Supporting Information

Engineering and Analysis of a Self-Sufficient Biosynthetic Cytochrome P450 PikC Fused to the RhFRED Reductase Domain

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1. Molecular cloning

The gene encoding the reductase domain of $P450_{RhF}$ (RhFRED) and the linker sequence was amplified by PCR under standard conditions using forward primer: 5'-GG*GAATTC*GTGCTGCACCGCCATCAACCG-3' (the italic bases represent *Eco*RI restriction site), reverse primer 1: 5'-TTAGAGCTCCAGAGGCGCAGGGCCAGGCG-3' (the *Sac*I cutting site is underlined) for amplifying *RhFRED* gene without the stop codon, and reverse primer 2: 5'-ACATCAAGCTTTCAGAGGCGCAGGGCCAG-3' (the *Hind*III cutting site is underlined) for cloning *RhFRED* gene retaining the stop codon. The cDNA without and with the stop codon were digested by *Nde*I/*Sac*I and *Nde*I/*Hind*III restriction enzyme pairs, respectively, and then ligated into the *Nde*I/*Sac*I digested pET21b(+) (Novagen) and *Nde*I/*Hind*III digested pET28b(+) (Novagen) correspondingly, generating the recombinant plasmid pET21b(+)-RhFRED and pET28b(+)-RhFRED. On the other hand, using previously prepared plasmid pET28a-*pikC* (Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Chem. Biol.* **1998**, 5, 661) as template, the *pikC* gene with stop codon removed was amplified by PCR under standard conditions using a pair of primers as follow: forward, 5'-GGAGTTC*CATATG*CGCCGTACCCAGCAG-3', reverse: 5'-GATAGAATTCACCGGTACGGCGGCCCGC-3' (The italic and underlined bases represent the introduced *Nde*I and *Eco*RI restriction sites for following cloning manipulation). The *Nde*I/*Eco*RI double digested *pikC* gene was then ligated into the previously *Nde*I/*Eco*RI-digested pET21b(+)-RhFRED and pET28b(+)-RhFRED to generate vectors pET21b(+)-pikC-RhFRED and pET28b(+)-pikC-RhFRED (Figure S1) for overexpressing C-His_6-tagged and N-His_6-tagged fusion protein PikC-RhFRED.

2. Overexpression and purification of fusion proteins

The *Escherichia coli* BL21 (DE3) transformant carrying certain overexpression vector was grown at 37 ^{o}C overnight in LB media containing 50 μg/ml of kanamycin (or ampicillin), then 10 ml of culture was used to inoculate 1 liter of LB media containing certain selective antibiotic (50 μg/ml), thiamin (1 mM), 10% glycerol and a rare salt solution. Cells were grown at 37 ^{o}C for 3~4 h until A_{600} reached 0.6~1.0. Then, isopropyl β-D-thiogalactoside (IPTG, 0.1 mM) and δ-aminolevulinic acid (1 mM) were added, and the cells were cultured at 18 ^{o}C overnight. The following protein purification was carried

out according to previously developed procedure (Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Chem. Biol.* **1998**, 5, 661).

