SI for: Towards environmentally acceptable synthesis of chiral α-hydroxy ketones via solvent-free oxidase-lyase cascades.

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Material and Methods

Materials

Unless stated otherwise all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany), New England Biolabs (Ipswich, MA, USA), Merck (Darmstadt, Germany) or Alfa Aesar (Karlsruhe, Germany) in the highest purity available and used without further purification. Alcohol oxidase from *Pichia pastoris* (PpAOX) was purchased from Sigma-Aldrich as buffered aqueous solution (1 KU). Lyophilized benzaldehyde lyase (≥ 1.6 U/mg) was initially obtained from evoxx (evo-1.4.106.S, Monheim, Germany).

Bacterial strain and plasmid

Escherichia coli BL21 (DE3) were purchased from New England Biolabs (Beverly, MA, USA). The plasmid pET28a containing the gene encoding the benzaldehyde lyase from *Pseudomonas fluorescens* (NCBI_Nucleotide accession-number: P51853) bearing an additional N-terminal His_6-tag was kindly provided by Dr. Dörte Rother (Forschungszentrum Jülich, Jülich, Germany). The plasmid was transformed into the appropriate *E. coli* strain by the heat shock method.[1]

Cultivation conditions

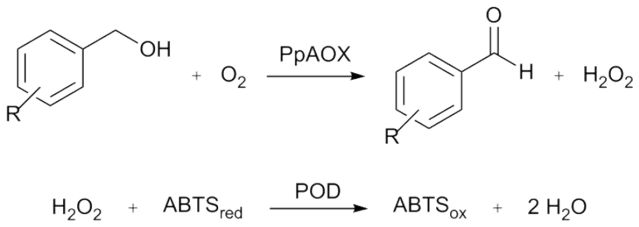
Expression of PfBAL was carried out by inoculation of 400 mL TB (terrific broth) medium supplied with the appropriate antibiotic (kanamycin) with an overnight culture to give an OD_{600} of 0.05. *E. coli* BL21 (DE3) cells were used as expression host. Cells were grown at 37°C in baffled shake flasks. PfBAL expression was induced at an OD_{600} of 0.6-0.8 with 1.0 mM IPTG. Cultivation was continued at 20°C for 24 hours. Cells were harvested (centrifugation at 1344 *g* at 4°C for 15 min) and washed twice in potassium phosphate buffer (pH 8.5, 50 mM). The bacterial cell (10.25 g) pellet was resuspended in the same buffer to give a wet cell weight (WCW) of 100 g/L and disrupted three times for 10 min by ultrasonication or directly frozen and freeze-dried afterwards. PfBAL was directly used as crude cell extract and was not further purified.

Determination of PfBAL activity

One unit of ligase activity is defined as the amount of PfBAL which catalyzes the formation of 1 µmol of benzoin per minute under standard conditions (30˚C, pH 8.5).
Initial rates of PfBAL-catalyzed benzoin formation were determined using HPLC (see details below in chapter Analytics). Samples containing 10-60 mM benzaldehyde in 50 mM potassium phosphate buffer, pH 8.5, 2.5 mM $MgSO_4$, 0.1 mM ThDP were incubated with 50 µL BAL at 30˚C in glass vessels. At appropriate time intervals, samples were taken to measure the amount of benzoin.

Determination of PpAOX activity

One unit is defined as the amount of PpAOX which oxidizes 1.0 µmole of benzyl alcohol to benzaldehyde per min at pH 7.5 at 25 °C.



Scheme S1 Spectrophotometric assay to determine the activity of PpAOX. PpAOX: alcohol oxidase from *Pichia pastoris*, ABTS™: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), POD: peroxidase from *Horseradish*.

The following solutions were prepared directly prior to the measurements: phosphate buffer (100 mM, pH 7.5 at 25 °C), ABTS solution (2 mM) in phosphate buffer which was bubbled with pure O_2 gas for ~5 minutes before use, alcohol solutions [1.0% (v/v)] in distilled water with 5% (v/v) of dimethyl sulfoxide (DMSO) or 2-methyltetrahydrofuran (2-Me-THF) if necessary, peroxidase solution (250 units/ml solution of peroxidase in phosphate buffer and an alcohol oxidase solution (7.35 unit/ml of alcohol oxidase in phosphate buffer).

