Supporting Information

Concurrent Oxidations with Tandem Biocatalysts in One Pot: Green, Selective and Clean Oxidations of Methylene Group to Ketone

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Content

1. Materials and methods

Chemicals

Tetralin **1a**, (*R*)-1-tetralol **2a**, (*S*)-1-tetralol **2a**, 1-tetralone **3a**, indan **1b**, (*R*)-1-indanol **2b**, (*S*)-1-indanol **2b**, 1-indanone **3b**, 1-benzyl-4-hydroxy-piperidine **5**, 1-benzyl-4-piperidone **6**, ampicillin (> 99%), kanamycin solution (50 mg/ml), NADH (> 99%) and NADPH (>99%) were purchased from Sigma-Aldrich. Isopropyl □-D-thiogalactopyranoside (ITPG, >99%) was bought from 1st BASE. Medium components tryptone and yeast extract were purchased from Biomed Diagnostics. *N*-benzyl-peperidine **4** was prepared according to the published procedures with 59.5% yield and >99.8% purity (GC).[1]

Biocatalysts

LKADH (~0.4 units/mg) was purchased from Sigma-Aldrich. *Pseudomonas monteilii* TA-5, *E. coli*($P450_{pyr}$), and *E. coli* (RDR) were obtained in our laboratory from our previous research.

Analytical methods

The concentrations of tetralin **1a**, 1-tetralol **2a**, 1-tetralone **3a**, indan **1b**, 1-indanol **2b**, 1-indanone **3b** and ee value of 1-tetralol **2a**, 1-indanol **2b** were analyzed by Shimadzu Prominence HPLC on a Chiralcel OB-H column (150 mm × 2.1 mm) at 25 ºC. UV detection: 210 nm; eluent: *n*-hexane/2-propanol (95:5); flow rate: 1 ml/min; retention time: 7.3 min for (*R*)-**2a**, 8.6 min for (*R*)-**2b**, 11.1 min for (*S*)-**2a**, 11.7 min for benzyl alcohol, 13.3 min for **3a**, 13.6 min for (*S*)-**2b**, and 20.5 min for 1-indanone **3b**.

The concentrations of *N*-benzyl-piperidine **4**, 1-benzyl-4-hydroxy-piperidine **5**, and 1-benzyl-4-piperidone **6** were analyzed by Shimadzu Prominence HPLC on a ZORBAX Eclipse plus C18 column (150 mm × 4.6 mm) at 25 ºC. UV detection: 210 nm; eluent: acetonitrile/10 mM KP buffer pH 7.7 (20:80); flow rate: 1 ml/min; retention time: 5.8 min for **5**, 10.0 min for 1-phenylethanol as internal

standard, 18.6 min for **4**, and 27.5 min for **6**.

2. Cultivation of Microorganisms

Pseudomonas monteilii TA-5

Agar plate culture: The strain was inoculated on M9 agar plate containing trace element, and grown at room temperature for 2 days with toluene vapor as carbon source.[2]

Preculture: A single colony from the M9 agar plate was inoculated into 10 mL of LB medium, and grown at 300 rpm and 30 ºC for 7 h.

Subculture: 2 mL of the above preculture was added in 100 ml M9 medium containing trace element in a 250 ml shaking flask with ventilated plastic stopper. 15 mL plastic tube with length of 9 cm containing 1 mL toluene was put into the flask, and the vapor of toluene was used as carbon source. The mixture was incubated at 250 rpm and 30 ºC for 18 h. Then the cells were harvested by centrifugation at 10,375 g for 10 min and the cells were directly used for biotransformation.

E. coli ($P450_{pyr}$): E. coli BL21 (DE3) - pRSFDuet P450pyr - pETDuet Fdx FdR1500

Agar plate culture: The recombinant strain was inoculated on LB agar plate containing ampicillin (100 μg/mL) and kanamycin (50 g/ml), and grown overnight at 37 ºC.[3]

Preculture: A single colony from the LB agar plate was inoculated into 10 mL of LB medium with ampicillin (100 μg/ml) and kanamycin (50 ug/ml), and grown at 300 rpm and 37 ºC for 12 h.