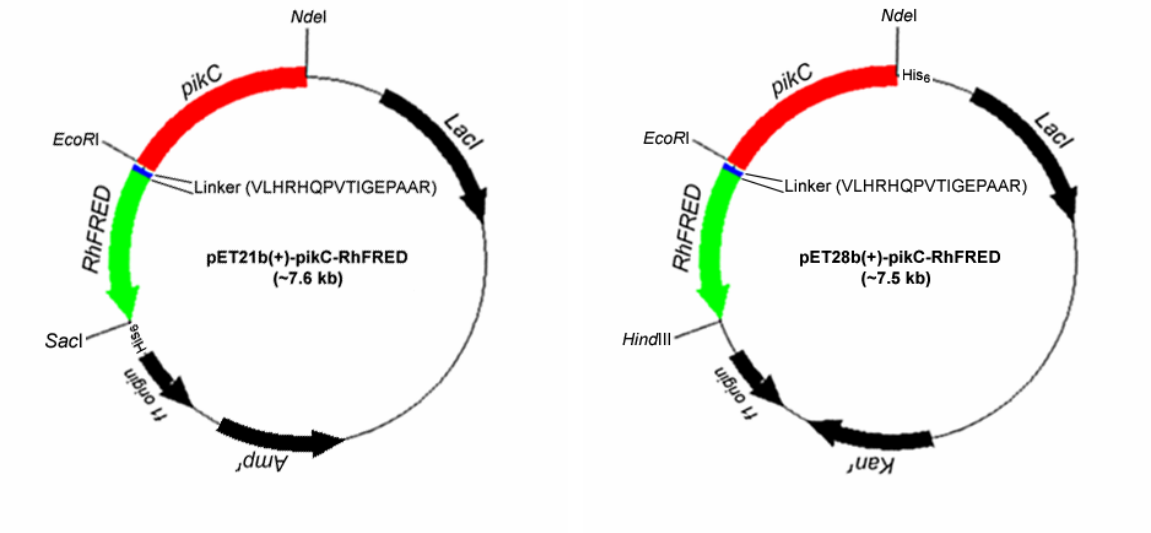


Figure S1. Maps for N-terminal (left) and C-terminal His_6-tagged (right) PikC-RhFRED overexpression vectors.

3. SDS-PAGE analysis

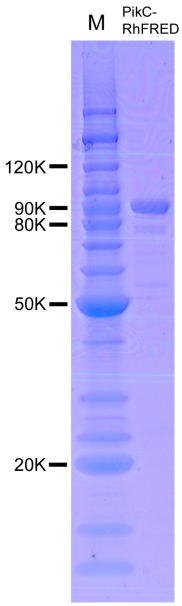


Figure S2. SDS-PAGE analysis of functional N-terminal His_6-tagged PikC-RhFRED. The calculated molecular mass is about 83 kDa.

4. UV-visible absorption spectrum

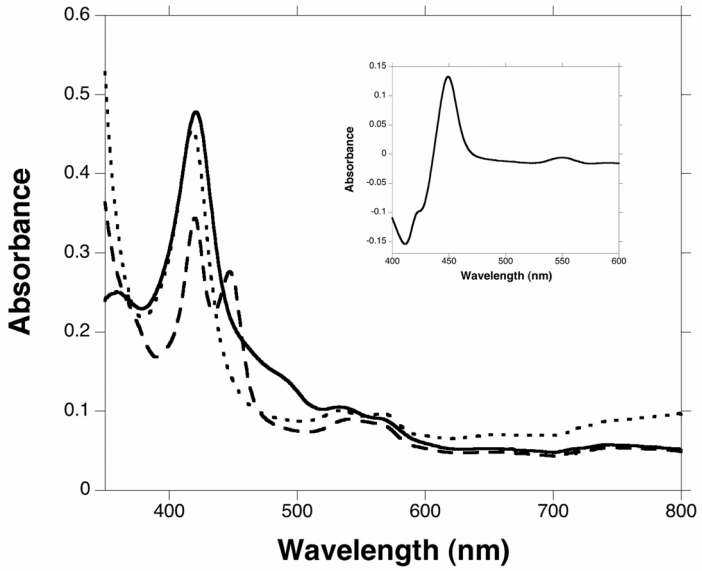


Figure S3 UV-visible absorption spectra for purified N-terminal His_6-tagged PikC-RhFRED (~1.6 μM): oxidized (solid line), sodium dithionite reduced (dotted line), and CO-reduced (dashed line) are shown. The CO-bound reduced difference spectrum is shown in inset. This assay is also employed to determine the concentration of functional P450 enzyme using the extinction coefficient of 91,000 $M^{-1}{\cdot}cm^{-1}$ (Omura, T., Sato, R. *J. Biol. Chem.* **1964**, 239, 2379.).

