Manipulating the Stereoselectivity of the Robust Baeyer-Villiger Monooxygenase TmCHMO by Directed Evolution

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Materials and chemicals

KOD Hot Start DNA Polymerase was obtained from Novagen. Restriction enzyme *Dpn* I was bought from NEB. The oligonucleotides were synthesized by Life Technologies. Plasmid preparation kit was ordered from Zymo Research, and PCR purification kit was bought from QIAGEN. DNA sequencing was conducted by GATC Biotech. All commercial chemicals were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI) or Alfa Aesar.

Methods

Docking 4-methycyclohexanone into WT TmCHMO

The X-ray structure of TmCHMO (5M10) was used as the basis for docking calculations. 4-methylcyclohexanone was prepared for docking using ChemDraw. Docking was performed using Autodock Vina. For this, the pdb file 5M10 was downloaded, hydrogens were added, and water, buffer and the ligand were removed. To prepare a suitable receptor for ligand docking, a simulation cell was defined by amino acids of the active site cavity (L145, L146, F248, G278, F279, R329, F434, T435, N436, L437, W492, and F507). The docking pose with the carbonyl atom closest to the flavin C(4a) was taken as the final docking pose.

PCR based methods for library construction of TmCHMO

Libraries were constructed using Over-lap PCR and megaprimer approach with KOD Hot Start polymerase. 50 μL reaction mixtures typically contained 30 μL water, 5 μL KOD hot start polymerase buffer (10×), 3 μL 25 mM $MgSO_4$, 5 μL 2 mM dNTPs, 2.5 μL DMSO, 0.5 μL (50~100 ng) template DNA, 100 μM primers Mix (0.5 μL each) and 0.5 μL KOD hot start polymerase. The PCR conditions for short fragment: 95 °C 3 min, (95 °C 30 sec, 56 °C 30 sec, 68 °C 40 sec) × 30 cycles, 68 °C 120 sec. For mega-PCR: 95 °C 3 min, (95 °C 30 sec, 60 °C 30 sec, 68 °C 6 min) × 28 cycles, 68 °C 10 min. The PCR products were analyzed on agarose gel by electrophoresis and purified using a Qiagen PCR purification kit. 2 μL NEB CutSmart™ Buffer and 2 μL *Dpn* I were added in 50 μL PCR reaction mixture and the digestion was carried out at 37 °C for 7 h. After *Dpn* I digestion, the PCR products 1.5 μL were directly transformed into electro-competent *E. coli*Top10 to create the final library for Quick Quality Control (QQC)[3] and screening.

Primer design and library creation of TmCHMO

Primer design and library construction depend upon the particular amino acid chosen, and the eleven single sites saturation mutagenesis libraries (L145，L146，F248， F279，R329， F434，T435， N436， L437，W492 and F507) of TmCHMO were constructed following the procedure: 1) Amplification of the short fragments of TmCHMO using mixed primers L145NNK-F/L145-R, L146NNK-F/L146-R, F248NNK-F/F248-R, F279NNK-F/F279-R, R329NNK-F/R329-R, F434NNK-F/F434-R, T435NNK-F/T435-R, N436NNK-F/N436-R, L437NNK-F/L437-R, W492NNK-F/W492-R and F507NNK-F/F507-R for libraries L145NNK，L146NNK，F248NNK， F279NNK， R329NNK， F434NNK，T435NNK， N436NNK， L437NNK，W492NNK and F507NNK, respectively; 2) Amplification of the whole plasmid TmCHMO using the PCR products of step1 as

megaprimers, leading to the final various plasmids for library generation.

For rational designed 5-residue randomization mutagenesis library: 1) Amplification of the short fragments of TmCHMO using mixed primers 146E/L-**F**/434I/F+435F/Y/W/T+437T/A/G/L-**R** and 434I/F+435F/Y/W/T+437T/A/G/L-**F**/507W/F-**R**, respectively; 2) Over-lap PCR using the PCR products of step 2 as template and mixed primers 146E/L-**F**/507W/F-**R**; 3) Amplification of the whole plasmid TmCHMO using the PCR products of step2 as megaprimers, leading to the final variety plasmids for library generation.