In a 1.0 or 1.505 ml reaction mix, the final concentrations were 1.86 mM ABTS, 0.00033% (v/v) benzyl alcohol, 1.25 (1.505 mL) or 0.83 (1mL) units POD, and 0.4935 (1.505 mL) or 0.3379 (1 mL) units alcohol oxidase.

First, the following reagents were pipetted into suitable cuvettes: 93 µL or 1.4 mL ABTS and 3.3 or 5 µL POD solution. The solutions were mixed with the pipette. The absorption at 405 nm was monitored until it was constant. After equilibration, 33.2 µL or 50 µL of the respective alcohol solution was added, mixed and the absorption at 405 nm was recorded again until it was constant. After addition of 33.2 µL or 50 µL PpAOX solution, the increase in A_{405} was recorded for 3 minutes. The ΔA_{405}/minute was obtained using the maximum linear rate for both, the sample and the blank.

The enzyme activities were calculated using the following formula:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{405}\text{/minute sample} - \Delta A_{405}\text{/minute Blank}) \times (1.0) \times (df)}{(36.8) \times (0.03322)}$$

where:
1.0 = Total volume (in milliliters) of assay
df = Dilution Factor
36.8 = Millimolar extinction coefficient of ABTS at 405 nm
0.3322 = Volume (in milliliters) of enzyme used

By using the same activity assay, the PpAOX was further investigated in terms of pH profile, temperature stability and temperature optimum, oxidative stability, product inhibition and substrate scope:

1) Temperature Optimum

To determine the temperature optimum, the phosphate buffer (100 mM, pH 7.5) and all reaction solutions were pre-incubated to the respective temperature (15-50˚C, in steps of 5˚C) until they reached the desired temperature (≈ 10 min). Afterwards the ABTS assay was performed as described above.

2) Temperature Stability

To determine the thermostability, the PpAOX (0.35 mg/mL) was incubated in phosphate buffer (100 mM, pH 7.5) at various temperatures ranging from 20-60° C for 10 min. Afterwards, the samples were immediately placed on ice and the activity was measured at room temperature using the ABTS.

3) Inactivation of PpAOX

Inactivation of the enzyme was determined by incubation of PpAOX (0.35 mg/mL) in phosphate buffer (100 mM, pH 7.5) at 30˚C. After defined time intervals samples were taken and immediately placed on ice. Residual activity was measured at room temperature using the ABTS assay.

4) Oxidative stability

PpAOX (0.35 mg/mL) was used to determine oxidative stability by incubating the enzyme in phosphate buffer (100 mM, pH 7.5) containing various concentrations of hydrogen peroxide (0-100mM) at 30˚C for 3 h. After the incubation time, 10 µL of a catalase solution (1 mg/mL in buffer) was added to the samples and incubated further on ice. Residual activity of the PpAOX was measured using the ABTS assay.

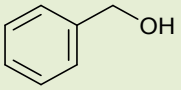
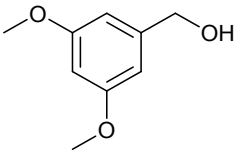
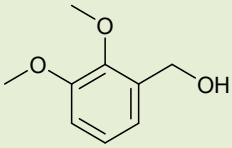
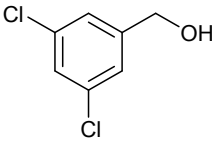
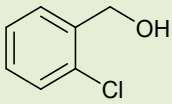
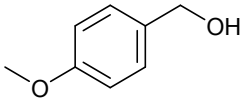
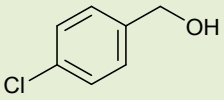
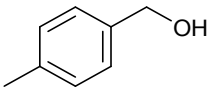
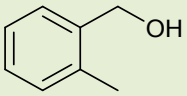
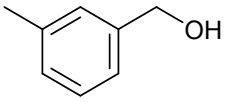
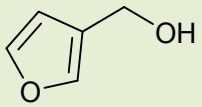
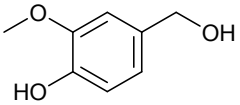
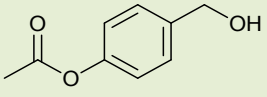
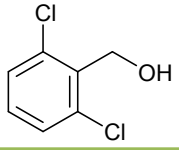
5) Product Inhibition