5. Gel-filtration analysis

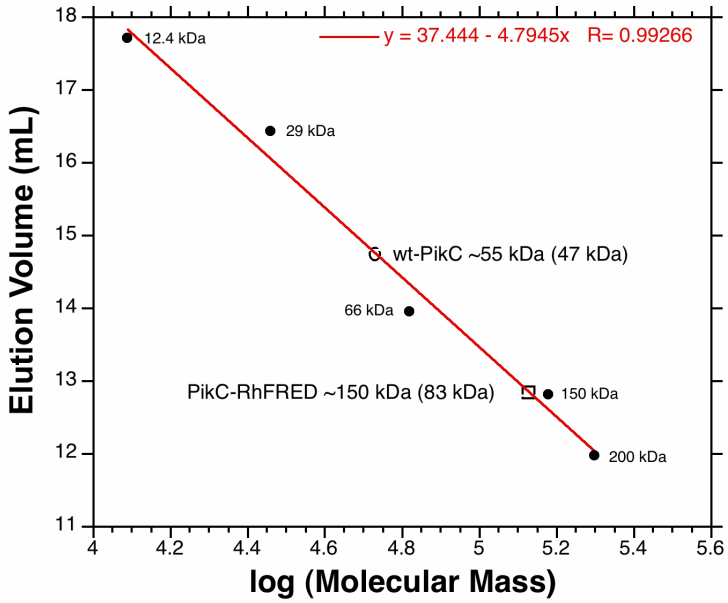


Figure S4. The calculation of the apparent molecular mass of wt-PikC and PikC-RhFRED on the basis of the gel-filtration standard curve. The deduced molecular mass of wt-PikC and PikC-RhFRED is approximately 55 kDa and 150 kDa, respectively, suggesting the monomeric (wt) and dimeric (fusion) form in 50 mM sodium phosphate pH 7.3 buffer containing 300 mM NaCl, 10% glycerol. The theoretical molecular mass of each monomer is shown in parentheses.

6. Spectral substrate binding assays

Spectral substrate binding assay was carried out on UV-visible spectrophotometer 300 Bio (Cary) at room temperature by titrating 30 mM substrate DMSO solution (blank DMSO for reference group) into 1 ml of 1 μM P450 sample in 1 μl aliquots, leading to substrate concentration ranging from 30 to 360 μM. The series of Type I difference spectra were used to deduce ΔA ($A_{peak(389)}$-$A_{trough(424)}$). Then, the data were fit to Michaelis-Menten equation to obtain the dissociation constant K_d.