For ISM, the libraries A and B were constructed as following procedure: 1) Amplification of the short fragments of TmCHMO using mixed primers F434NDT/T435NDT-**F**/F434/T435-**R** and L146NDT-**F**/F507NDT-**R** for Libraries A and B, respectively; 2) Amplification of the whole plasmid TmCHMO using the PCR products of step1 as megaprimers, leading to the final various plasmids for library generation. All the primers used are listed in Table S5. The PCR products were digested by *Dpn* I and transformed into electro-competent *E. coli*Top10 to create the library for screening.

Screening procedures

Colonies were picked up and transferred into deep-well plates containing 300 μL LB medium with 50 ug/mL carbenicillin and cultured overnight at 37 °C with shaking. An aliquot of 120 μL was transferred to glycerol stock plate and stored at −80°C. Subsquently, 800 μL TB medium containing 0.02% (0.2g/L) L-arabinose and 50 μg/mL carbenicillin was added directly to the culture plate, then continued to culture it for 16 h at 25 °C with shaking for protein expression. The cell pellets were harvested, then washed with 400μL of 50 mM, pH 7.4 potassium phosphate buffer. The cell pellets were resuspended in 400 μL of the same buffer and 4-methycyclohexanone (final concentration 10 mM in reaction system) in 20 μl methanol was added. The plates were incubated at 30 °C, 800 rpm, 18h. The product and remaining substrate were extracted using equal volumes of ethyl acetate (EtOAc) for GC analysis by chiral column (Supplementary Table S 6)

Protein expression and purification

All enzymes were expressed using *E. coli* Top10 cells in the presence of L-arabinose and purified using Ni-sepharose resin, as previously described.[1] The purified HisTag-SUMO-TmCHMO fusion protein was incubated overnight with SUMO protease. Subsequently, a Ni^{2+}-Sepharose column was used to capture the SUMO-His-Tag protein yielding isolated TmCHMO in the flow through.

Determination of kinetic parameters

Enzyme activity for kinetic parameters was measured by monitoring the consumption of NADPH at 340 nm. The activity assay was performed in a mixture containing 0.15 mM NADPH and varying concentration of 4-methylcyclohexanone (0-50mM) with 5% (final) methanol as cosolvent. It should be noted that 0.05μM WT (reacting too fast in higher concentration) and 2 μM mutants were used in each reaction.

To determine NADPH affinity, varying amounts of NADPH were added to mixture with constant 2.5 mM substrate. The limitations of the assay are the quick consumption of the NADPH at low concentrations and too high absorbance in high concentrations.

Determination of thermostability by ThermoFAD method

The T_m was determined using the ThermoFAD method. Specifically, 25 µl samples containing 1 mg/ml purified enzyme were prepared in a 96-well thin wall PCR plate, and then the plate was heated from 20°C to 90°C, increasing temperature by 0.5°C/10 seconds, using an RT-PCR machine (CFX96-Touch, Bio-Rad Laboratories), which could measure fluorescence using a 450–490 excitation filter and a 515–530 nm emission filter. The melting point was defined as the temperature when the first derivative of the observed fluorescent signal showed maximum value.

Biotransformation reactions for tested substrates

1mL PBS buffer (pH 7.4, 50mM) containing recombinant expressed cells (OD_{600}=35) and 10mM substrate [final concentration, methanol as co-solvent (5% of total volume)] were added into 25mL flask (providing enough oxygen), and the reaction was performed at 30°C with shaking (220 rpm) for 24h. The product was extracted with ethyl acetate containing 0.1 mM Methylbenzoate as the internal standard for GC analysis.

Chemical reference reactions

Chemical reactions were conducted for the substrates 2-benzylcyclohexanone, 4-phenylcyclohexanone, 4tertbutylcyclohexanone and bicycle[4.2.0)Octan-7-one, which we could not find their enantiomers through enzymatic reference reactions. Specifically, substrate (4.0 µmol, 8 µl of a 0.5 M stock solution in dioxane) and 3-chloroperbenzoic acid [8.9 µmol, 20 µl of a 10%w/v stock solution of reagent grade 3-chloroperbenzoic acid (77% w/w) in dichloromethane, 2.2 equiv] were combined in a micro-inlay for GC vials. This results in a final concentration of 0.160 M ketone and 0.360 M peracid. The clear colorless solution was shaken at room temperature for 18 h. The solution was diluted with dichloromethane (100 µl) and transferred into a 1.5 ml Eppendorf tube. A solution of triethylamine (ca. 45 µmol, 1000 µl of 0.6% v/v solution in dichloromethane, ca. 9 equiv.) and water (500 µl). The biphasic mixture was shaken for 30 min and centrifuged for 30 s at 10 kRCF at room temperature. The aqueous layer was removed and the organic phase was dried over Na_2SO_4. Methylbenzoat as standard was added and the solution was analyzed *via* GC.

