A quinone mediator drives oxidations catalysed by alcohol dehydrogenase-containing cell lysate

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I. General Methods

GC-MS analysis was carried out on an Agilent 6890N Network GC system equipped with a 5973 Network Mass Selective Detector (both Agilent Technologies, Santa Clara, USA) and a DB-5ms column (length = 30 m, diameter = 0.25 mm, film thickness = 0.25 µm) (Agilent Technologies). A carrier gas (helium) flow of 1 mL min^{-1} was used and the injection volume was 1 µL with a split ratio of 41.7:1.

The detected mass range was 50–300 amu. As temperature gradients, $T_{0\ min}$ = 60 °C, $T_{3\ min}$ = 60 °C, $T_{5\ min}$ = 100 °C, $T_{8.6\ min}$ = 280 °C, $T_{11.6\ min}$ = 280 °C [cyclohexanol (**3**)/cyclohexanone (**4**)] and $T_{0\ min}$ = 60 °C, $T_{3\ min}$ = 60 °C, $T_{14\ min}$ = 280 °C, $T_{19\ min}$ = 280 °C [1-phenylethanol (**5**)/acetophenone (**6**)] were used.

High-performance liquid chromatography (HPLC) was performed on a HP 1100 chromatography system (Agilent Technologies) equipped with a photodiode array detector. A Chiralcel OD-H column (250 mm × 4.6 mm) with guard column (50 mm × 4.6 mm) (both Daicel Inc., West Chester, USA) was used as the chiral stationary phase.

Nuclear magnetic resonance (NMR) spectra were recorded on a DRX 400 spectrometer (Bruker BioSpin, Rheinstetten, Germany) (^{1}H: 400 MHz, ^{13}C: 100.6 MHz). $CDCl_3$ (Euriso-Top, Saint-Aubin, France) was used as the solvent. Chemical shifts (δ) of ^{1}H NMR spectra are reported in ppm with a solvent resonance as an internal standard (^{1}H NMR: $CHCl_3$ 7.26 ppm).

Photometric assays were carried out on a UV 1650 PC spectrophotometer with a temperature-controlled sample chamber CPS-240A CE (both Shimadzu, Kyoto, Japan).

Chemical reagents used in the activity assays or in enzymatic oxidations were purchased from Alfa Aesar (Ward Hill, USA), AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerland) and Carl Roth GmbH (Karlsruhe, Germany). Alcohol dehydrogenase from horse liver [HLADH(N)his] was obtained from Evocatal (Monheim am Rhein, Germany). Catalase from bovine liver, horse radish peroxidase (HRP) and Ampliflu Red for H_2O_2 quantification were from Sigma-Aldrich Chemie GmbH (Munich, Germany). Restriction enzymes were obtained from New England Biolabs (Frankfurt, Germany). The solvent ethyl acetate was obtained in technical grade from Allchem GmbH (Breisach, Germany) and was distilled before use.

II. Molecular cloning and bacterial expression

Bacteria, DNA and expression vector

The strains *E. coli* TG1 and BL21(DE3) (both Agilent Technologies) were used for cloning and expression. The expression vector pET19b was purchased from Merck KGaA (Darmstadt, Germany). The gene for NfsB was provided by Prof. Dr. A. Stolz (University of Stuttgart) in the plasmid pUC118 (NfsB:pUC118).

PCR amplification, cloning and sequence analysis

All restriction, PCR and cloning steps were performed according to standard procedures.[1] Enzymes from commercial suppliers were used as recommended in the standard kit protocols. The NfsB gene was amplified by PCR from NfsB:pUC118 and inserted into the vector pET19b using the restriction sites for NdeI (5') and BamHI (3'). The resulting expression construct NfsB(N)his:pET19b was transformed in TG1 competent *E. coli* cells for amplification.