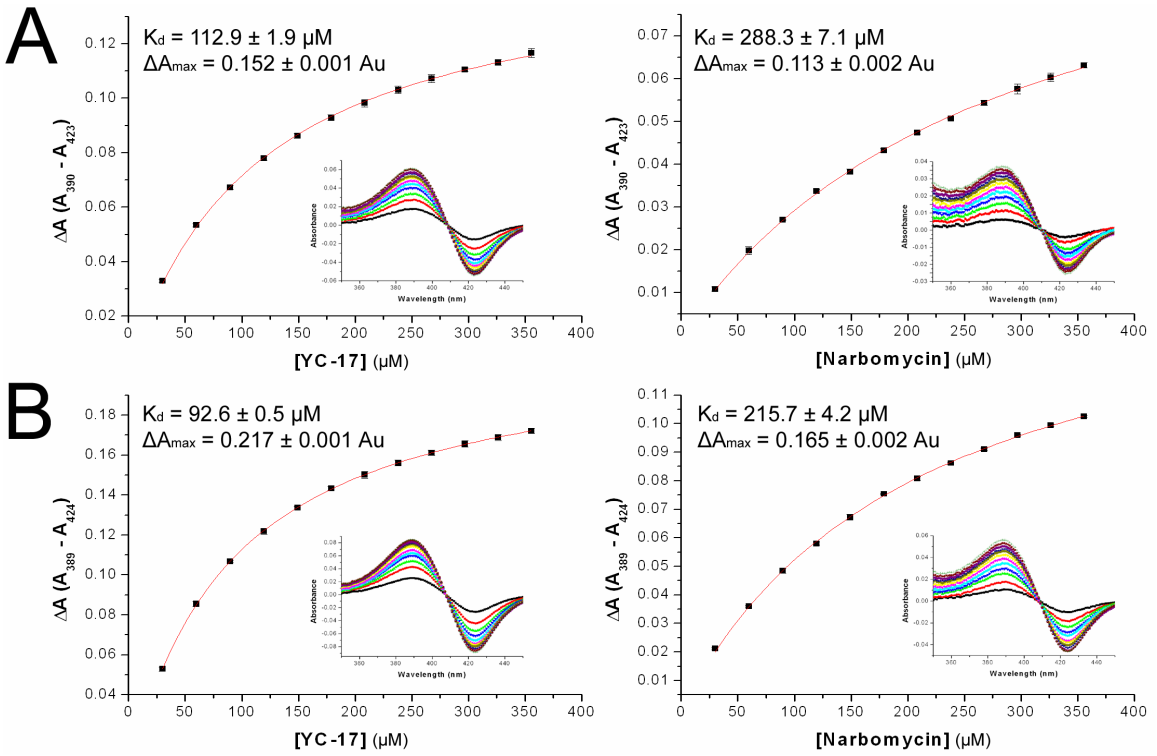


Figure S5. The substrate binding affinity (K_d) of YC-17 (**1**) and narbomycin (**4**) toward wt-PikC (A) and PikC-RhFRED (B). The insets show the Type I binding spectra. All experiments were performed in duplicate.

7. HPLC analysis of reactions catalyzed by PikC enzymes

The reactions for PikC enzymes were carried out using previously developed assays with minor modifications (Xue, Y.; Wilson, D.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Chem. Biol.* **1998**, 5, 661). The typical assay contains 1 μM PikC-RhFRED (or 1 μM PikC with 3.5 μM spinach ferredoxin and 0.1 U/ml spinach ferredoxin-$NADP^+$ reductase), 200 μM YC-17 (**1**) or narbomycin (**4**), and 0.5 mM NADPH in 100 μl of desalting buffer (50 mM NaH_2PO_4, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, 10% glycerol). The reaction was stopped and extracted after 1 h of incubation at 30 ^oC by addition of 2×200 μl of chloroform. The extracts were dried, dissolved in 120 μl of methanol and analyzed by Xbridge C18 5 μm 250 mm reverse-phase column at 230 nm, using 10-70% solvent B (A: deionized water + 10 mM ammonium acetate, B: acetonitrile) at 1 ml/min over 30 min. The peak identity in each HPLC trace was determined by mass spectrometry and comparison with authentic compounds regarding HPLC retention time and UV spectrum.

8. Steady-state kinetics

The standard reaction contains 100 nM of PikC-RhFRED (or 150 nM of wt-PikC with 3.5 μM spinach ferredoxin and 0.1 U/ml spinach ferredoxin-$NADP^+$ reductase), 20~250 μM substrate in 200 μl of desalting buffer (50 mM NaH_2PO_4, pH 7.3, 1 mM EDTA, 0.2 mM DTE, 10% glycerol). After pre-incubation at 30 ^oC for 5 min, the reaction was initiated by adding 1 μl of 50 mM NADPH and 50 μl aliquots were taken at 0s, 20s, and 40s (or 0s, 30s, and 60s when substrate concentration greater than 100 μM) to thoroughly mix with 2×100 μl of chloroform. The nitrogen-dried samples were redissolved in 150 μl of methanol for subsequent HPLC analysis. The HPLC conditions were: Xbridge C18 5 μm 250 mm reverse-phase column, 20-80% solvent B (A: deionized water + 0.1% trifluoroacetic acid, B: acetonitrile + 0.1% trifluoroacetic acid) at 0.8 ml/min over 20 min, UV wavelength 230 nm. The initial velocity of substrate consumption was deduced from decreased area under the curve (AUC) of specific substrate peaks. Finally, the data from duplicated experiments were fit to Michaelis-Menten equation.