Chemical synthesis of 5-methyloxepan-2-one

Synthesis of 5-methyloxepan-2-one: To a solution of 4-Methylcyclohexanone (200 mg, 1.78 mmol) in 10 mL CH_2Cl_2 was added at 0°C *m*-CPBA (800 mg, 4.64 mmol) and TFA (136 µL, 1.78 mmol). The reaction mixture was allowed to reach room temperature and left to react 24h. In continuation, 10% $Na_2S_2O_3$ (5 mL) was added and the mixture was further stirred for another 2h. The organic layer was extracted with CH_2Cl_2 (3 x 20 mL), washed twice with saturated sol. $NaHCO_3$, and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum, and the crude reaction mixture was purified using column chromatography (EA : PE=1:4) to afford *5-methyloxepan-2-one* a colorless oil (175 mg, 77%). NMR data are in concordance with the ones reported in the literature.[2]

Schemes, Tables and Figures

Table S1. Screening results of single site saturation mutagenesis at positions (L145，L146，F248，F279，R329， F434，T435， N436， L437，W492 and F507) towards 4-methylcyclohexanone.

List	Mutations	ee（%）	Favored enantiomer	Conversion rate
LGY1-146-H11	L146E	95%	*S*	70%
LGY1-434-C4	F434I	94%	*S*	74%
LGY1-435-B12	T435F	95%	*S*	95%
LGY1-435-C12	T435Y	95%	*S*	97%
LGY1-435-D9	T435W	96%	*S*	97%
LGY1-437-C6	L437G	85%	*S*	44%
LGY1-437-E7	L437T	77%	*S*	33%
LGY1-437-E12	L437A	74%	*S*	65%
LGY1-507-D3	F507W	95%	*S*	64%

Table S2. The code used for the construction of 5-residue randomization mutagenesis library.

Positions	Code
146	E/L
434	I/F
435	F/Y/W/T
437	T/A/G/L
507	W/F

Table S3. Screening results of 5-residue randomization mutagenesis library towards 4-methycyclohexanone.

List	Mutations	ee （%）	Favored enantiomer	Conversion rate
LGY-R1-C10	T435F/L437A/F507W	60%	*R*	70%
LGY-R2-A7	T435F/L437A	50%	*R*	96%
LGY-R2-F7	T435W/L437A/F507W	66%	*R*	50%

Table S4. Screening results of libraries A and B towards 4-methylcyclohexanone.

Library	Code	Mutations	ee (%)	Favored enantiomer	Conversion rate
template	LGY437-E12	L437A	74%	*S*	65%
LGY437-E12→**A**	LGY2-1-A10	F434L/T435F/L437A	54%	*R*	68%
	LGY2-2-B3	F434I/T435F/L437A	53%	*R*	62%
	LGY2-2-G4	F434I/T435L/L437A	64%	*R*	77%
template	LGY2-2-G4	F434I/T435L/L437A	64%	*R*	77%
A→B	LGY3-1-D12	F434I/T435L/L437A/F507L	86%	*R*	93%
	LGY3-1-E2	L146V/F434I/T435L/L437A/F507L	87%	*R*	60%
	LGY3-4-D11	L146F/F434I/T435L/L437A/F507C	94%	*R*	86%
	LGY3-4-E5	F434I/T435L/L437A/F507V	91%	*R*	96%

Table S5. List of primers for TmCHMO libraries.

