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Supporting information for:

H_2 as a fuel for flavin- and H_2O_2-dependent biocatalytic reactions

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1. Materials

FAD and FMN were purchased from Carl Roth. Ethylbenzene, (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, acetophenone, 2-methylcyclohexenone, 2-methylcyclohexanone, cyclohexane and cyclohexanol were purchased from Sigma–Aldrich in the highest purity. Amberlite™ FPA54 was purchased from DuPont. The production and purification of NAD^+-reducing hydrogenase (SH) was performed as described in Lauterbach *et al.*,[1] SH was immobilized on Amberlite™ FPA54 as described in Herr *et al.* [2] using a ratio of (1:1000) of enzyme to carrier. Unspecific peroxygenase (UPO), ene reductase (*Ts*OYE) and styrene monooxygenase (StyA) were produced and as described in Molina-Espeja *et al.*,[3] Lee *et al.*,[4] and van Schie *et al.*,[5] respectively.

2. H_2-driven biotransformation

2.1. Coupled with UPO

Preliminary experiments were conducted in a reaction volume of 1 ml 50 mM KPi buffer at pH 7.5 containing 200 µM FMN and 1 ml ethylbenzene was used in a two-phase system. The reaction was performed in explosive secured vials from (Reacti-Vial™, Thermo Scientific) sealed with a gas tight rubber septum with 7 ml excess of headspace according to Al-Shameri *et al.*[6] The reaction was performed at 30 °C for 16 hours. The gas mixture in buffer was set on 12 % O_2, 38 % N_2 and 50 % H_2. The gas mixture was set by purging the organic phase with H_2 for 20 min. Then the same volume of an air-saturated buffer was added, and 3.5 ml of air was injected into the headspace. Finally, the mixture was left for 30 min to equilibrate before measuring the gas content.

The biotransformation with the immobilized SH was performed using 210 mg of Amberlite FPA54-SH (= 15 µg) and UPO (2 µM). The reaction volume was 0.75 ml with 50 mM KPi buffer at pH 7.5 containing 200 µM FMN and 0.75 ml ethylbenzene/cyclohexane. The gas mixture was set empirically to 40 % O_2 and 60 % H_2 by the following procedure: The biotransformation reaction was purged with H_2 for 20 min. 3.5 ml of O_2 was injected into the headspace. Finally, the mixture was left for 30 min to equilibrate before measuring the gas content. The gas content was measured using GC-TCD. The GC analysis showed 40 % ± 2 O_2 and 60 % H_2 ± 2 gas composition. Purified SH (2 µM) and UPO (1 µM) were added to start the reaction.

Samples were taken from the organic phase and were extracted in ethyl acetate with *n*-octanol as internal standard.

2.2. Coupled with *Ts*OYE

Reaction volume of 1 ml 50 mM KPi buffer at pH 7.5 containing: 200 µM FMN, 20 mM substrate. The buffer was pre-purged with H_2. SH (2.5 µM) and *Ts*OYE (8 µM). The reaction was performed at 30 °C for 16 hours. Samples extracted in ethyl acetate with *n*-dodecane as internal standard

2.3. Coupled with StyA

Reaction volume of 1 ml 50 mM KPi buffer at pH 7.5 containing: 1 mM FAD, 300 mM substrate, the gas mixture in buffer was set on 10 % O_2, 30 % N_2 and 60 % H_2 as described above. Purified SH (3 µM) and styrene monooxygenase (5µM) were added. The reaction was performed at 30 °C for 16 hours. The positive control was done by using 1-benzyl-1,4-dihydronicotinamide-reduced FAD according to a previous procedure.[7] Samples were extracted in ethyl acetate with *n*-dodecane as internal standard.

3. GC analyses

3.1. GC analyses of the gases

To analyze the gases compositions, a GC-TCD (Shimadzu GC-2014AT) was used, which is equipped for the detection of small gases with Hayesep N columns and a 13X molecular sieve column with argon as the gas carrier.[7] 200 µL sample was taken from the gas phase (headspace) of the septum-sealed vial using gas tight Hamilton syringes, injected immediately in the GC and measured.

