appended to the propionate group(s) have been successfully introduced into the myoglobin scaffold.[17] Experimentally, we found that iron-chlorin e6 (**2**) could be readily incorporated into the hemin-free form of wild-type sperm whale myoglobin (apoMb) to give the desired cofactor-substituted metalloprotein (Mb[Fe(Ce6)]). Most conveniently, this process was carried out by adapting a protocol introduced by Watanabe and co-workers,[18] in which *E. coli* cells expressing apoMb are lysed in the presence of **2**, followed by purification of the reconstituted metalloprotein via Ni-affinity chromatography (yield: 2.5 mg protein/L culture). Upon incorporation into the Mb protein scaffold, the Soret band of the cofactor undergoes a red-shift ($\lambda_{max}$: 395 → 413 nm) and increase in intensity ($\varepsilon_{413}$ = 188 130 $M^{-1}$ $cm^{-1}$ vs $\varepsilon_{395}$ = 33 840 $M^{-1}$ $cm^{-1}$; Figure 1b). As observed for proteins containing metallochlorin cofactors,[19] Mb[Fe(Ce6)] exhibits a green color as opposed to the characteristic red color of Mb (Figure 1c), further evidencing the different electronic properties of the non-native cofactor compared to hemin.

Building upon these results, we prepared a Fe(Ce6)-containing variant of Mb(H64V,V68A), which we previously identified as a highly active and stereoselective catalyst for olefin cyclopropanation.[6a] The Mb(H64V,V68A) scaffold was expected to provide a model system to both examine the relative performance of the non-native cofactor ([Fe(Ce6)]) versus the heme group within the protein matrix and evaluate the impact of cofactor substitution on the stereoselectivity of the metalloprotein. In initial experiments, the effect of [Fe(Ce6)] incorporation on the structure and stability of Mb(H64V,V68A) was investigated by circular dichroism (CD). The CD spectrum of Mb(H64V,V68A)[Fe(Ce6)] was found to exhibit a profile similar to that of the heme-containing counterpart (Figure S2), indicating the lack of major perturbations in the secondary structure of the protein upon incorporation of the non-native cofactor. Furthermore, like the heme-containing counterpart, the visible-range CD spectrum of the reconstituted protein in the ferric, CO-bound form showed a single, positive absorption band in correspondence to the Soret region, indicating the occurrence of a single orientation for the protein-embedded [Fe(Ce6)] cofactor (Figure S3).[20] Thermal denaturation experiments further revealed that Mb(H64V,V68A)[Fe(Ce6)] has reduced stability compared to Mb(H64V,V68A) ($\Delta T_m$ = −7.6 °C) (Figure 1d and S4). This difference notwithstanding, the apparent melting temperature ($T_m$) of the artificial metalloprotein (58.4 °C) remains comparable or higher than that of heme-dependent enzymes from mesophilic organisms ($T_m$ ∼ 45−55 °C).[21]

To examine the cyclopropanation activity of the Fe(Ce6)-based Mb variants, initial reactions were carried out using styrene (**3**) as the substrate and ethyl α-diazoacetate (**4**, EDA) as the carbene donor under "standard reaction conditions" (0.01 M olefin, 0.02 M EDA, 0.01 M $Na_2S_2O_4$, 0.1 mol % catalyst). Under anaerobic conditions, Mb[Fe(Ce6)] produced (*E*)-ethyl-2-phenylcyclopropane-1-carboxylate (**5**) in low yield (16%; 160 catalytic turnovers (TON)) and enantioselectivity (82% de, −20% ee). Comparable yields (15%; 150 TON) but lower diastereoselectivity (69% de) and no enantioselectivity were obtained in parallel reactions with the cofactor alone (Fe(Ce6)). In stark contrast to the poor activity of the wild-type Mb-derived variant, the reaction with Mb(H64V,V68A)-[Fe(Ce6)] furnished quantitative yields of **5** along with excellent diastereo- and enantioselectivity (99.6% de; 98.5% ee). Such catalytic activity and stereoselectivity compare well with those of Mb(H64V,V68A), indicating that (a) the iron-chlorin cofactor is catalytically competent toward carbene transfer and (b) the stereoinductive effect provided by the two active site mutations could be transferred to the [Fe(Ce6)]-based biocatalyst.