Library	Primers	Sequence (5' to 3')
L145NNK	L145NNK-F	CCGCGCTGGGT**NNK**CTGAGCCGTAGCAACA
	L145-R	CGTTGCCGCTCGGAACGCAGT
L146NNK	L146NNK-F	CCGCGCTGGGTCTG**NNK**AGCCGTAGCAACA
	L146-R	CGTTGCCGCTCGGAACGCAGT
F248NNK	F248NNK-F	ACCGTTGCG**NNK**GGCTTTGAGGAAAGCA
	F248 -R	TTCGCGTACAGATCGGTCGGGGTC
F279NNK	F279NNK-F	AAAGGCAACGGT**NNK**CGTTTTATGTTCGG
	F279-R	TTCGCGTACAGATCGGTCGGGGTC
R329NNK	R329NNK-F	CTGTACGCGAAA**NNK**CCGCTGTGCAACG
	R329-R	GCAGGTTCATCGCACGGTAGTTA
F434NNK	F434NNK-F	AACGGTCCG**NNK**ACCAACCTGCCGCCGA
	F434-R	ACCCGGAATGTTCGCGCCGAAGA
T435NNK	T435NNK-F	CGGTCCGTTT**NNK**AACCTGCCGCCGAGCA
	T435-R	ACCCGGAATGTTCGCGCCGAAGA
N436NNK	N436NNK-F	CGGTCCGTTTACC**NNK**CTGCCGCCGAGCA
	N436-R	ACCCGGAATGTTCGCGCCGAAGA
L437NNK	L437NNK-F	GGTCCGTTTACCAAC**NNK**CCGCCGAGCAT
	L437-R	ACCCGGAATGTTCGCGCCGAAGA
W492NNK	W492NNK-F	AGGCGGATAGC**NNK**ATCTTCGGCGCGAA
	W492-R	CTTGCGCACGTTCACCACGCAGTTGGA

F507NNK	F507NNK-F		CGCGGTTATG**NNK**TACCTGGGCGGTC
	F507-R		CTTGCGCACGTTCACCACGCAGTTGGA
Rational designed 5-residue randomization mutagenesis library	146E/L-F	ee-146L-F	ACCGCGCTGGGTCTG**CTG**AGCCGTAGCAACATCCCGGA
		ee-146E-F	ACCGCGCTGGGTCTG**GAG**AGCCGTAGCAACATCCCGGA
	434I/F+435F/Y/W/T+437T/A/G/L-F	ee-434I/F-435F/Y-437T/A-F	TGGGCCCGAACGGTCCG**WTTTWT**AAC**RCG**CCGCCGAGCATTGAGGC
		ee-434I/F-435W-437T/A-F	TGGGCCCGAACGGTCCG**WTTTGG**AAC**RCG**CCGCCGAGCATTGAGGC
		ee-434I/F-435T-437T/A-F	TGGGCCCGAACGGTCCG**WTTACC**AAC**RCG**CCGCCGAGCATTGAGGC
		ee-434I/F-435F/Y-437G-F	TGGGCCCGAACGGTCCG**WTTTWT**AAC**GGT**CCGCCGAGCATTGAGGC
		ee-434I/F-435F/Y-437L-F	TGGGCCCGAACGGTCCG**WTTTWT**AAC**CTG**CCGCCGAGCATTGAGGC
		ee-434I/F-435W-437G-F	TGGGCCCGAACGGTCCG**WTTTGG**AAC**GGT**CCGCCGAGCATTGAGGC
		ee-434I/F-435W-437L-F	TGGGCCCGAACGGTCCG**WTTTGG**AAC**CTG**CCGCCGAGCATTGAGGC
		ee-434I/F-435T-437G-F	TGGGCCCGAACGGTCCG**WTTACC**AAC**GGT**CCGCCGAGCATTGAGGC
		ee-434I/F-435T-437L-F	TGGGCCCGAACGGTCCG**WTTACC**AAC**CTG**CCGCCGAGCATTGAGGC
	434I/F+435F/Y/W/T+437T/A/G/L-R	ee-434I/F-435F/Y-437T/A-R	GCCTCAATGCTCGGCGG**CGY**GTT**AWAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435W-437T/A-R	GCCTCAATGCTCGGCGG**CGY**GTT**CCAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435T-437T/A-R	GCCTCAATGCTCGGCGG**CGY**GTT**GGTAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435F/Y-437G-R	GCCTCAATGCTCGGCGG**ACC**GTT**AWAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435F/Y-437L-R	GCCTCAATGCTCGGCGG**CAG**GTT**AWAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435W-437G-R	GCCTCAATGCTCGGCGG**ACC**GTT**CCAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435W-437L-R	GCCTCAATGCTCGGCGG**CAG**GTT**CCAAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435T-437G-R	GCCTCAATGCTCGGCGG**ACC**GTT**GGTAAW**CGGACCGTTCGGGCCCA
		ee-434I/F-435T-437L-R	GCCTCAATGCTCGGCGG**CAG**GTT**GGTAAW**CGGACCGTTCGGGCCCA
	507W/F-R	ee-507F-R	CCAGACCGCCCAGGTA**GAA**CATAACCGCGTGACGT
		ee-507W-R	CCAGACCGCCCAGGTA**CCA**CATAACCGCGTGACGT
ISM	LGY437-E12→**A**	F434NDT/T435NDT-F	GCCCGAACGGTCCG**NDTNDT**AAC**GCG**CCGCCGAGCATTG
		F434/T435-R	ACCCGGAATGTTCGCGCCGAAGA
	A→B	L146NDT-F	CCGCGCTGGGTCTG**NDT**AGCCGTAGCAACA
		F507NDT-R	GACCGCCCAGGTA**AHN**CATAACCGCG