Product Inhibition of the enzyme was determined by incubation of PpAOX (0.35 mg/mL) in phosphate buffer (100 mM, pH 7.5) at 30˚C with different benzaldehyde concentrations (0-60mM). After 3h, samples were taken and immediately placed on ice. Residual activity was measured at room temperature using the ABTS assay.

6) Substrate scope

The substrate scope of PpAOX was determined using the ABTS assay according to the description above. All investigated substrates and their final assay concentrations were given in Table S1.

Table S1 Various alcohol substrates which were investigated to determine the PpAOX activity.

Entry	Substrate	Structure	Final Assay Concentration [mM] (Co-Solvent)
1	Benzyl alcohol		1
2	3,5-Dimethoxybenzyl alcohol		1 (DMSO)
3	2,3-Dimethoxybenzyl alcohol		1
4	3,5-Dichlorobenzyl alcohol		1 (2-Me-THF)
5	2-Chlorobenzyl alcohol		1 (2-Me-THF)
6	4-Methoxybenzyl alcohol		1
7	4-Chlorobenzylalcohol		1 (DMSO)
8	4-Methylbenzyl alcohol		1 (DMSO)
9	2-Methylbenzyl alcohol		1 (DMSO)
10	3-Methylbenzyl alcohol		1
11	Furan-3-methanol		1
12	Vanillyl alcohol		1 (DMSO)
13	4-Acetoxybenzyl alcohol		1
14	2,6-Dichlorobenzyl alcohol		Not soluble

General protocol for the biocatalytic oxidation-lyase reactions on analytical scale

Benzyl alcohol (2.16 mg, 20 mM) was dissolved in a mixture of phosphate buffer (50 mM, pH 8.5) containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and catalase from bovine liver (2.5 mg/mL). After addition of PfBAL (3.12 mg, 39.13 µM, 5 U) and PpAOX (17.75 µL, 788.89 nM, 25 U) to a total reaction volume of 1 mL, the reaction vial was covered with an oxygen gas-filled balloon and the mixture was shaked at 800 rpm in an Eppendorf shaker at 30˚C for 24 h. The reaction mixture was extracted with dichloromethane (3 x 1 mL), and the organic layer washed with water (3 x 1 mL) and brine (1 x 1 mL) and dried over Na_2SO_4 vacuum. The solvent was evaporated in vacuum. The reactions were followed by ^{1}H NMR and the enantiomeric excesses were determined by chiral-phase HPLC (AD-H column, UV detection at 210 and 254 nm).

Slurry-to-slurry reactions

The liquid alcohol substrates (350 mM, Table S1) were dissolved in a mixture of phosphate buffer (50 mM, pH 8.5) containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and catalase from bovine liver (2.5 mg/mL). In contrast, the solid alcohol substrates were directly used in their solid form (350 mM) in a slurry-to-slurry mixture with phosphate buffer (50 mM, pH 8.5) containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and catalase from bovine liver (2.5 mg/mL). To the reactions, PfBAL (5 mg, 62.72 µM, 8 U) and PpAOX (17.75 µL, 788.89 nM, 25 U) to a total reaction volume of 1 mL were added and the reaction vials were covered with an oxygen gas-filled balloon and shaked at 800 rpm in an Eppendorf shaker at 30˚C for 24 h. The reactions were followed by ^{1}H NMR and the enantiomeric excesses were determined by chiral-phase HPLC (AD-H column, UV detection at 210 and 254 nm).
After 72 hours, the reaction mixtures were centrifuged for 10 min at 1344 *g*. The supernatant was removed from the solid pellet, but kept for HPLC analysis. The solid pellet was resuspended in 1 mL distilled water and centrifuged again for 10 min at 1344 *g*. The supernatant was again removed and the solid pellet washed with distilled water. This process was repeated 5 times to wash the enzyme and all other reaction components away. The solid pellets were analyzed by ^{1}H NMR and the enantiomeric excesses were determined by chiral-phase HPLC (AD-H column, UV detection at 210 and 254 nm).