3.2. GC analyses of biotransformations

Compound analyses were carried out on Shimadzu GC-2010 gas chromatographs (Shimadzu, Japan) with an AOC-20i Auto injector equipped with a flame ionization detector (FID), using nitrogen or helium as the carrier gas. Products were confirmed by reference standards. Details of the columns and temperature programs used are given in Table S1. All measurements were calibrated with calibration curves. Time between experiments and GC measurements was several days which explained the poor enantiomeric excess obtained (racemization of certain products occurred).

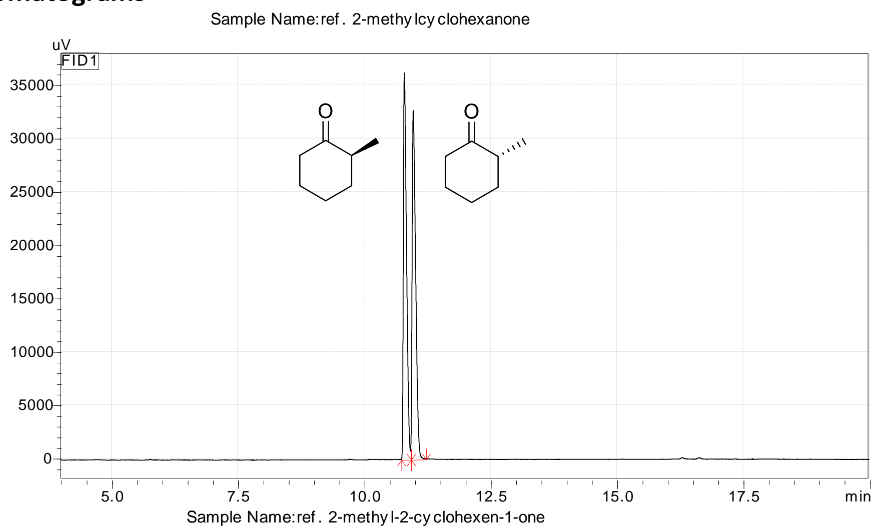
3.2.1. GC methods

Table S1. GC columns and oven programs used for analysis

Column	Temperature gradient	Analyte (retention time)
CP Wax 52 CB (Agilent) (50 m × 0.53 mm × 2 µm) carrier gas: N_2	130 °C hold 2 min 25 °C.min^{-1} to 175 °C hold 2.5 min 30 °C.min^{-1} to 245 °C hold 1 min	ethylbenzene (1.49 min) octanol (3.64 min) phenylethanol (5.82 min)
CP-Chirasil-Dex CB (Agilent) (25 m × 0.32 mm × 0.25 µm) carrier gas: H_2	120° C hold 2.6 min 15 °C.min^{-1} to 135°C hold 3.3 min 25 °C.min^{-1} to 225°C hold 1.0 min	2.40 min ethylbenzene 6.20 min (*R*)-1-phenylethanol 6.47 min (*S*)-1-phenylethanol
CP Wax 52 CB (Agilent) (50 m × 0.53 mm × 2 µm) carrier gas: N_2	65 °C hold 2.5 min 10 °C.min^{-1} to 120 °C hold 3.5 min 30 °C.min^{-1} to 230 °C hold 1 min	cyclohexane (1.22 min) cyclohexanol (8.95 min) octanol (12.22 min)
CP Sil 5 CB (Agilent)	80 °C hold 3 min	cyclohexane (6.64 min)