Table S6. Conditions for GC analyses.

Entry	Procedure	Retention time	Comment
1a	155°C, 5°C /min, 170°C (1 min), 50°C/min, 200°C (1 min). H_2: 1.5bar Column: Hydrodex-β-TBDAc, 25 m x 0.25 mm ID, 0.15 µm.	**1a**: 0.67 min, (*S*)-**2a**: 3.44 min (*R*)-**2a**: 3.52 min	All chemicals were identified by comparison with authentic samples. This method was used for Screening
1a	80°C (2min), 5°C/min, 160°C(1min), 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**1a**: 6.08 min (*S*)-**2a**: 14.33 min (*R*)-**2a** : 14.75 min	
1b	80°C (2min); 2°C/min, 220°C (8min), Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**1b**: 34.8 (*S*)-**2b**: 50.997 (*R*)-**2b**: 51.282	
1c	80°C (2min), 5°C/min, 160°C(1min), 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**1c**: 13.143 min (*R*)-**2c**: 22.512 min (*S*)-**2c**: 22.583 min	
1d	80°C (2min), 5°C/min, 160°C(1min), 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**1d**: 21.855 min **(+)-2d**: 27.8 min **(-)-2d**: 28.073 min	
3	80°C (2min); 5°C/min, 160°C(1min); 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**3**: 5.342 min (4R;6S)-**4**: 18.53 min (4S;6R)-**4**: 18.62 min	
5a	80°C (2min); 2°C/min, 220°C (8min), Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**5a**: 26.187 min (*S*)-**6a**: 46.767 min (*R*)-**6a**: 46.997min	
5b	80°C (2min); 2°C/min, 220°C (8min), Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**5b**: 41.713 min (*S*)-**6b**: 60.803 min (*R*)-**6b**: 61.1 min	
7	80°C (2min), 5°C/min, 160°C(1min), 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**7**: 4.647 min **N(-)-8a**: 14.233 min **N(+)-8a**: 14.31 min **ABN(-)-8b**: 13.133 min **ABN(+)-8b: -**	
9	80°C (2min), 5°C/min, 160°C(1min), 10°C/min, 220°C(8min). Helium: 2ml/min. Column: BGB173, 30 m x 0.25 mm ID, 0.25 µm.	**9**: 7.357 min **N(-)-10a**: 16.37 min **N(+)-10a**: 16.22 min **ABN(-)-10b**: 15.28 min **ABN(+)-10b**: 17.19 min	
11	80°C (2min), 5°C/min, 160°C(1min), 10°C/min,	**11**: 7.305 or 7.492 min **P(-)-12a**: 19.508 min	

	220°C(8min). Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**P(+)-12a**: 19.073 min **D(-)-12b**: 19.41 min **D(+)-12b**: 19.635 min	
13	110°C (2min); 10°C/min, 118°C;2°C/min 122°C; 25°C/min, 200°C (1min); 50°C/min, 220°C (4min), Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**13:** 9.1min (*S*)**-14a**: 11.1 min (*R*)**-14a**: 11.5 min	
15	80°C (2min); 2°C/min, 220°C (8min). Helium: 2ml/min. Column: BGB175, 30 m x 0.25 mm ID, 0.25 µm.	**15**: 39.83 min (*S*)**-16a**: 57.6 min (*R*)**-16a**: 57.88 min	