Figure S1 Picture of a representative slurry-to-slurry reaction after 72 hours.

Preparative scale synthesis of (R)-Benzoin

Preparation of the feeding solution: Benzyl alcohol (10.36 mL) was dissolved in 84.64 mL phosphate buffer (50 mM, pH 8.5) and 5 mL (5% (v/v)) 2-methyl tetrahydrofuran (2-Me-THF) to give a total volume of 100 mL. In the original reaction vessel, a mixture of 87.5 mL phosphate buffer (50 mM, pH 8.5), ThDP (0.15 mM), $MgCl_2$ (2.5 mM), catalase from bovine liver (2.5 mg/mL), PfBAL (350 µL crude cell extract, 1 mM, 130.5 U) and PpAOX (300 µL, 13.33 µM, 392 U) to a volume of 100 mL were mixed. The reaction mixture was stirred using a KPG® stirrer (IKA Labortechnik) equipped with a Teflon stirrer and the temperature was kept at 30˚C using a jacketed beaker connected to a thermo-stated water bath (Colora Messtechnik GmbH). The reaction vessel was tightly closed with septa. One outlet of the reaction vessel was connected to an oxygen gas-filled balloon whereas the other was connected via the pump (BPT 8538, Watson-Marlow Limited, UK) to the feeding solution.

Due to the limited solubility of benzyl alcohol, the feeding solution (1 M benzyl alcohol in 5% (v/v) 2-Me-THF) was continuously stirred to enable the formation of an even emulsion. The total reaction volume was 200 mL. Therefore, the feeding solution was added in defined time intervals to the main reaction to avoid insolubility problems of the benzyl alcohol. The complete feed was finished after 6 hours and 20 minutes. The reaction was further continued for 96 hours at 30˚C.

The reaction mixture was filtered applying vacuum. The filtration residue was washed three times with ultrapure water (3 x 50 mL) and then the amount of crude product was determined (4.6 g). For further purification, this crude product was dissolved in 50 mL dichloromethane. The aqueous reaction solution was additionally extracted three times with dichloromethane (3 x 200 mL) and the organic layer was washed with brine. The two organic phases were combined and dried over the appropriate amount sodium sulfate (approx. 5 g) and the solvent was evaporated under vacuum. The white crystalline product was yielded in 4.48 g (83 % isolated yield).

An appropriate amount of product was dissolved in 600 µL $CDCl_3$ or DMSO and the ^1H NMR spectra were recorded on a Bruker 400 MHz NMR unit.