The primers used for PCR (forward: 5'-GAGCGTCATATGGATATCATTTCTGTCGC-3'; reverse: 5'-TTC-AGGGATCCTTACACTTCGGTTAAGGTG-3') were synthesised by Eurofins Genomics (Ebersberg, Germany).

Protein production and purification

For production of NfsB(N)his in *E. coli* BL21(DE3), 800 mL of ampicillin containing (100 µg mL^{-1}) LB-Lennox medium (10% tryptone, 0.5% yeast extract, 0.5% NaCl; Carl Roth GmbH) was inoculated with an 8 mL overnight starter culture and shaken at 160 rpm and 37 °C until an optical density (λ = 600 nm) of 0.6 was reached. Expression was induced by addition of IPTG (1 mM). After incubation for 4 h (37 °C, 160 rpm), cells were harvested by centrifugation (30 min, 4618 × *g*, 4 °C) and resuspended in KPi buffer (50 mM, pH = 7.5; 5 mL per 1 L culture medium). The cells were disrupted by sonication (6 × 15 s, Branson Sonifier Cell Disruptor 250, Branson Ultrasonics, Danbury, USA) and cell debris was removed by centrifugation (20 min, 12857 × *g*, 4 °C).

NfsB(N)his and HLADH(N)his were purified by affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany). Ni-NTA agarose was equilibrated with KPi buffer (50 mM, pH = 7.5) containing 25 mM imidazole. Crude enzyme preparation was added to the Ni-NTA agarose (2 mL enzyme preparation per 1 mL Ni-NTA agarose) and incubated on ice for 30 min. Nonspecifically bound proteins were washed off with KPi buffer (50 mM, pH = 7.5) containing 25 mM, 50 mM and 100 mM imidazole until no eluted protein could be detected in a colorimetric assay with 1X Roti-Quant protein staining solution (Carl Roth GmbH). Desired enzymes were eluted with KPi buffer (50 mM, pH = 7.5) containing either 500 mM imidazole, in the case of NfsB(N)his, or 250 mM imidazole, in the case of HLADH(N)his. The volume of the elution fractions was reduced to 5 mL by ultrafiltration (Vivaspin 20 centrifugal concentrator, 10000 MWCO PES; Sartorius Stedim Biotech GmbH, Göttingen, Germany) at 4500 × *g* and desalted by size exclusion chromatography (PD-10 Desalting Columns, GE Healthcare Bio-sciences AB, Uppsala, Sweden). Purified NfsB(N)his was stored in KPi buffer (50 mM, pH = 7.5) containing 20% (v/v) glycerol at −20 °C. Purified HLADH(N)his was stored at 4 °C.

LBADH was produced in *E. coli* as described elsewhere[2] and used as a cell-free raw extract.

Activity measurements

Activities of all enzyme preparations used were measured photometrically as a decrease or increase in the concentration of either substrate or cofactor. Activities were calculated as enzyme units (1 U = 1 µmol substrate conversion per minute). In order to avoid nonlinear time-dependent absorbance changes, diluted enzyme preparations were used. All measurements were performed at least in triplicate.

LBADH assay:

970 µL of isopropyl alcohol solution (200 mM) in $MgCl_2$-containing KPi buffer (50 mM, pH = 8.0, 1 mM $MgCl_2$) were mixed with 20 µL of $NADP^+$ solution (9.5 mM), also in $MgCl_2$-containing KPi buffer, in a cuvette (path length: 1 cm). The reaction was started by the addition of 10 µL of the enzyme preparation at 25 °C. After gentle mixing, the increase in absorbance at λ = 340 nm was measured for 60 s. For calculation of the enzymatic activity, a molecular extinction coefficient of ε_{340nm} = 6.22 mL µmol^{-1} cm^{-1} was used for NADPH.

HLADH assay:

100 µL of benzaldehyde solution (30 mM) and 20 µL of NADH solution (12.5 mM), both in KPi buffer (50 mM, pH = 7.0), were added to 870 µL of KPi buffer. 10 µL of the enzyme preparation was added at 30 °C. The decrease in absorption at λ = 340 nm was measured for 60 s [ε_{340nm}(NADH) = 6.22 mL µmol^{-1} cm^{-1}].