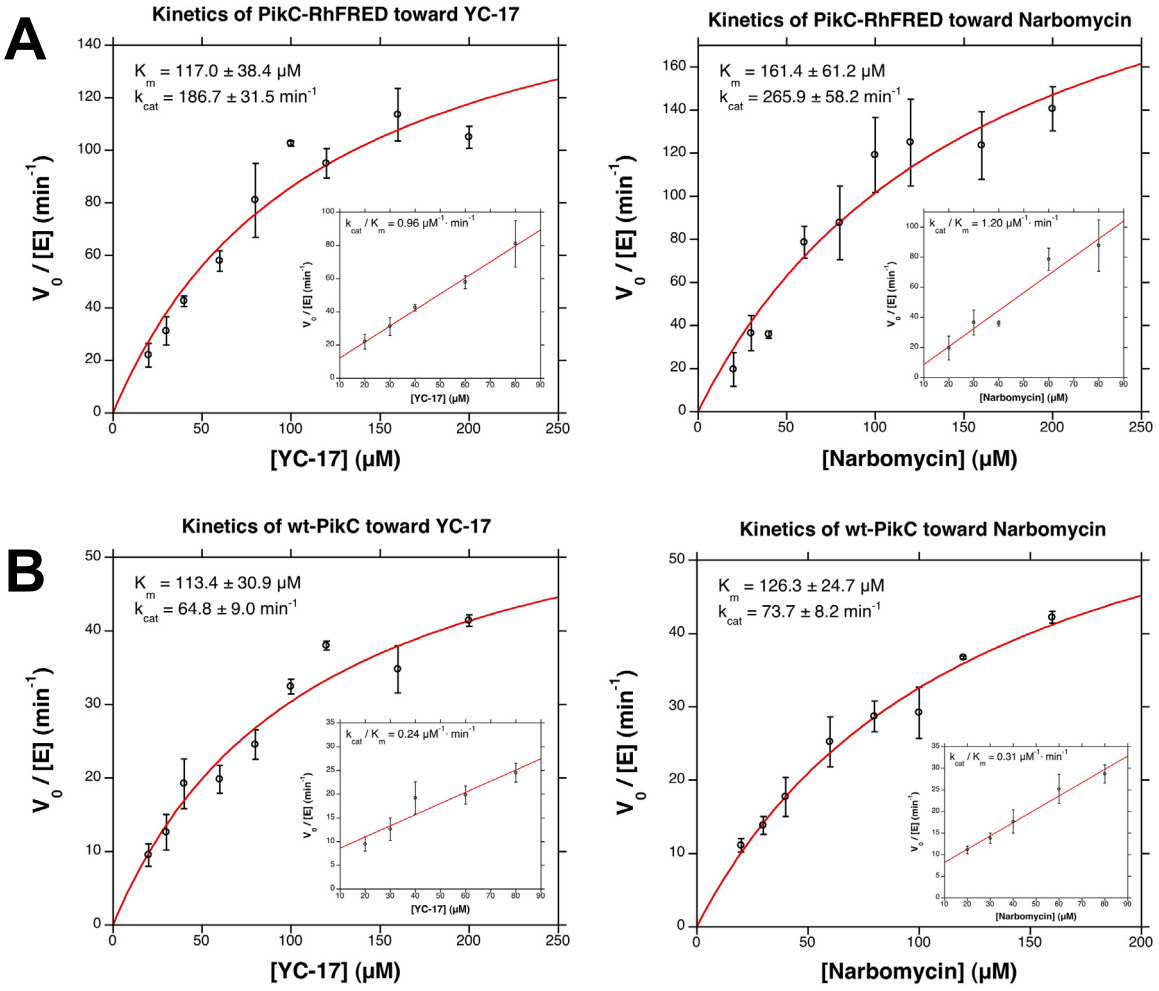
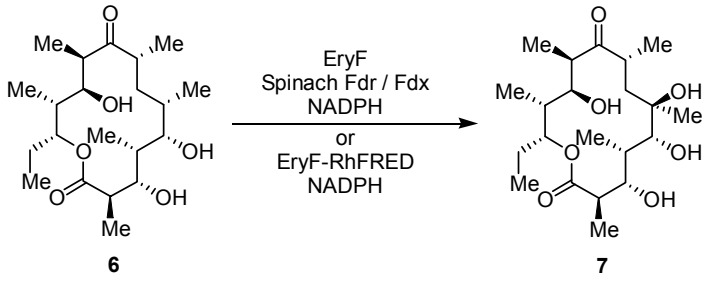


Figure S6. Michaelis-Menten curves of PikC-RhFRED (A) and wt-PikC (B) using YC-17 (**1**) or narbomycin (**4**) as substrate. The apparent K_m and k_{cat} values are deduced from the unsaturated Michaelis-Menten curve. The k_{cat}/K_m values deduced from the linear fit of low-concentration data are shown in insets.