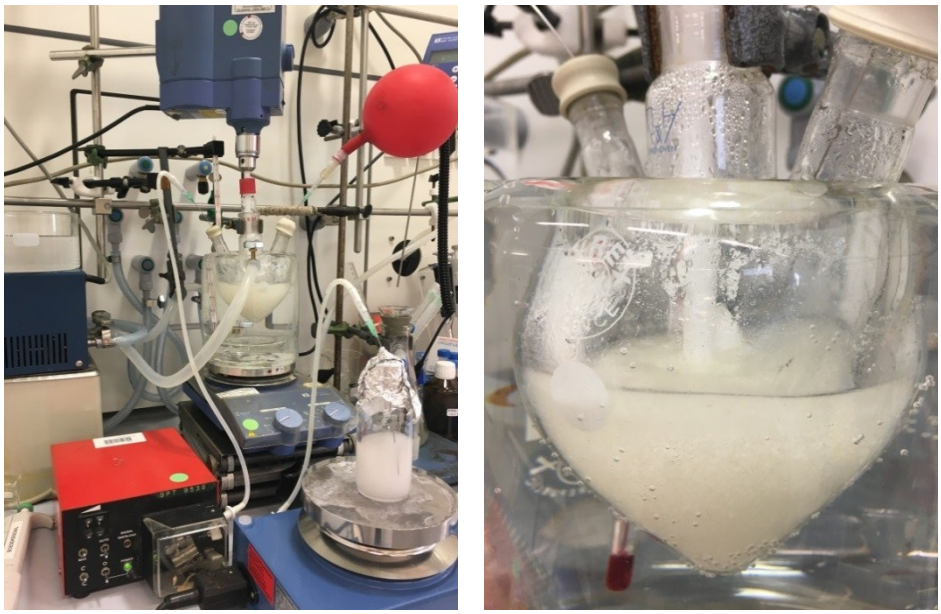


Figure S2 Picture of the reaction setup. Left: Whole reaction setup including the pump for substrate feed from the substrate reservoir. Right: Close-up of the reaction solution after 3 hour of reaction. The product (*R*)-benzoin is visible as white precipitate in the solution.

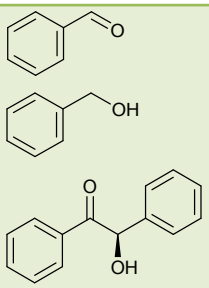
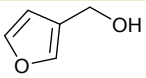
Analytics

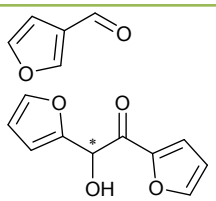
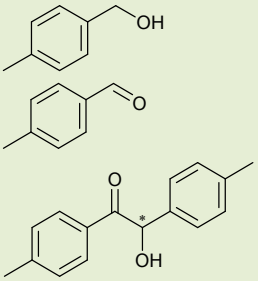
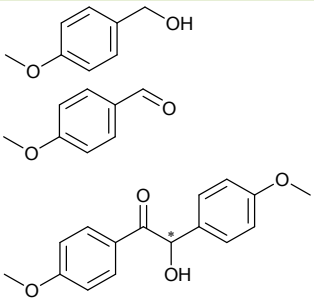
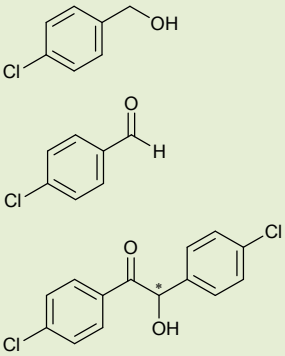
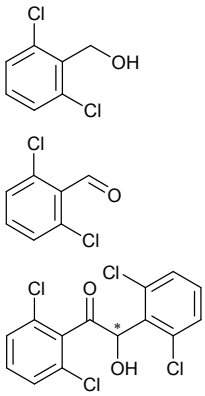
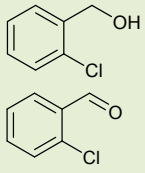
1) HPLC

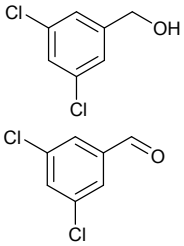
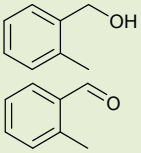
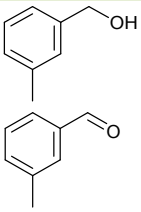
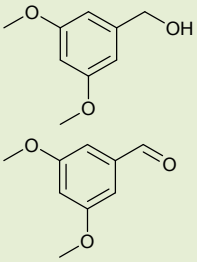
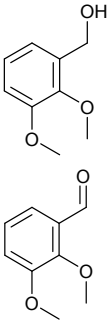
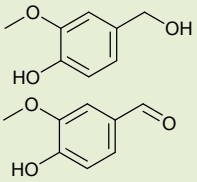
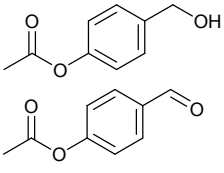
Enantiomeric excesses were determined by HPLC using the in the following described conditions. Retention times for all alcohol substrates, aldehyde intermediates and condensation products were summarized in Table S2.