(25 m × 0.25 mm × 1.2 µM) carrier gas: N_2	25 °C.min^{-1} to 345 °C hold 1 min	cyclohexene (7.08 min) dodecane (9.36 min)
Lipodex E (Macherey-Nagel) (50 m × 0.25 mm × 0.25 µm) carrier gas: H_2 split ratio 50, linear velocity 36.9 cm/s, column flow 2.12 mL/min	80 °C hold 2 min 5 °C.min^{-1} to 110 °C hold 5 min 5 °C.min^{-1} to 130 °C hold 5 min 20 °C.min^{-1} to 220 °C hold 1 min	dodecane (10.4 min) (+)-(2*S*,5*S*)-dihydrocarvone (15.0 min) (+)-(2*R*,5*R*)-dihydrocarvone (15.23 min) (-)-(2*R*,5*S*)-dihydrocarvone (15.94 min) (-)-(2*S*,5*R*)-dihydrocarvone (16.5 min) (*R*/*S*)-carvone (18.0 min)
CP-Chirasil-Dex CB (Agilent) (25 m × 0.32 mm × 0.25 µm) carrier gas: H_2 split ratio 30, linear velocity 22.1 cm/s, column flow 1.00 mL/min	110 °C hold 4 min 5 °C.min^{-1} to 130 °C hold 1 min 25 °C.min^{-1} to 225 °C hold 1 min	dodecane (8.35 min) ketoisophorone (10.21 min) (*R*)-levodione (10.68 min) (*S*)-levodione (10.87 min)
CP-Chirasil-Dex CB (Agilent) (25 m × 0.32 mm × 0.25 µm) carrier gas: H_2 split ratio 150, linear velocity 30 cm/s, column flow 1.59 mL/min	70 °C hold 2 min 5 °C.min^{-1} to 90 °C hold 2 min 5 °C.min^{-1} to 110 °C hold 2 min 5 °C.min^{-1} to 130 °C hold 5 min 20 °C.min^{-1} to 220 °C hold 1 min	2-methylcyclohexenone (11.9 min) (*S*)-2-methylcyclohexanone (10.9 min) (*R*)-2-methylcyclohexanone (11.0 min) dodecane (15.6 min)