Subculture: 2 mL of the above preculture was added in 100 mL LB medium containing ampicillin (100 μg/ml) and kanamycin (50 ug/ml), and the mixture was shaken at 300 rpm and 37 ºC. IPTG (0.5 mM) was added when OD_{600} reached around 0.8~1.0 at 2 h, and then the mixture was shifted to 300 rpm, 30 ºC, and shaken for another 3 h. Then the cells were harvested by centrifugation at 10,375 g for 10 min and the cells were directly used for biotransformation.

E. coli(RDR): E. coli pET28a histag-RDR

Agar plate culture: The recombinant strain was inoculated on LB agar plate containing kanamycin (50 g/ml), and grown overnight at 37 ºC. [3]

Preculture: A single colony from the LB agar plate was inoculated into 10 ml of LB medium with kanamycin (50 g/ml), and grown at 250 rpm and 37 ºC for 12 h.

Subculture: 1 mL of the above preculture was added in 100 ml TB medium (12 g Bacto tryptone, 24 g Bacto yeast extract, 4 mL glycerol, KH_2PO_4 2.3 g, K_2HPO_4 12.54 g in 1 liter deionized water) containing kanamycin (50 ug/ml), and the mixture was shaken at 250 rpm and 37 ºC. IPTG (0.25 mM) was added when OD_{600} reached around 0.6~0.8 at 2 h, and then the mixture was shifted to 250 rpm, 25 ºC, and shaken for another 16~20 h. Then the cells were harvested by centrifugation at 10,375 g for 10 min and the cells were directly used for biotransformation or enzyme purification.

3. Purification of histag-RDR

Preparation of cell lysate

The above freshly harvested *E. coli* pET28a histag-RDR was resuspended in 10 mM imidazole buffer to a cell density of 20 g cdw/L, and passed through a homogenizer twice (Constant cell disruption system) at 20 kpsi. The cell debris was removed by centrifugation at 14,000 g for 30 minutes. The supernatant (i.e., lysate) was reserved for further purification.

Purification with Ni-NTA column

The above cell lysate was filtrated through 0.2 μM membrane filter. 5 ml (6 g protein/L) cell lysate was incubated with 0.5 ml Ni-NTA at 4 ºC for 1 h. The incubated mixture was loaded to column and filtrated. Droplets were collected. The column was washed with 10 mM (10 ml), 20 mM (10 ml), 30 mM (10 ml), 50 mM (0.5ml) imidazole buffer; and then eluted with 250 mM imidazole buffer 5 × 0.5 ml. All filtrates were separately collected. The protein concentration of each fraction was determined by

Bradford protein content assay[4] with bovine serum albumin as a standard. The fractions 5-9 (Fig. S1) of 250 mM imidazole buffer wash were collect together, exchanged with potassium phosphate (KP) buffer (100 mM; pH 8.0), and directly used for biotransformation

SDS-PAGE gel

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading on a gel containing 0.1% SDS and 12% acrylamide, staining the gel with a 0.1% solution of Coomassiebrilliant blue R-250 in methanol-acetic acid-water (4:1:5, vol/vol/vol), and destaining the gel by soaking it in deionized water overnight (Fig. S1).