HPLC conditions:
Column: Daicel AD-H Chiralpak 4.6 x 250 mm 5µ
Eluent: 93% heptane, 7% isopropanol (v/v)
Flowrate: 1.0 mL/min
Detection wavelengths: 210 and 254 nm

Table S2 Retention times of the reaction components.

Entry	Method	Name Substrate	Compounds	Retention time [min]
1	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	Benzyl alcohol		4.6 7.5 17.5
2	7% isopropanol, flow 1 mL/min,	Furan-3-methanol		4.1 -

	30 min, 254 nm			E1: 17.6 E2: 21.7
3	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	4-Methyl benzyl alcohol		7.5 4.7 E1: 19.6 E2: 21.2
4	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	4-Methoxy benzyl alcohol		6.4 4.1 E1: 15.4 E2: 22.5
5	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	4-Chloro benzyl alcohol		7.5 4.7 E1: 19.6 E2: 20.9
6	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	2,6-Dichloro benzyl alcohol		4.1 7.4 E1: 11.8 E2: 13.0
7	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	2-Chloro benzyl alcohol		7.3 4.2

8	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	3,5-Dichloro benzyl alcohol		6.3 4.5
9	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	2-Methyl benzyl alcohol		6.8 -
10	7% isopropanol, flow 1 mL/min, 30 min, 254 nm			6.8 4.4
11	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	3,5-Dimethoxy benzyl alcohol		12.9 5.9
12	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	2,3-Dimethoxy benzyl alcohol		10.4 5.5
13	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	Vanillyl alcohol		22.1 11.1
14	7% isopropanol, flow 1 mL/min, 30 min, 254 nm	4-Acetoxy benzyl alcohol		12.3 7.4 E1: 23.7 E2: 26.9

Representative HPLC chromatograms

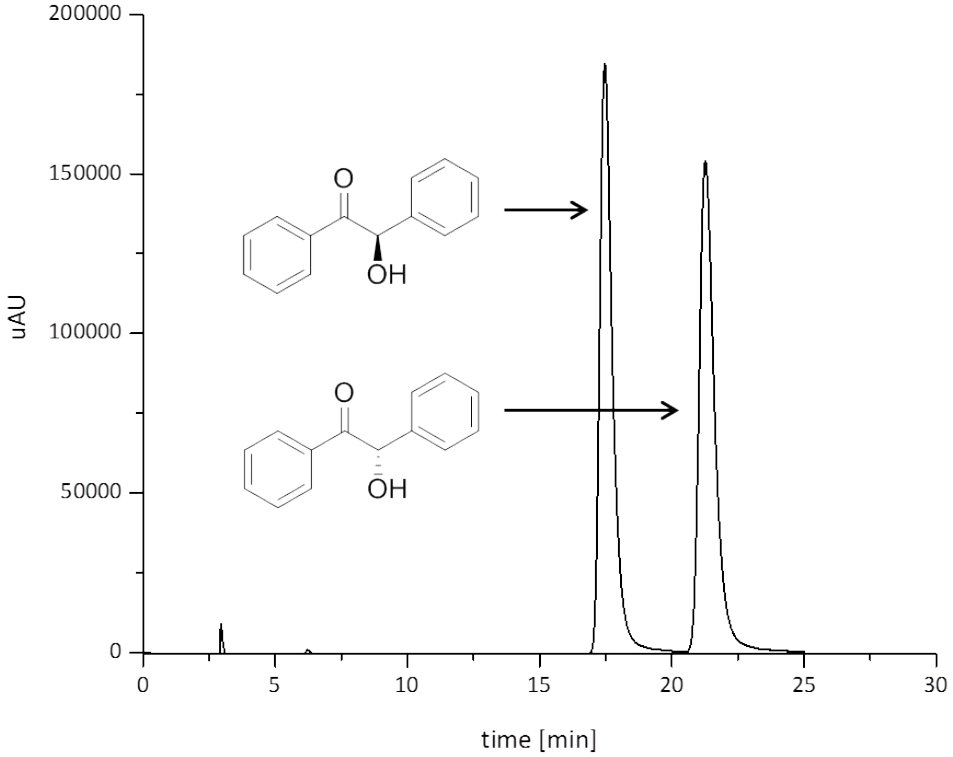


Figure S3 The separation and determination of the (*R*)-and (*S*)-enantiomers of benzoin.