3.2.2. GC chromatograms



Sample Name:ref. 2-methyl-2-cyclohexen-1-one

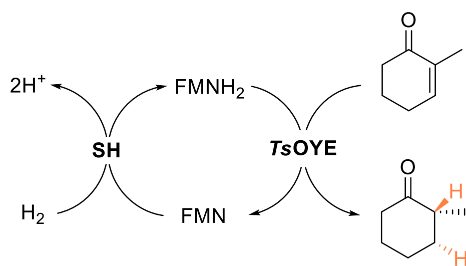
uV

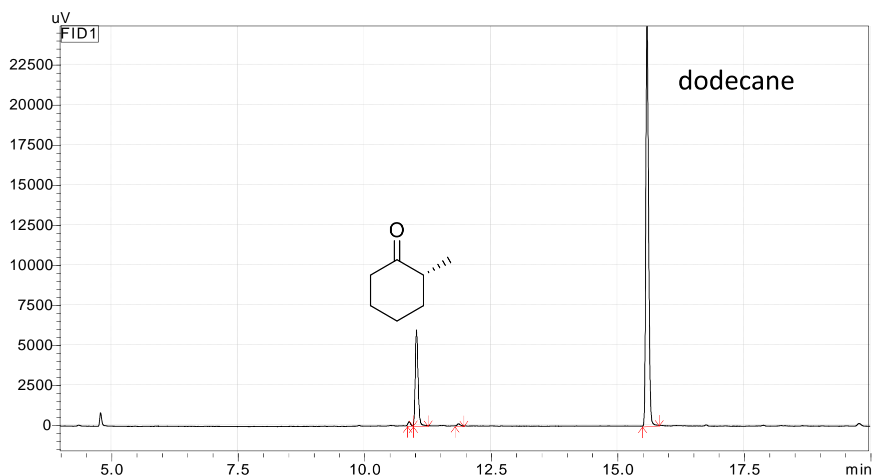
FID1

60000 50000 40000 30000 20000 10000 0

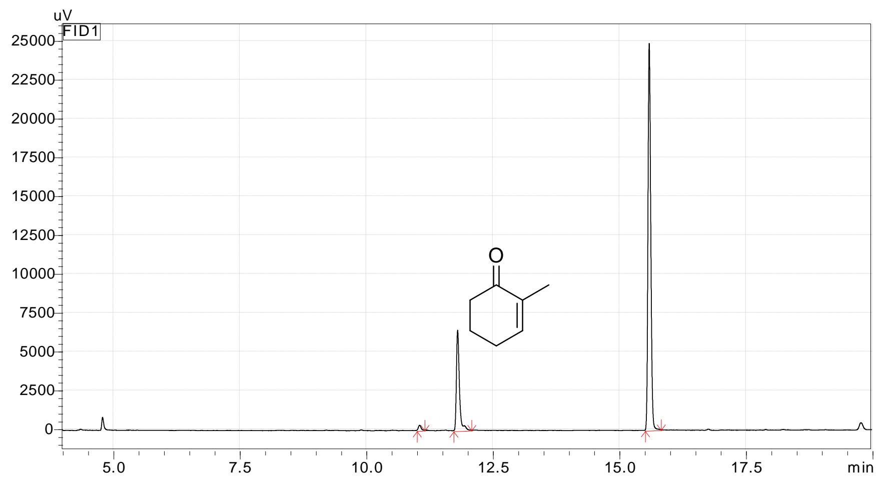
5.0 7.5 10.0 12.5 15.0 17.5 min

Example of the H_2-driven *Ts*OYE-catalyzed reduction of 2-methylcyclohexenone:

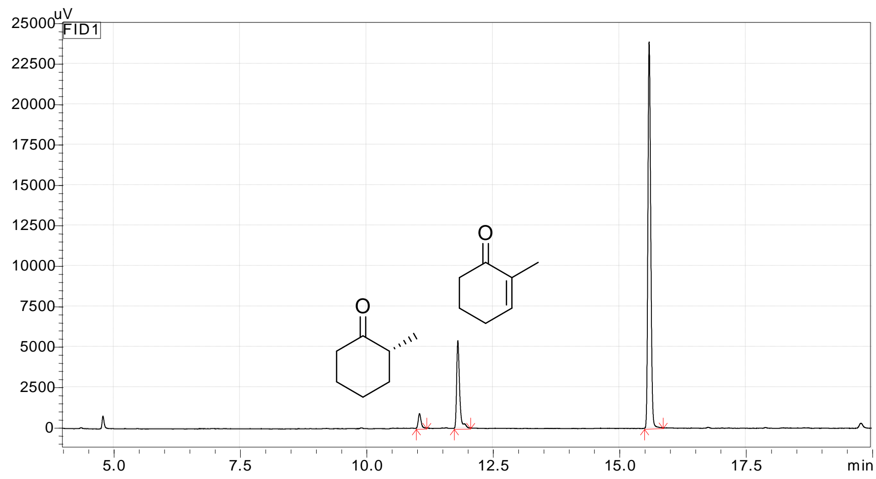




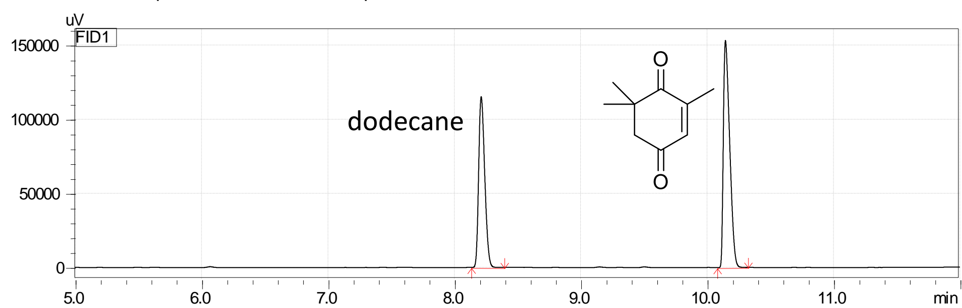
Negative control without SH:



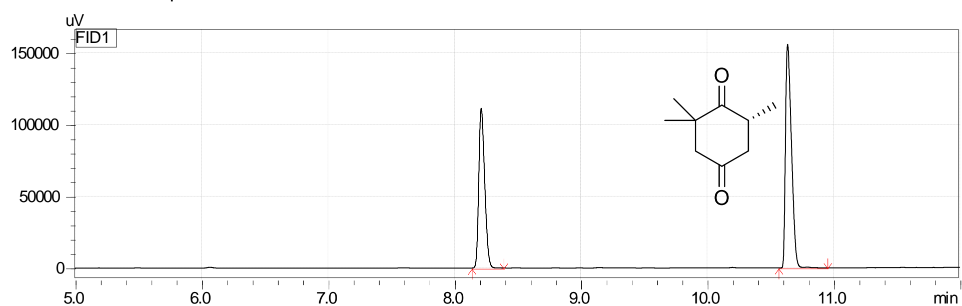
Negative control without added FMN:



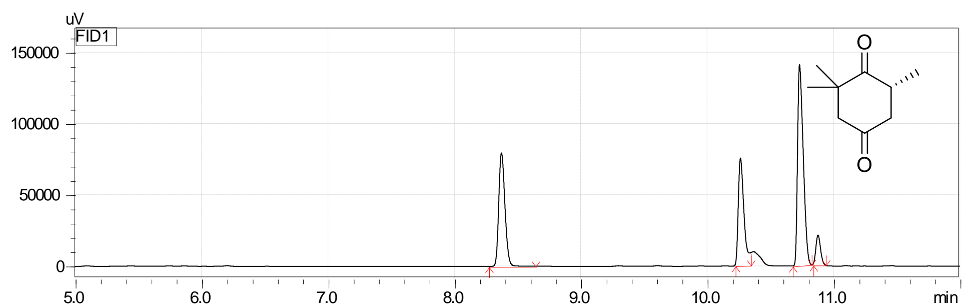
Ketoisophorone as a substrate:



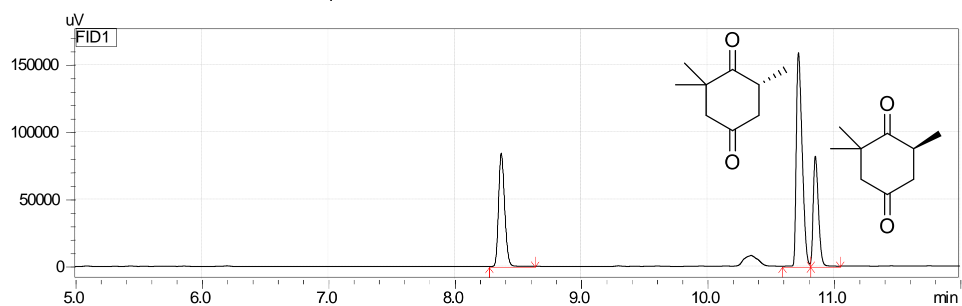
(6*R*)-Levodione:



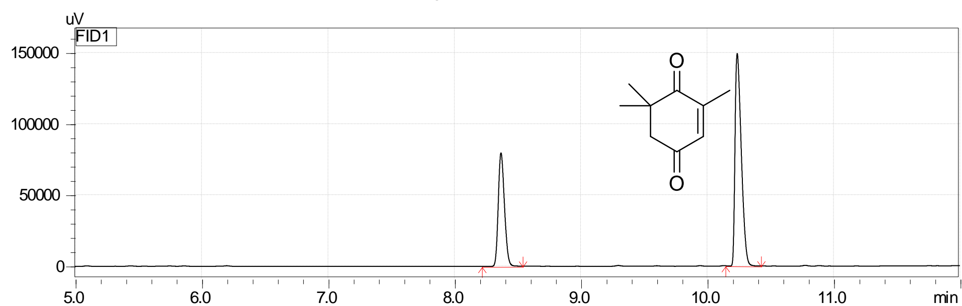
4 h reaction:



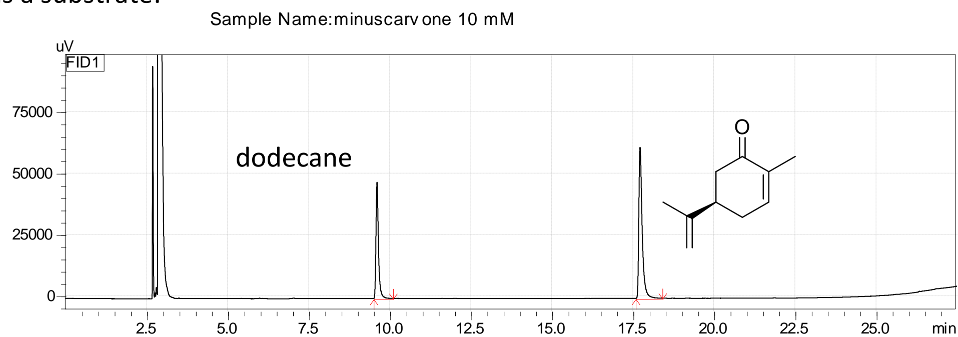
Overnight reaction:

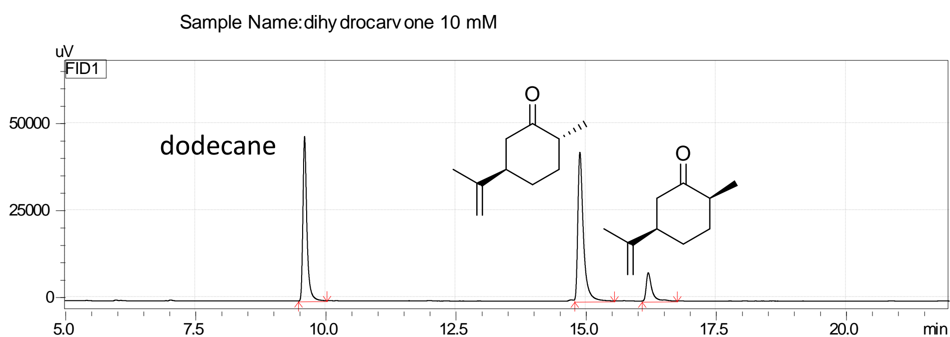


Negative control:

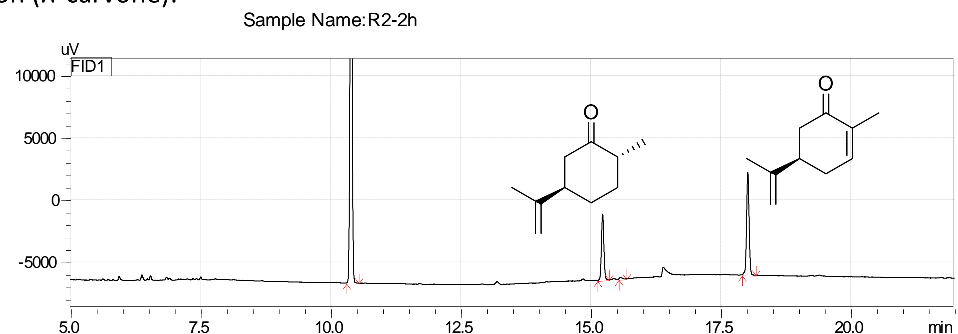


Carvone as a substrate:

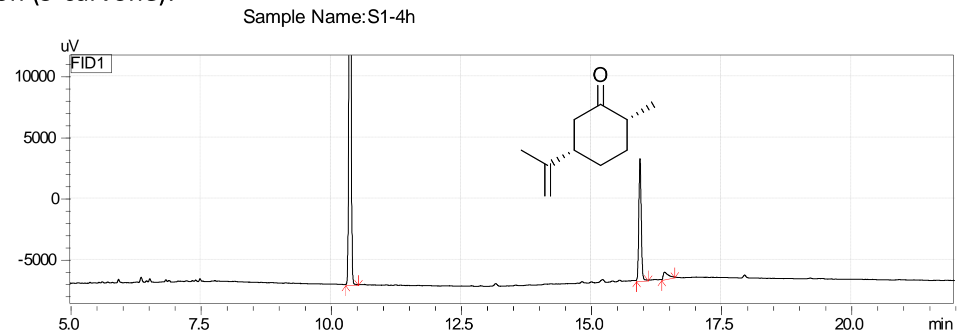




2 h reaction (*R*-carvone):



4 h reaction (*S*-carvone):



4. Additional results

4.1. Kinetic parameters of SH for FMN and FAD

Activity measurements of SH were performed spectrophotometrically using the Agilent Technologies Cary 50 UV-Vis spectrophotometer (equipped with a multi cell Peltier accessory) by monitoring the FMN_{ox} absorbance decrease at 500 nm in 2 mL cuvettes after purging with H_2. The activity of SH was measured in Tris-HCl (50 mM) at 30 ˚C. The specific activities were calculated using the extinction coefficient of FMN at 500 nm. The extinction coefficient of FMN and FAD at 500 nm ε = 2.55 m M^{-1} cm^{-1}.