NfsB assay:

100 µL of NADPH solution (10 mM) and 10 µL of $K_3[Fe(CN)_6]$ solution (100 mM), both in KPi buffer (50 mM, pH = 8.0), were mixed thoroughly with 880 µL of KPi buffer. After addition of 10 µL of the enzyme preparation and gently mixing, the decrease in absorption at λ = 420 nm was measured for 60 s at 25 °C [$\varepsilon_{420nm}(K_3[Fe(CN)_6])$ = 1.00 mL µmol^{-1} cm^{-1}].

III. Photometric and fluorescence assays

Time-dependent depletion of NAD(P)H in presence of NfsB(N)his and lawsone

10 µL of a lawsone solution (2 mM) and 200 µL of a 1 mM solution of either NADH or NADPH, all in KPi buffer (50 mM, pH = 7.5), were added to 780 µL of KPi buffer. The reaction was started by addition of 10 µL of a solution of purified NfsB(N)his and gentle mixing. The decline of the NAD(P)H concentration was monitored photometrically over time (λ = 340 nm; 25 °C; path length: 1 cm). The concentration of the enzyme solution was chosen to not exceed an initial absorbance decay of 0.4 min^{-1} for both cofactors.

Control:

Either the lawsone (**1**) solution or the NfsB(N)his solution were replaced by 10 µL KPi buffer (50 mM, pH = 7.5).

Determination of hydrogen peroxide formation

Hydrogen peroxide was determined in analogy to a previously described protocol.[3] Lawsone (**1**) and varying amounts of NADH were dissolved in KPi buffer (50 mM, pH = 7.5) to obtain solutions with a fixed concentration of **1** (2 µM) and increasing concentrations of NADH (0 µM – 10 µM). 20 µL of each solution were placed in a well on a 384-well microtiter plate. After addition of purified NfsB(N)his solution (8 U mL^{-1}) the plate was incubated at room temperature for 10 min. Subsequently, the reaction buffer containing HRP (1 U mL^{-1}) and Ampliflu Red (50 µM) was added. Fluorescence intensity after HRP-catalysed oxidation of Ampliflu Red was measured with a polarstar microplate reader (BMG Labtech GmbH, Ortenberg, Germany). The excitation/emission wavelength ($\lambda_{ex}/\lambda_{em}$) filters were set at λ_{ex} = 510 nm/λ_{em} = 615 nm. The experiments were carried out in triplicate for each NADH concentration.

Control: 20 µL of solutions either containing NADH, lawsone or NfsB(N)his in KPi buffer (50 mM, pH = 7.5) were placed in separate wells on a 384-well microtiter plate and incubated at room temperature for 10 min. 20 µL of the reaction buffer were added and the fluorescence intensity was measured as described above. No significant fluorescence was detected.

Calibration curve: 20 µL of hydrogen peroxide solutions (0.5 µM to 24 µM) were placed in separate wells on a 384-well microtiter plate. The fluorescence intensity after incubation at room temperature (10 min) and addition of the reaction buffer was measured as described above and plotted against the H_2O_2 concentration. As the relationship becomes non-linear for higher concentrations of H_2O_2, only data points corresponding to concentrations ≤10 µM were taken into account for generating the calibration curve by linear regression (Figure S1).