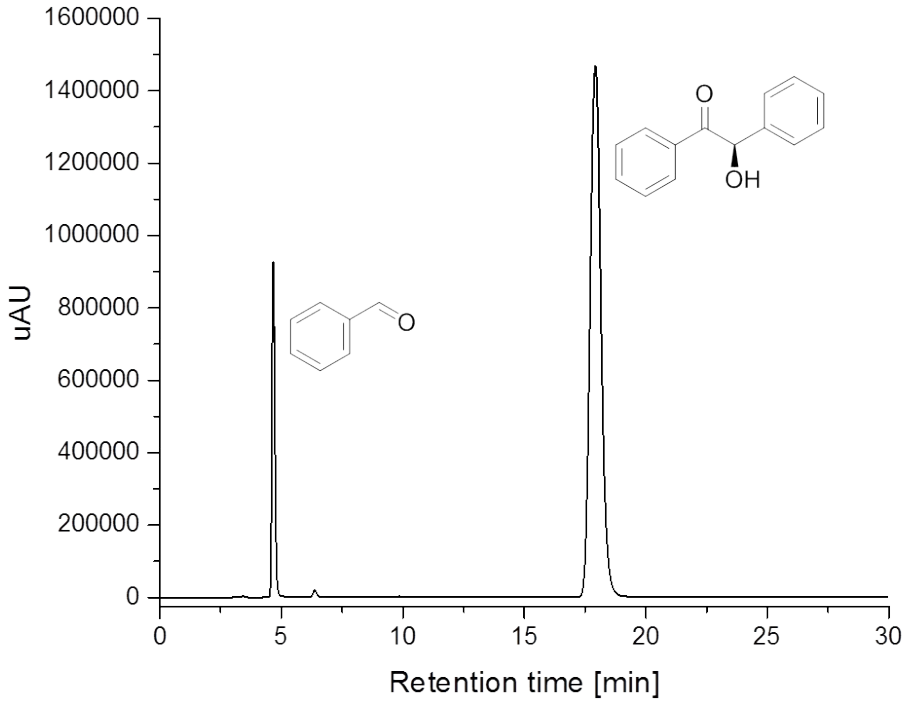


Figure S4 Representative HPLC-chromatogram for the cascade reaction starting from 500 mM benzyl alcohol showing the enantioselective formation of (R)-benzoin after 72 hours. Benzaldehyde (RT = 4.6 min) remained with 6 mM.