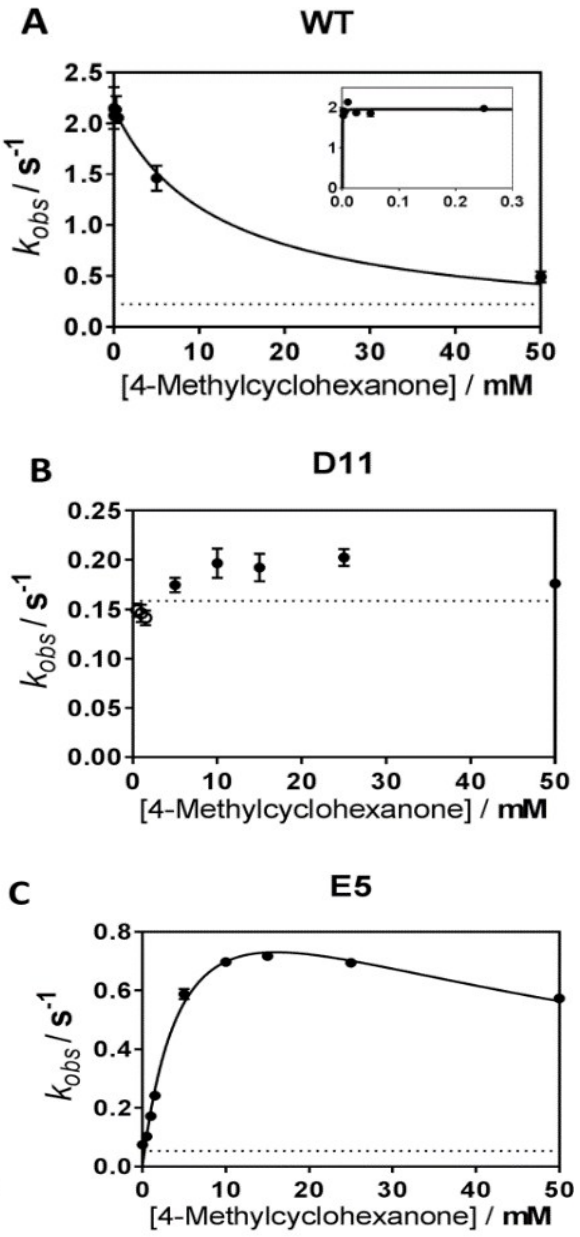

Figure S1. Plots of the substrate concentration dependency of the observed rates of NADPH consumption. The curves (solid lines) correspond to fitting the data with the Michaelis-Menten formula with substrate inhibition for 4-methylcyclohexanone. The dotted line indicates the uncoupling rate (the observed NADPH oxidase rate in absence of the substrate). The WT enzyme (A) displays a very low K_M ($K_M < 1$ μM, the inset shows rates in the μM range) while at high concentrations significant substrate inhibition is observed. The LGY3-4-D11 mutant (B) shows a maximal rate of around 0.2 s^{-1} and the rate is only slightly higher than the uncoupling rate. The LGY3-4-E5 mutant (C) displays the lowest uncoupling rate and a relatively high K_M, while the k_{cat} and K_I are similar to the WT enzyme.