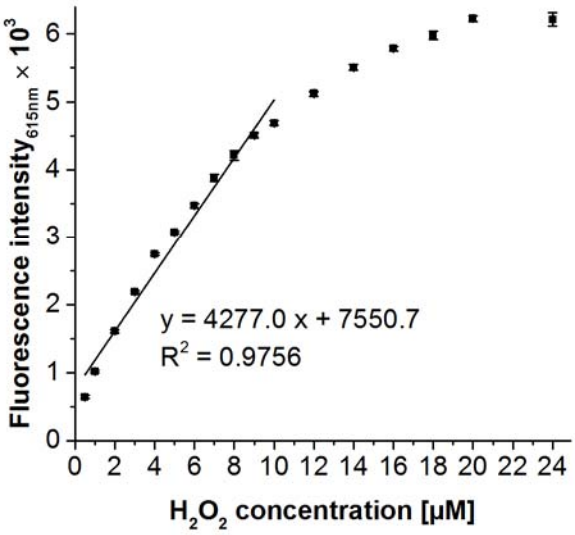

Figure S1: Calibration curve for the determination of the H_2O_2 concentration by the use of the HRP/Ampliflu Red assay.

IV. Enzymatic oxidations

HLADH(N)his-catalysed oxidation of cyclohexanol (3)

The substrate cyclohexanol (**3**, 100 mg, 1 mmol), lawsone (**1**, 8.7 mg 50 µmol) and NAD^+ (33.2 mg, 50 µmol) were dissolved in 50 mL Tris-HCl buffer (50 mM, pH = 8). HLADH-containing *E. coli* lysate (200 U) were added to start the reaction. The reaction mixture was stirred at room temperature for 20 h. Afterwards it was extracted three times with 50 mL ethyl acetate. Combined organic phases were filtered through Celite 535 filter aid, washed with 0.01 M NaOH and H_2O and dried over Na_2SO_4. Vacuum evaporation afforded the crude product. A conversion of 87% into product **4** was determined by ^{1}H NMR spectroscopy.

^{1}H NMR:

Cyclohexanol (**3**): (400 MHz, $CDCl_3$), δ = 1.10 – 1.34 (m, 5H), 1.49-1.58 (m, 2H), 1.67 – 1.78 (m, 2H), 1.84-1.93 (m, 2H), 3.54-3.65 (m, 1H).
Cyclohexanone (**4**): (400 MHz, $CDCl_3$), δ = 1.68 – 1.75 (m, 2H, H-4), 1.81 – 1.89 (m, 4H, H-3 and H-5), 2.33 (t, ^3J = 6.7 Hz, 4H, H-2 and H-6).

Reaction monitoring

General procedure – "standard conditions":
Cyclohexanol (**3**) was dissolved in Tris-HCl buffer (50 mM, pH = 8.5) to give a 20 mM solution. 5 mol% of both NAD^+ and lawsone (**1**) were added. 10 U mL^{-1} of purified NfsB(N)his and 4 U mL^{-1} of purified HLADH(N)his were added to start the reaction. The typical total volume of the reaction mixture was 1 mL. After defined periods of time (15 min, 30 min, 1 h, , 2 h, 4 h, 8 h, 20 h and 24 h), samples of 50 µL were taken and immediately extracted with 100 µL of ethyl acetate. The conversion was determined by GC-MS analysis of the organic phase. All reactions were carried out in triplicate, unless otherwise stated.

GC-MS:
Cyclohexanol (**3**): t_R = 4.39 min, *m/z* (%) = 100 (4) $[M]^+$, 82 (68), 67 (32), 57 (100).
Cyclohexanone (**4**): t_R = 4.50 min, *m/z* (%) = 98 (74) $[M]^+$, 83 (14), 69 (38), 55 (100).

Protocols differing from the general procedure:

"w/o NfsB(N)his":
The NfsB(N)his preparation was substituted by Tris-HCl buffer (50 mM, pH = 8.5).

"w/o lawsone":
No lawsone (**1**) was added to the reaction mixture.

"2 bar O_2":
The reaction was carried out in a Miniclave pressure reactor system (Büchi AG, Uster, Switzerland) supplied with oxygen at a gauge pressure of 2 bar.

"H_2O_2 addition":

Immediately after starting the reaction H_2O_2 was added to a final concentration of 20 mM.