To evaluate the ligand effect on the oxygen sensitivity of these biocatalysts, the same reaction was then carried out under aerobic conditions (i.e., in open-vessel reactions). In agreement



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**Table 1. Catalytic Activity and Stereoselectivity of Fe(Ce6)- and Heme-Containing Mb Variants in Styrene Cyclopropanation with EDA under Aerobic Conditions**[a]


3 (1.0 equiv) 4 (2.0 equiv) 5

| entry | protein | cofactor | yield[b] | TON | de[c] | ee[c] |
|---|---|---|---|---|---|---|
| 1 | - | Fe(Ce6) | 1% | 14 | 63% | −5% |
| 2 | Mb | Fe(Ce6) | 6% | 57 | 77% | 22% |
| 3 | Mb(H64V,V68A) | Fe(ppIX) | 43% | 434 | 98% | 95% |
| 4 | Mb(H64V,V68A) | Fe(Ce6) | >99% | >990 | 99.6% | 98% |
| 5 | Mb(H64V,V68A) | Fe(Ce6) | >99%[d] | >990 | 99.8% | 99% |
| 6 | Mb(H64V,V68A,H93F) | Fe(Ce6) | 22% | 222 | 52% | 12% |
| 7 | Mb(H64V,V68A,H93A) | Fe(Ce6) | 9% | 90 | 73% | 19% |
| 8 | Mb(H64V,V68A) | Fe(ppIX) | <1%[e] | <5 | - | - |
| 9 | Mb(H64V,V68A) | Fe(Ce6) | 57%[e] | 572 | 95% | 92% |
| 10 | Mb(H64V,V68A) | Fe(Ce6) | 70%[f] | 1390 | 99.7% | 98% |
| 11 | Mb(H64V,V68A) | Fe(Ce6) | 35%[g] | 6970 | 99.6% | 99% |

[a]Standard reaction conditions: 10 mM styrene, 20 mM EDA, 10 mM $Na_2S_2O_4$, 10 $\mu$M catalyst in KPi buffer (50 mM, pH 7), room temperature. [b]Based on GC conversion using calibration curves with isolated **5**. Error is within 15%. [c]For *trans*-(1*S*,2*S*) stereoisomer as determined with chiral GC. [d]Reaction time: 5 min. [e]No reductant ($Na_2S_2O_4$) added to reaction. [f]With 20 mM styrene, 40 mM EDA. [g]With 1 $\mu$M Mb variant, 20 mM styrene, 40 mM EDA.

with previous observations,[9] the presence of oxygen significantly reduces the catalytic activity of Mb(H64V,V68A), which exhibits only ~430 TON for the formation of **5** (entry 3, Table 1) compared to >10 000 TON[6a] under oxygen-free conditions. The stereoselectivity of the transformation is also affected (98% de and 95% ee vs >99.9% de and ee). In stark contrast, the reaction with Mb(H64V,V68A)[Fe(Ce6)] afforded the cyclopropanation product **5** in quantitative yields and excellent diastereomeric and enantiomeric excess (99.6% de; 98% ee) also in the presence of oxygen (entry 4). Product yields remained high (70%) even after reducing the catalyst loading to 0.05 mol % (entry 10). The Mb(H64V,V68A)[Fe(Ce6)]-catalyzed reactions proceed cleanly, that is, without formation of carbene dimerization byproducts (i.e., diethyl maleate/fumarate), even upon single addition of the diazo reagent to the reaction mixture. Time-course experiments showed that Mb(H64V,V68A)[Fe(Ce6)] is also a remarkably fast catalyst, featuring an initial product formation rate of over 2000 turnovers per minute and enabling the reaction to reach completion within 5 min (entry 5, Table 1). The Mb(H64V,V68A)[Fe(Ce6)]-catalyzed cyclopropanation of styrene with EDA follows Michaelis−Menten kinetics (Figure S5) and from the corresponding curves an apparent $K_{M(EDA)}$ of 32 mM and apparent $k_{cat}$ of 2840 $min^{-1}$ was estimated.[22] These parameters correspond to a catalytic efficiency ($k_{cat}/K_M$) of 1.4 × $10^3$ $M^{-1}$ $s^{-1}$, which falls within the range of $k_{cat}/K_M$ values ($10^3$−$10^6$ $M^{-1}$ $s^{-1}$) observed for the majority (60%) of naturally occurring enzymes.[23] For the same reaction, Mb(H64V,V68A) exhibits a comparable $K_{M(EDA)}$ value (41 mM) but a significantly lower turnover number ($k_{cat}$: 545 $min^{-1}$), likely due to the inhibitory effect of oxygen. The superior performance of the iron-chlorin e6 containing variant became apparent also from its catalytic efficiency in terms of total turnovers. Under catalyst-limited conditions, Mb(H64V,V68A)[Fe(Ce6)] supports over 6900 total turnovers (TTN) in the styrene cyclopropanation reaction (entry 11), as opposed to <500 TTN for the heme-containing counterpart. Altogether, these results indicated that the chlorin ligand is able to confer the Mb-based variant with high cyclopropanation reactivity in the presence of oxygen.