9. LC-MS analysis of *in vitro* activity of fusion enzyme EryF-RhFRED

Scheme S1. Hydroxylation catalyzed by EryF



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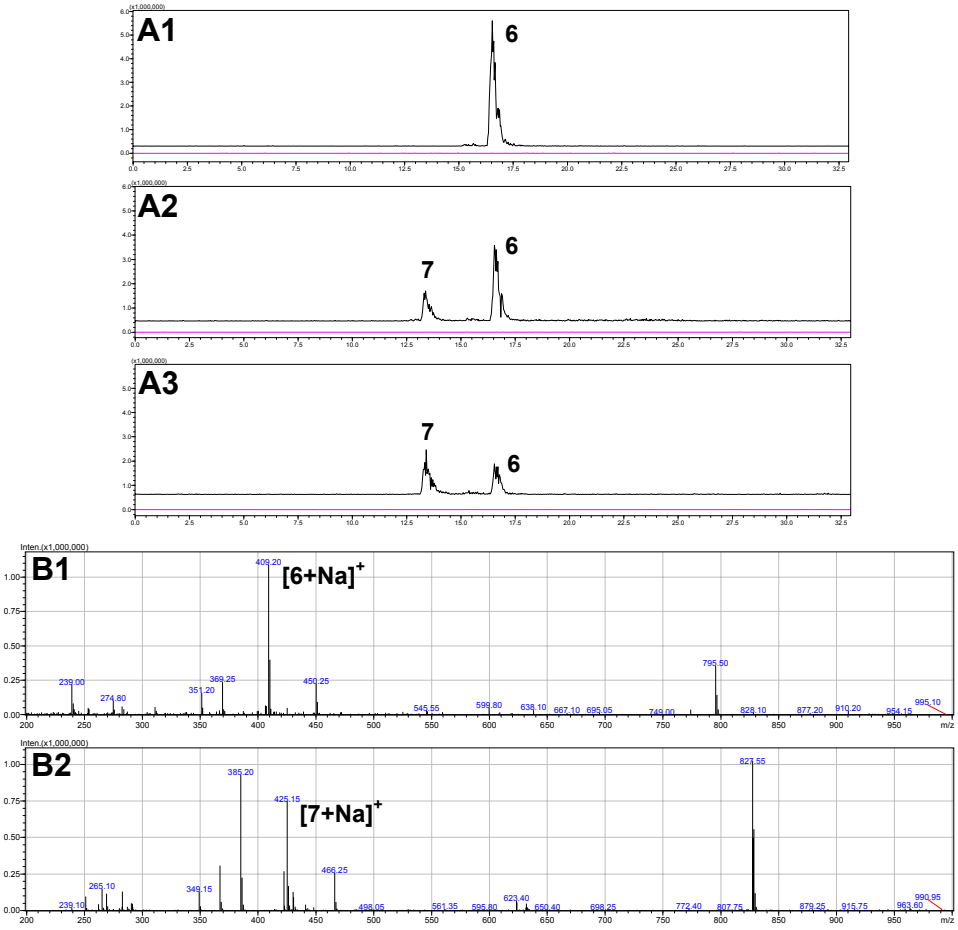

Figure S7. LC-MS analysis of *in vitro* activity of EryF-RhFRED: A1. Negative control of 6-deoxyerythronolide B (**6**) in absence of P450. A2. **6** with wt EryF in presence of Fdr, Fdx, and NADPH. A3. **6** with EryF-RhFRED in presence of only NADPH. B1. Mass spectrum of **6**. B2. Mass spectrum of erythronolide B (**7**). The sample preparation was similar to that for typical PikC assay. The LC conditions were: Xbridge C18 3.5 µm 150 mm reverse-phase column, 20-100% solvent B (A: deionized water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid) at 0.2 ml/min over 20 min.