"catalase addition":

295 U of catalase were added to the reaction mixture, before the reaction was started.

"catalase addition at 2 bar O_2":

Catalase (295 U) was added to the reaction mixture before a 2 bar O_2 gauge pressure was applied using the Miniclave pressure reactor system.

"addition of catalase and H_2O_2"

Catalase (295 U) was added to the reaction mixture as well as H_2O_2 was added to a final concentration of 20 mM.

"NAD^+ excess":

The reaction was carried out in the absence of NfsB(N)his and lawsone (**1**), and in the presence of excess NAD^+ (60 mM). The conversion was determined after 24 h by GC-MS analysis; no conversion was detected. The reaction was carried out twice.

blank:

Instead of purified HLADH(N)his, Tris-HCl buffer (50 mM, pH = 8.5) was added to the reaction mixture. The conversion was determined after 24 h by GC-MS analysis; no conversion was detected. The reaction was carried out twice.

GC-MS quantification

Intensities in GC-MS correspond to the integrated abundance of charged stable fragments after electron impact ionization. The conversion of cyclohexanol (**3**) into cyclohexanone (**4**) was calculated from the linear relationship between concentration and chromatogram peak areas determined from calibration curves (Figure S2).

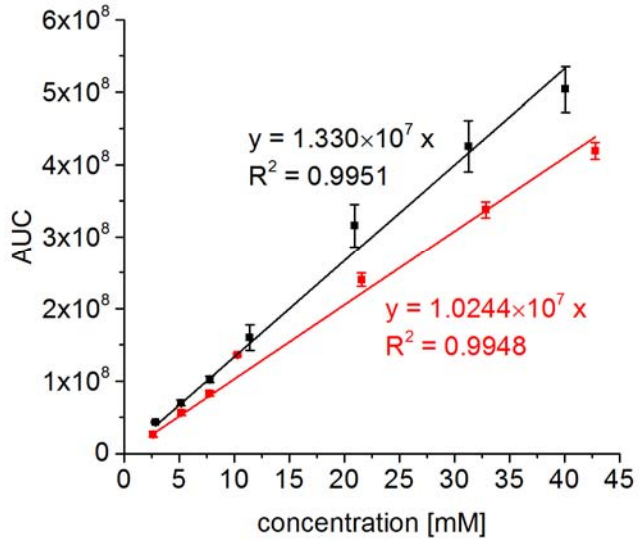

Figure S2: Calibration curves for different concentrations of cyclohexanol (3; —) and cyclohexanone (4; —) in ethyl acetate. Area under curve (AUC) values were determined in triplicate (error bars represent the standard deviation).

Kinetic resolution of racemic 1-phenylethanol (*rac*-5)

1-Phenylethanol (*rac*-**5**, 122 mg, 1 mmol), lawsone (**1**, 8.7 mg, 50 µmol) and $NADP^+$ (38 mg, 50 µmol) were dissolved in Tris-HCl buffer (50 mM, pH = 8.0) containing 1 mM $MgCl_2$. The reaction was started by the addition of LBADH-containing *E. coli* lysate (200 U) and the total volume was adjusted to 50 mL with buffer. The solution was stirred at room temperature for 24 h before it was extracted three times with 50 mL of ethyl acetate. The collected organic phase was washed twice with 100 mL of 0.01 M NaOH solution, then dried over $MgSO_4$. Ethyl acetate was removed under reduced pressure to obtain the crude product.

A conversion of 49% was determined by ^{1}H NMR spectroscopy. The *ee* = 97% for (*S*)-1-phenylethanol [(*S*)-**5**] was determined by chiral-phase HPLC. Commercial enantiopure (*R*)-1-phenylethanol [(*R*)-**5**] and racemic 1-phenylethanol (*rac*-**5**) were used as standards for chiral-phase HPLC.

GC-MS:

1-Phenylethanol (**5**): t_R = 6.48 min, *m/z* (%) = 122 (35) $[M]^+$, 107 (100), 79 (84), 77 (48), 51 (15).