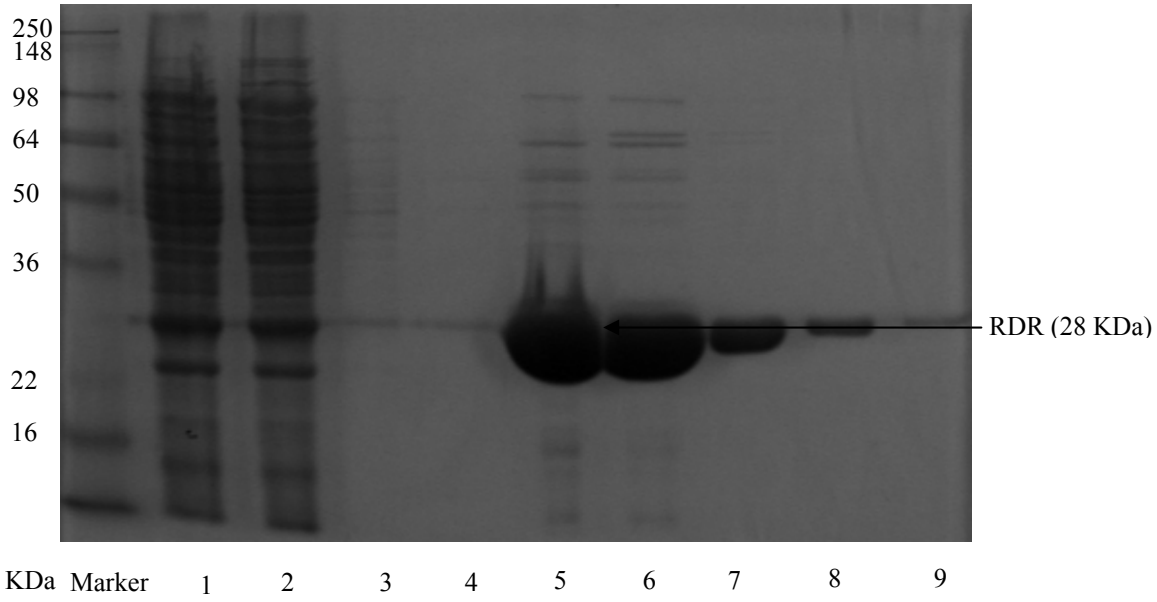
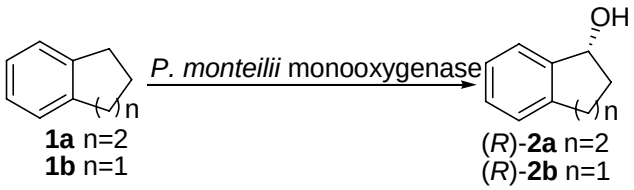


Figure S1. SDS-PAGE of cell lysate (lane 1); loading filtrate (lane 2); 10 mM imidazole buffer wash sample (lane 3); 50 mM imidazole buffer wash sample (lane 4); 250 mM imidazole buffer wash fraction one (lane 5); 250 mM imidazole buffer wash fraction two (lane 6); 250 mM imidazole buffer wash fraction three (lane 7); 250 mM imidazole buffer wash fraction four (lane 8); 250 mM imidazole buffer wash fraction five (lane 9)..

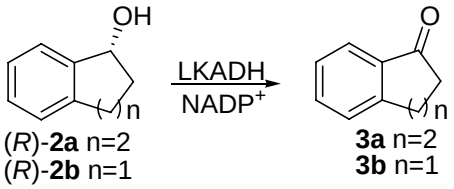
4. Activity assays



Scheme S1

Bioconversion of 1a to (*R*)-2a: To 990 µL 1.5 g/L *P. monteilii* TA-5 cell suspension in Tris-HCl buffer (100 mM; pH 7.0) was added 2 mM (10 µL 0.2 M stock solution in methanol) **1a**. The mixture was shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 mL ethyl acetate containing 1 mM benzyl alcohol was added and mixed. The organic phase was separated after centrifugation, dried over anhydrous Na_2SO_4, and subjected to HPLC analysis for quantifying the product formation.

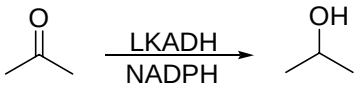
Bioconversion of 1b to (*R*)-2b: To 990 µL 1.5 g/l *P. monteilii* TA-5 cell suspension in Tris-HCl buffer (100 mM; pH 7.0) was added 2 mM (10 µL 0.2 M stock solution in methanol) **1b**. The mixture was shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 mL ethyl acetate containing 1 mM benzyl alcohol was added and mixed. The organic phase was separated after centrifugation, dried over anhydrous Na_2SO_4, and analysed by normal phase HPLC.