Interestingly, Mb(H64V,V68A)[Fe(Ce6)] was found to maintain significant cyclopropanation activity also in the absence of reductant (572 TON, entry 9). Under identical conditions, negligible product formation is observed with the heme-containing Mb(H64V,V68A) (<5 TON, entry 8), a result consistent with our previous observations that the ferrous species is the catalytically competent form of the hemoprotein in carbene transfer reactions.[6a] To determine whether ferrous Mb(H64V,V68A)[Fe(Ce6)] is formed during the reaction (e.g., upon reduction by EDA),[11a,b,24] the reaction with ferric Mb(H64V,V68A)[Fe(Ce6)] was carried out in the presence of carbon monoxide, which binds and potently inhibits the ferrous but not the ferric form of the protein (Figure 1b). Under these conditions, the cyclopropanation product accumulated in lower but still significant amounts (240 TON), indicating that ferric Mb(H64V,V68A)[Fe(Ce6)] is indeed responsible for at least part of the catalytic activity observed in the absence of reductant. Thus, these results suggest that, in addition to providing tolerance toward oxygen inhibition, the chlorin ligand enables the metalloprotein to be catalytically active also in the ferric form. Fe(III)-based catalysts active in cyclopropanation reactions have been documented before, but these systems were characterized by low diastereoselectivity (<2.5:1 for *trans:cis*) and no stereoselectivity.[11c,25]

Incorporation of [Fe(Ce6)] into the protein enhances its cyclopropanation reactivity (entry 2 vs 1, Table 1), and further enhancements in both activity and stereoselectivity derive from the active site mutations (entry 4 vs 2). To gain further insight into structural determinants of activity in Mb(H64V,V68A)[Fe(Ce6)], two variants of this protein were prepared in which the proximal histidine (His93) is mutated to Phe or Ala. The H93F mutation replaces the iron-binding imidazolyl group with an isosteric but non-Lewis basic phenyl group, whereas the H93A substitution creates a cavity at the proximal side of the cofactor,[26] likely favoring metal coordination by a water molecule. Both mutations were found to dramatically reduce the cyclopropanation activity of the Mb variants (entries 6−7,



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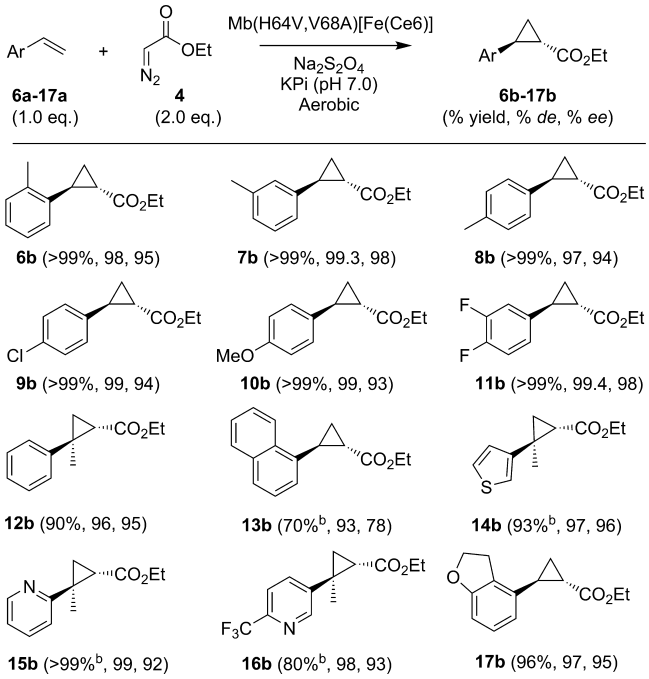
Table 1), indicating that axial ligation of the metal center by the histidine residue is critical for optimal catalytic reactivity.