Acetophenone (**6**): t_R = 6.55 min, *m/z* (%) = 120 (35) $[M]^+$, 105 (100), 77 (60), 51 (15).

Chiral HPLC (Figure S2): (Chiralcel OD-H, 25 °C, *n*-hexane/2-propanol = 90:10, 0.5 mL min^{-1})

(*R*)-1-Phenylethanol [(*R*)-**5**]: t_R = 11.6 min.

(*S*)-1-Phenylethanol [(*S*)-**5**]: t_R = 13.1 min.

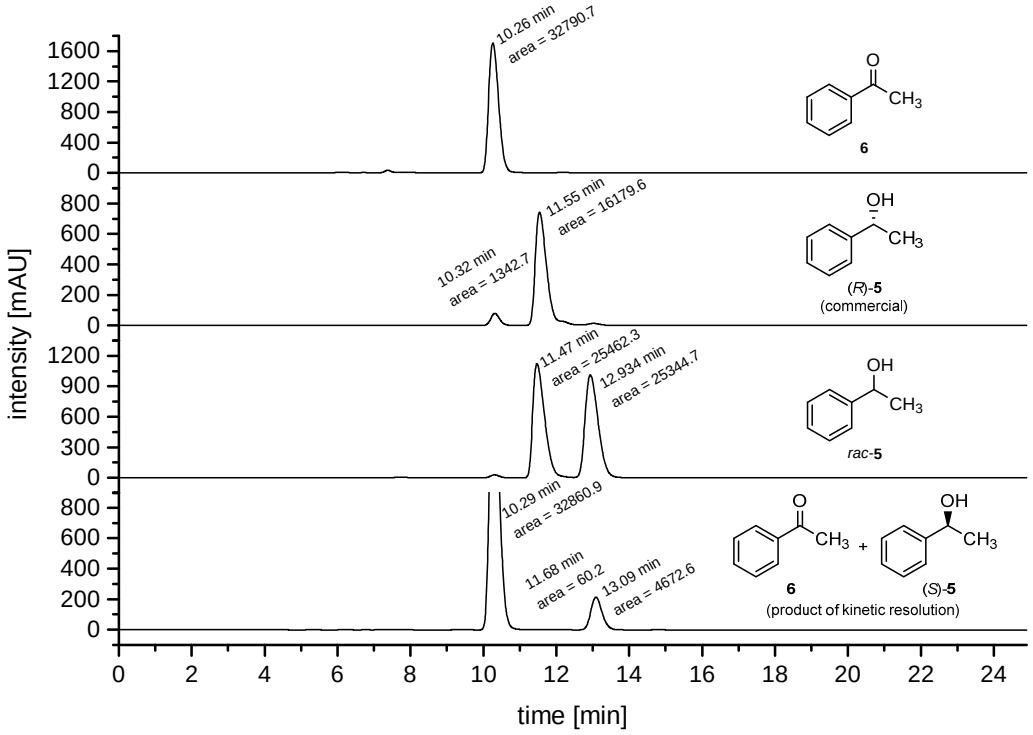

Figure S3: HPLC chromatograms (chiral phase: Chiralcel OD-H) of acetophenone (6); commercial (*R*)-1-phenylethanol [(*R*)-5]; racemic 1-phenylethanol (*rac*-5); and the products of the enzymatic kinetic resolution, (*S*)-1-phenylethanol [(*S*)-5] and acetophenone (6).

^{1}H NMR (Figure S3):

1-Phenylethanol (**5**): (400 MHz, $CDCl_3$), δ = 1.51 (d, ^3J = 6.4 Hz, 3H, CHOHC*H*$_3$), 1.8 (br s, 1H, O*H*), 4.91 (q, ^3J = 6.4 Hz, 1H, C*H*OHCH$_3$), 7.26–7.30 (m, 1H, H-4), 7.33–7.40 (m, 4H, H-2, H-3, H-5 and H-6).