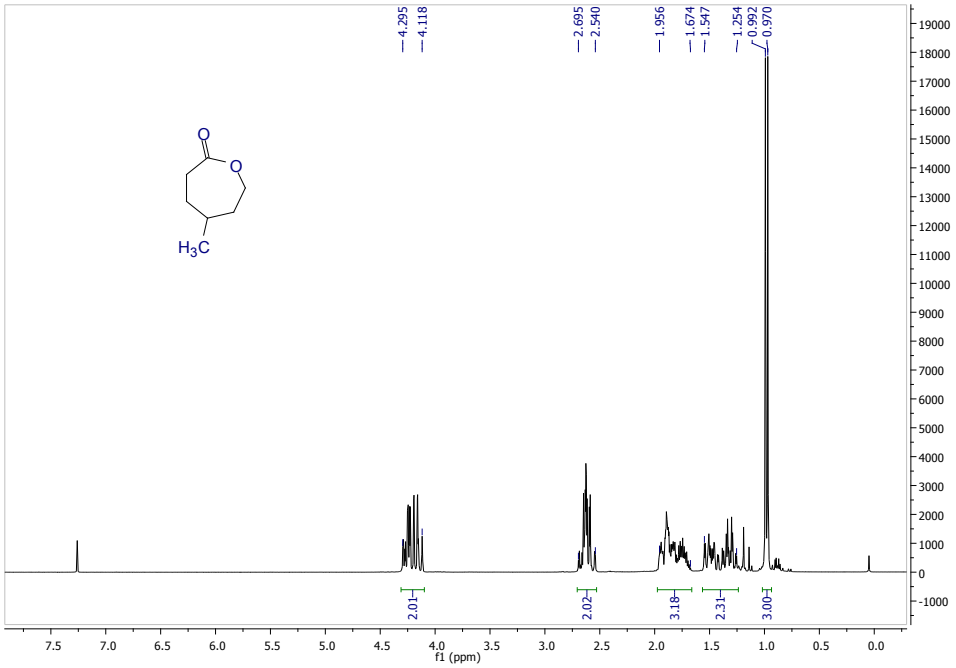

Figure S2. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.29 – 4.11 (m, 2H), 2.69 – 2.54 (m, 2H), 1.95 – 1.67 (m, 3H), 1.54 – 1.25 (m, 2H), 0.98 (d, J = 6.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.12 (s), 77.58 (s), 77.16 (s), 76.74 (s), 68.18 (s), 37.38 (s), 35.38 (s), 33.33 (s), 30.91 (s), 22.22 (s).^{1}H NMR spectrum (300 MHz, $CDCl_3$) of lactone