Table S2. Kinetic parameters for H_2-driven FMN reduction.

V_{max} (U/mg)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($sec^{-1}mM^{-1}$)	R^2
5.8 ± 0.4	0.682 ± 0.106	20.3 ± 1.4	30	0.993

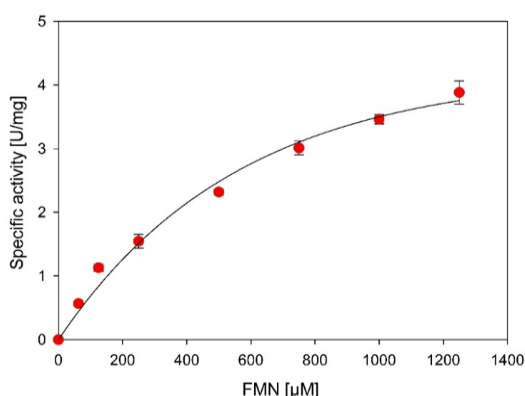


Figure S1: Kinetic parameters of SH for FMN

4.2. Formation of H_2O_2

The formation of H_2O_2 was verified when O_2 was purged to the FMN, which was before reduced by the SH. H_2O_2 detection was achieved using Amplex^{TM} Red and horseradish peroxidase by measuring the formation of resorufin at 570 nm (Figure S2).

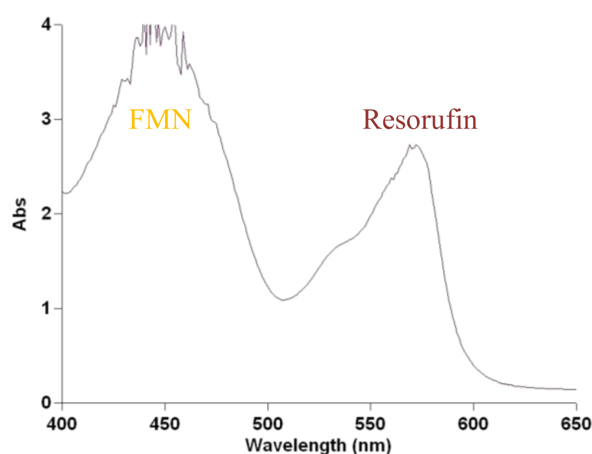


Figure S2: H_2O_2 is detected by the formation of resorufin at 570 nm after adding O_2 to the reduced FMN

4.3. Biotransformations

Table S3. H_2-driven alkene reduction with *Ts*OYE. n. d. not determined

Time /h	Concentration /mM				
	levodione	cyclohexenone	(2*R*,5*R*)-dihydrocarvone	(2*R*,5*S*)-dihydrocarvone	2-methhylcyclo-hexenone
2	7.4 ± 1.8 (84% *R*)	0.4 ± 0.01	1 ± 0.1	1 ± 0.0	n.d.
4	15.3 ± 0.6 (78% *R*)	1.3 ± 0.01	1.1 ± 0.1	1.9 ± 0.2	n.d.
16	21.8 ± 0.4 (37% *R*)	9.7 ± 0.18	0.48 ± 0.0	0.7 ± 0.02	17.8 ± 0.5 (>94% (*R*)

Table S4. H_2-driven hydroxylation with UPO

Time /h	Concentration /mM		
	Phenylethanol	Cyclohexanol	Control without H_2
1.25	4.2 ±1.8	3.1 ± 1.8	0
3	4.5 ± 1.7	3.6 ± 1.7	0
4	4.4 ± 0.6	3.7 ± 1.8	0

5. References

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