Acetophenone (**6**): (400 MHz, $CDCl_3$), δ = 2.61 (s, 3H, COC*H*$_3$), 7.44–7.50 (m, 2H, H-3 and H-5), 7.55–7.60 (m, 1H, H-4), 7.94 –7.99 (m, 2H, H-2 and H-6).

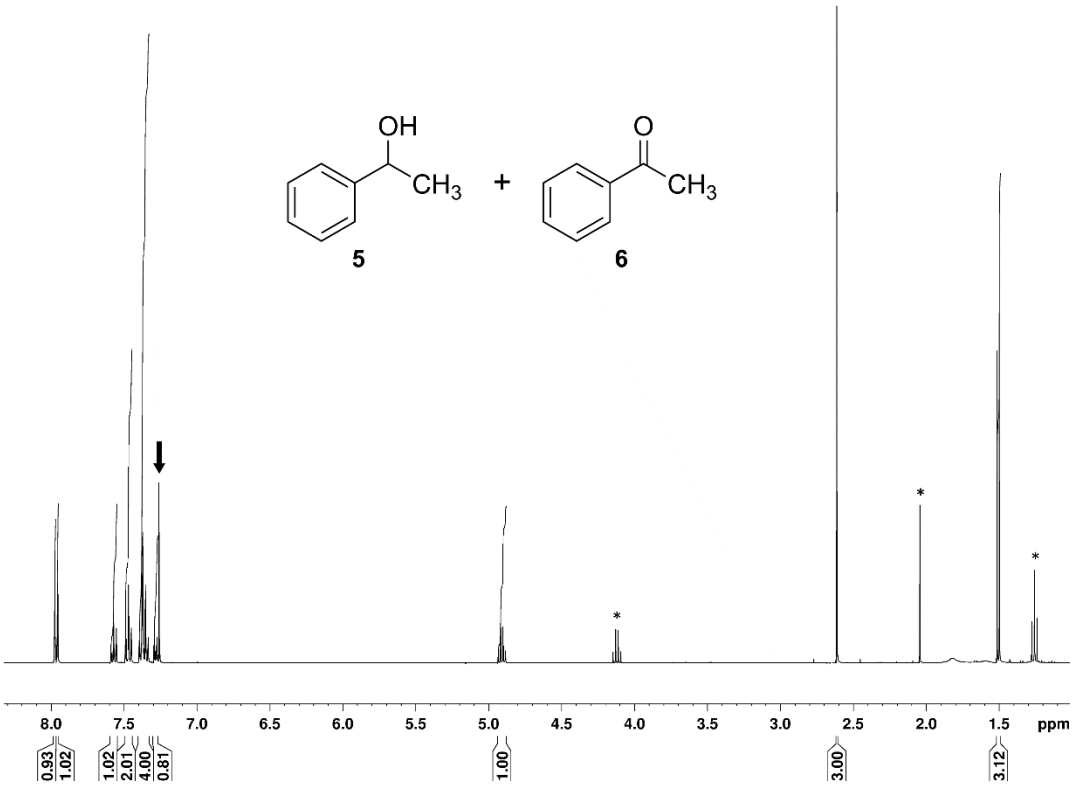

Figure S4: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of the products of the kinetic resolution [1-phenylethanol (5) and acetophenone (6)]. The solvent signal (chloroform) is marked with an arrow. Signals corresponding to residual ethyl acetate from extraction are marked with asterisks.

V. References

1 D. W. R. J. F. Sambrook, *Molecular cloning: A laboratory manual*, Cold Spring Harbor, New York, 3rd edn., 2000.
2 W. Hummel, *Adv. Biochem. Eng. Biotechnol.*, 1997, **58**, 145.
3 J. G. Mohanty, J. S. Jaffe, E. S. Schulman and D. G. Raible, *J. Immunol. Methods*, 1997, **202**, 133.