Next, the substrate scope of the oxygen-tolerant cyclopropanation biocatalyst Mb(H64V,V68A)[Fe(Ce6)] was probed using a range of different vinylarene substrates (**6a–17a**; Scheme 1). Using this variant, various styrene derivatives

**Scheme 1. Substrate Scope of Mb(H64V,V68A)[Fe(Ce6)] in the Aerobic Cyclopropanation of Vinylarenes with EDA**[a]




[a]Reaction conditions: 10 mM olefin, 20 mM EDA, 10 mM $Na_2S_2O_4$, 10 $\mu$M protein in KPi buffer (pH 7), room temperature. [b]With 20 $\mu$M protein.

including *ortho-*, *meta-*, and *para*-substituted, as well as disubstituted styrenes could be converted to the desired cyclopropanation products (**6b–11b**) in excellent yields (>99%) and high diastereo- and enantiomeric excess (93–99% de; 97–99% ee) in open-vessel reactions. Notably, both electron-donating (**8b, 10b**) and electron-withdrawing (**9b, 11b**) groups in the phenyl ring were well tolerated by the [Fe(Ce6)]-based biocatalyst. $\alpha$-Methylstyrene (**12a**) was also converted with high efficiency (90%) and with high diastereo- and enantioselectivity (96% de, 95% ee). Compounds **13a–17a** were then tested to explore the scope of the reaction across other types of aryl-substituted olefins. For these substrates, high conversions (70–99%) could be achieved using a 2-fold higher catalyst loading (0.2 instead of 0.1 mol %). As an exception, cyclopropanation of 5-vinyl-2,3-dihydrobenzofuran **17a** proceeded with high efficiency using our standard reaction conditions. Importantly, high enantio- and diastereoselectivity (93–99% de, 78–96% ee) were achieved in all cases, which further demonstrated the broad substrate scope of the Mb(H64V,V68A)[Fe(Ce6)] catalyst.

We recently established that a variety of metallo-substituted heme analogues can be incorporated into myoglobin by recombinant means,[9,12a] a strategy that bypasses the need for in vitro reconstitution protocols.[12b] Notably, this approach could be readily extended to enable the expression of Mb(H64V,V68A)[Fe(Ce6)] in *E. coli* cells. Interestingly, coexpression of the heterologous heme transporter ChuA[12a] was not required for facilitating the uptake of the non-native [Fe(Ce6)] cofactor by the cells. Following these results, a preparative-scale whole-cell cyclopropanation reaction was carried out using Mb(H64V,V68A)[Fe(Ce6)]-expressing cells in the presence of 0.1 g of styrene and a 2-fold molar excess of EDA. From this reaction, the cyclopropanation product **5** was isolated in high yield (93%) and stereoselectivity (96% de, 90% ee), thereby demonstrating the scalability of this biocatalytic transformation.

In summary, we have described the development of a highly efficient and stereoselective metalloenzyme for olefin cyclopropanation reactions under aerobic conditions. The oxygen-tolerance, broad substrate scope, and scalability of this biocatalyst support its utility and operational simplicity for asymmetric synthesis. The possibility to produce this artificial enzyme in bacterial cells enabled its application in whole-cell biotransformations and makes it amenable to further optimization via protein engineering and directed evolution. From a catalyst design standpoint, this work provides the first example of a hemoprotein-based carbene transferase incorporating a non-native cofactor other than a protoporphyrin IX analogue.[9,12b,27] The superior performance of the iron-chlorin containing Mb variant compared to the heme-based counterpart under aerobic conditions highlight the opportunities made available through modification of the metal-coordinating ligand toward modulating the reactivity of these biocatalysts. These studies pave the way to the future investigation of chlorin-based metalloproteins, also in combination with different metals,[9] for obtaining novel and/or improved catalytic properties in the context of olefin cyclopropanation and other synthetically valuable abiological transformations.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.7b02583.

Detailed description of the experimental procedures, supplementary figures and graphs, and compound characterization data (PDF)

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### Notes

The authors declare no competing financial interest.

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