Scheme S2

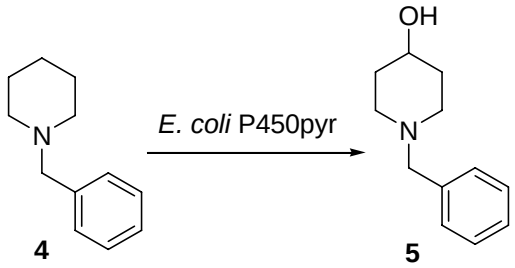
Bioconversion of (*R*)-2a to 3a: To 980 µL Tris-HCl buffer (100 mM; pH 7.0) was added 4 mM (*R*)-**2a**, 4 mM (20 µL 0.2 M stock solution in deionized water) $NADP^+$ and 2 mg LKADH. The mixture was shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 ml ethyl acetate containing 1 mM benzyl alcohol was added and mixed. The organic phase was separated after centrifugation, dried over anhydrous Na_2SO_4, and analyzed by normal phase HPLC.

Bioconversion of (*R*)-2b to 3b: To 980 µL Tris-HCl buffer (100 mM; pH 7.0) was added 4 mM (*R*)-**2b**, 4 mM (20 µL 0.2 M stock solution in deionized water) $NADP^+$ and 2 mg LKADH. The mixture was shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 mL ethyl acetate containing 1 mM benzyl alcohol was added and mixed. The organic phase was separated after centrifugation and dried over anhydrous Na_2SO_4, followed by analysis of normal phase HPLC.



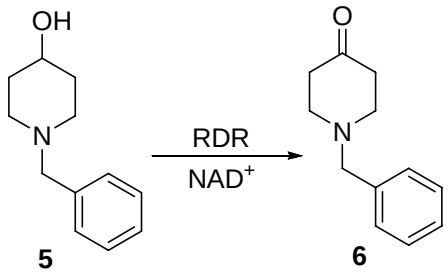
Scheme S3

Bioconversion of acetone to *iso*-propanol with NADPH as cofactor: To 2.975 mL Tris-HCl buffer (100 mM; pH 7.0) was added 10 mM acetone, 1 mM (15 µl 0.2 M stock solution in deionized water) NADPH, and 1 mg LKADH. The decrease of NADH was monitored by determining the UV absorption at 340 nm at 30 °C, and the concentration was calculated by using a ε340 of 6.22 liters $mmol^{-1}$. The specific activity was expressed in units/g protein, and 1 U was defined as the decrease of 1 µmol NADPH/min.



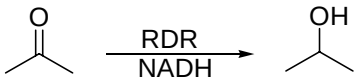
Scheme S4

Bioconversion of 4 to 5: To 950 µL 1.5 g/l cell suspension in KP buffer (100 mM; pH 8.0) was added 2% (w/v) glucose (40 µl 50% stock solution) and 2 mM (10 µL 0.2 M stock solution in methanol) **4**. The mixtures were shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 mL methanol with 2 mM 1-phenylethanol was added, mixed, centrifuged, and the supernatant was taken for reverse phase HPLC analysis.



Scheme S5

Bioconversion of 5 to 6: To 980 µL 0.2 g protein/l solution in KP buffer (100 mM; pH 8.0) was added 2 mM (10 µl 0.2 M stock solution in methanol) **5** and 2 mM (10 µl 0.2 M stock solution in deionized water) NAD^+. The mixtures were shaken at 1000 rpm and 30 ºC for 15 min on benchtop thermomixer. At 15 min, 1 mL methanol with 2 mM 1-phenylethanol was added, mixed, centrifuged, and the supernatant was taken and analyzed by reverse phase HPLC.



Scheme S6

Bioconversion of acetone to *iso*-propanol with NADH as cofactor: To 2.975 mL 0.2 g protein/l solution in KP buffer (100 mM; pH 8.0) was added 10 mM acetone, and 1 mM (15 μL 0.2 M stock solution in deionized water) NADH. The decrease of NADH was monitored by determining the UV absorption at 340 nm at 30 °C, and the concentration was calculated by using a ε340 of 6.22 liters $mmol^{-1}$. The specific activity was expressed in units/g protein, and 1 U was defined as the decrease of 1 μmol NADH/min.