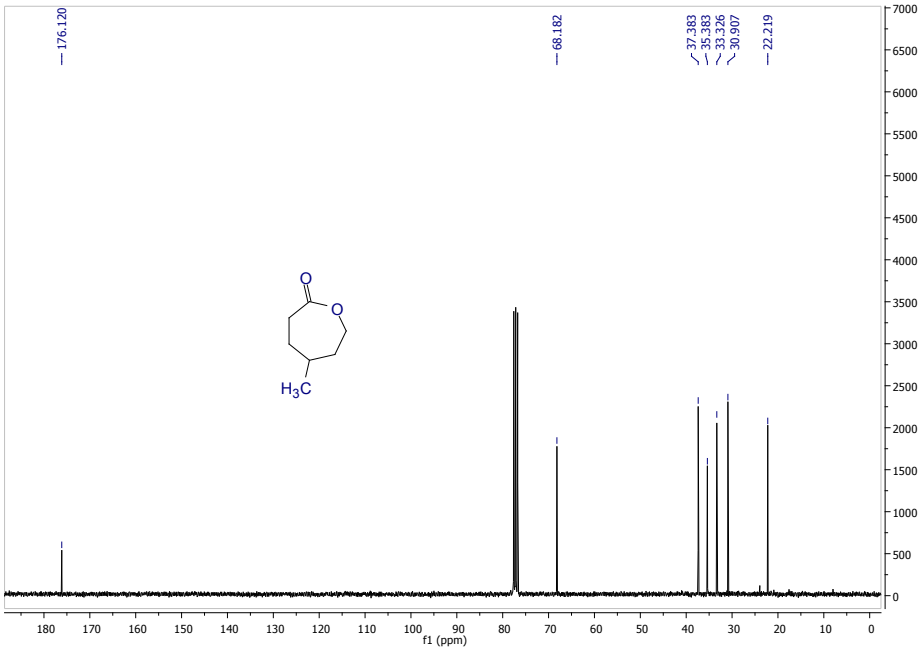

Figure S3. ^{13}C NMR (75 MHz, $CDCl_3$) of lactone

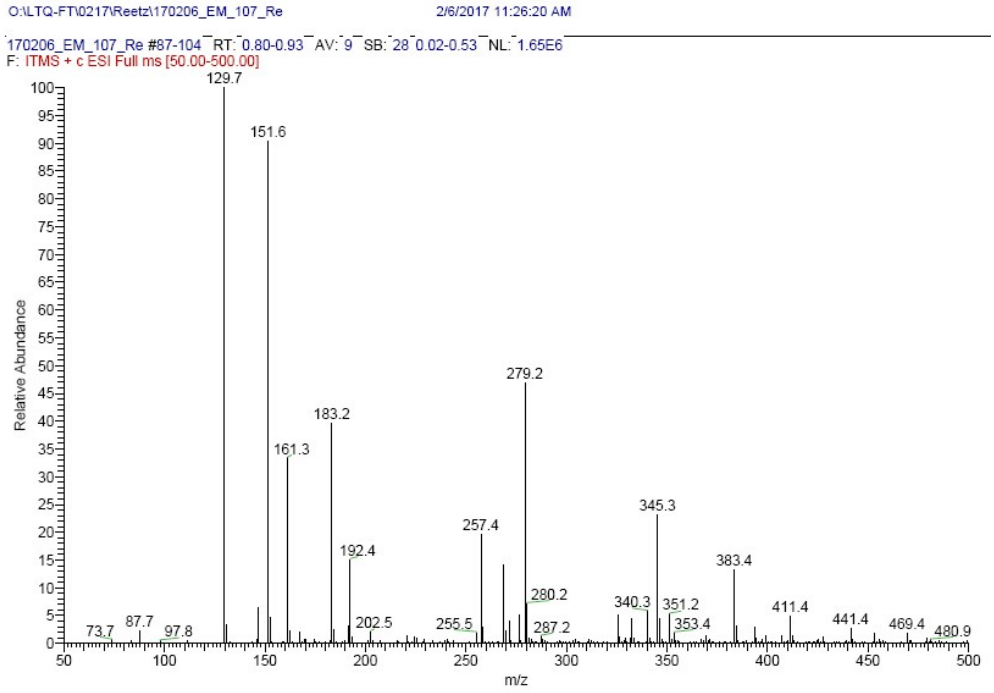

Figure S4. ESI (+) MS spectrum of lactone

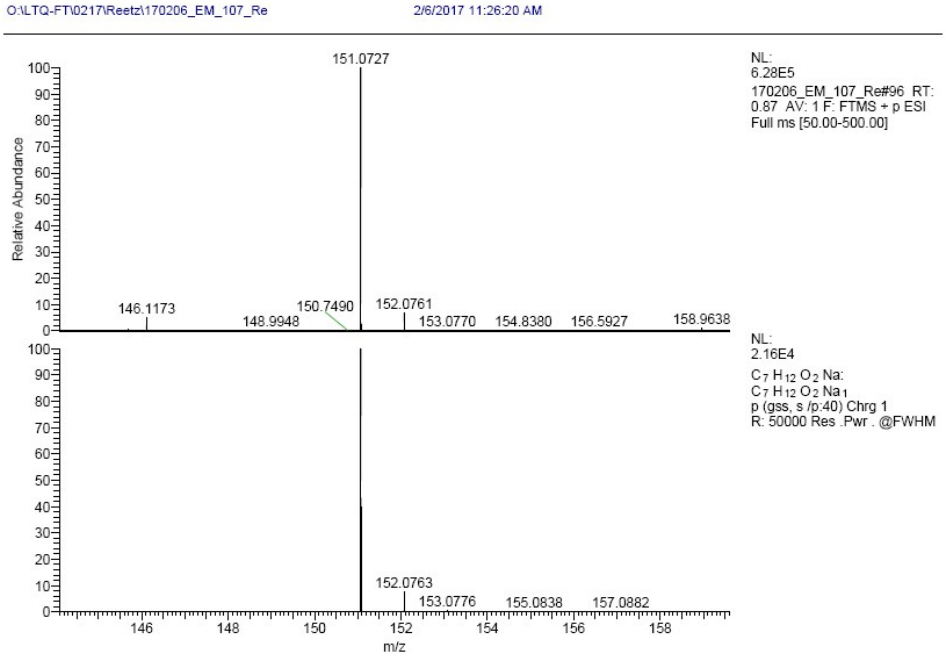

Figure S5. HRMS spectrum of lactone

1. H. L. van Beek, H. J. Wijma, L. Fromont, D. B. Janssen, M. W. Fraaije, *FEBS Open Bio.,* **2014**, *4*, 168-174.
2. M. G. Beaver, T. M. Buscagan, O. Lavinda, K. A. Woerpel, *Angew. Chem. Int. Ed*., **2016**, *55*, 1816–1819.