5. Concurrent oxidations of methylene group to ketone with tandem biocatalysts in one pot

Typical procedure for concurrent oxidations of tetralin 1a to 1-tetralone 3a with tandem biocatalysts in one pot: To 5.0 mL suspension (10 g cdw/L) of resting cells of *P. monteilii* TA-5 in Tris-HCl buffer (100 mM; pH 7.0) containing 1 mM $MgCl_2$ was added 17.5 mg LKADH, 2.5 μL $NADP^+$ stock solution (0.002 M), and 25 μL acetone. 4.0 mg (0.03 mmol) tetralin **1a** was then added to a concentration of 6.0 mM, and the mixture was shaken at 250 rpm and 30 ºC. At 2 h, cell pellets (24 mg cdw) of *P. monteilii* TA-5 were directly added to reaction system. Aliquots (100 μL) were taken out at different time points and mixed with 100 μL ethyl acetate containing 1 mM benzyl alcohol as internal standard. The cells were removed by centrifugation, and organic phase was separated and dried over anhydrous Na_2SO_4. The samples were analyzed by normal phase HPLC. At 30 h, 4.98 mM **3a** was produced as clean product in 86%, with the TTN of $NADP^+$ recycling of 4100.

Typical procedure for concurrent oxidations of *N*-benzyl-piperidine 4 to 1-benzyl-4-piperidone 6 with tandem biocatalysts in one pot: To 5.0 mL suspension (10 g cdw/L) of resting cells of *E. coli* ($P450_{pyr}$) in KP buffer (100 mM; pH 8.0) was added 4.0 g protein/L freshly purified histag-RDR, 2.5 μL NAD^+ stock solution (0.002 M), 25 μL acetone, and 50 μL glucose stock solution (50%). 4.4 mg (0.025 mmol) *N*-benzyl-piperidine **4** was added to a concentration of 5.0 mM, and the mixture was shaken at 250 rpm and 30 ºC. Aliquots (100 μL) were taken out at different time points and mixed with 100 μL methanol containing 2 mM 1-phenylethanol as internal standard. The cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC. At 25 h, 4.02 mM **6** was produced as clean product in 80%, with the TTN of NAD^+ recycling of 4000.

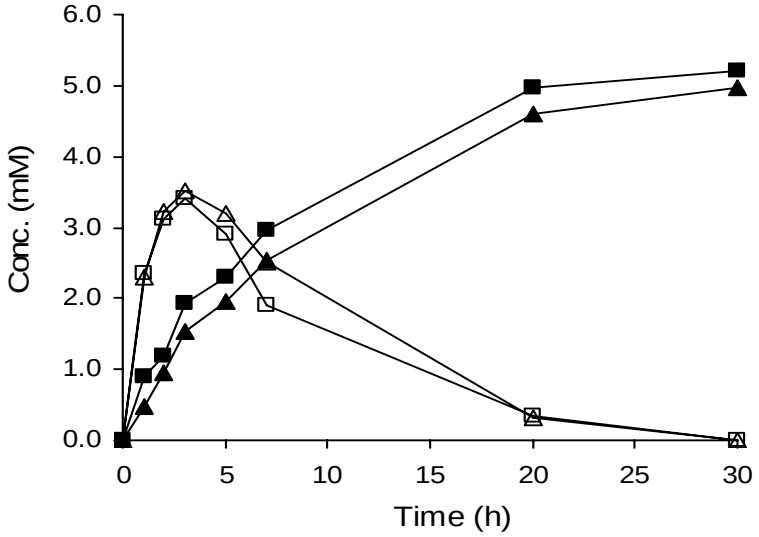


Fig. S 2. Time course of biooxidations of tetralin **1a** to 1-tetralone **3a** and of indan **1b** to 1-indanone **3b** with tandem biocatalysts in one pot. **3a** (▲), (*R*)-**2a** (△), **3b** (■) and (*R*)-**2b** (□). Reaction conditions: see Table 1, entry 5 and 7.

References

(1) Ferrer, M.; Sánchez-Baeza, F.; Messeguer, A. *Tetrahedron* **1997**, *53*, 15877-15888.

(2) Lie, F.; Chen, Y. Z.; Wang, Z. S.; Li, Z. *Tetrahedron: Asymmetry* **2009**, *20*, 1206-1211.

(3) Tang, W. L.; Li, Z.; Zhao, H. *Chem. Commun.* **2010**, *46*, 5461-5463.

(4) Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248–254.