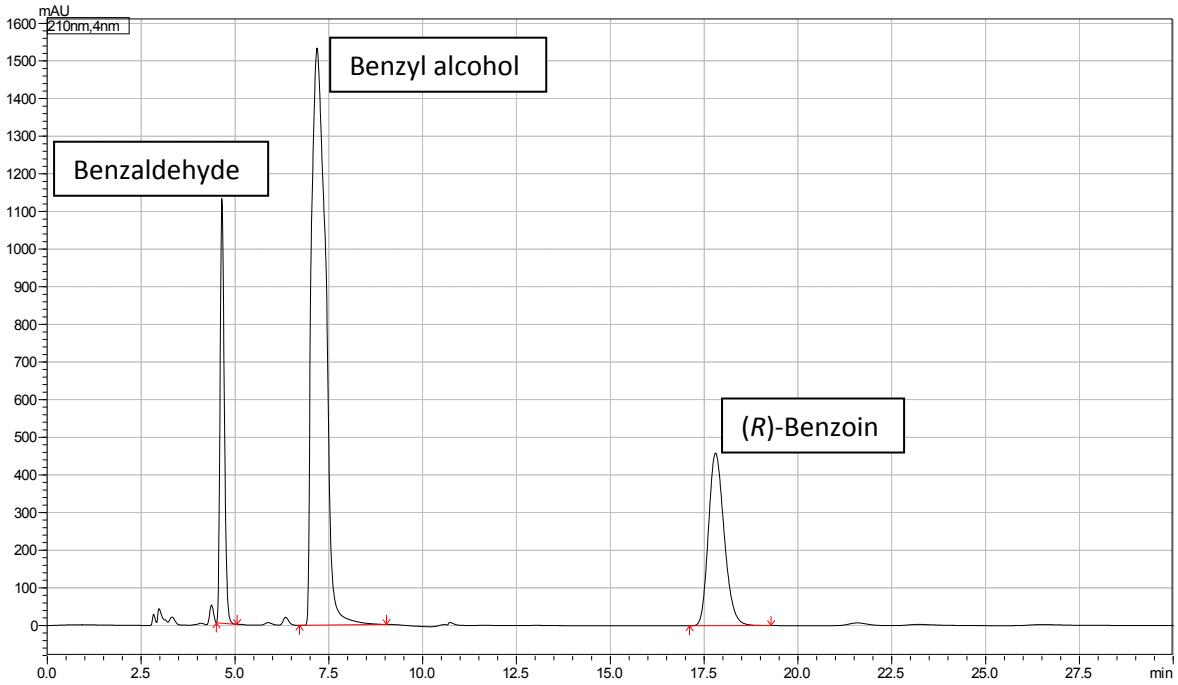


Figure S5 Representative HPLC-chromatogram for the biotransformation starting from benzyl alcohol showing all reaction components.

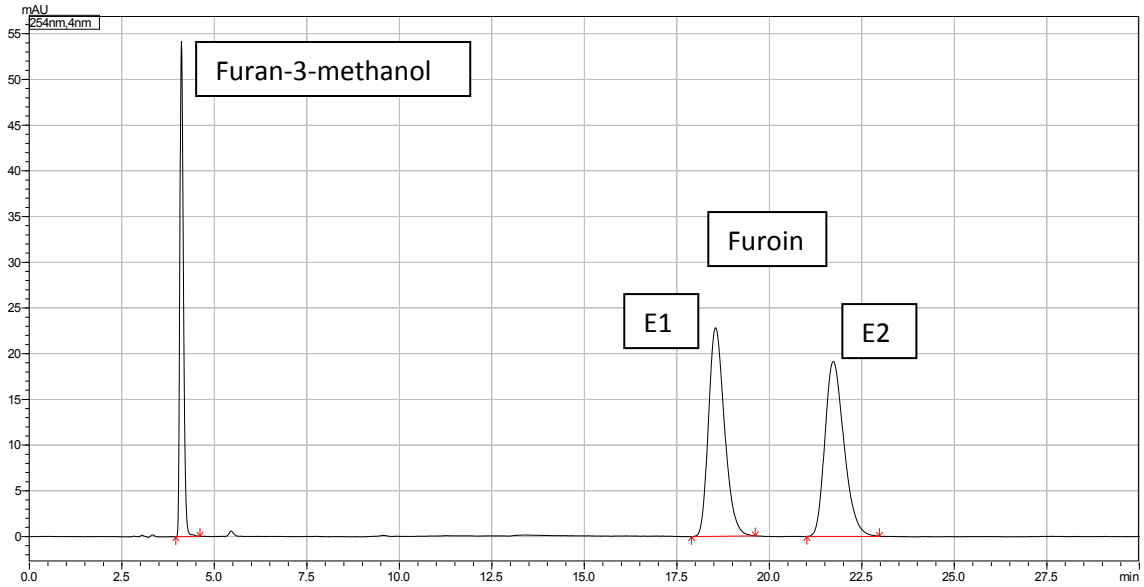


Figure S6 Representative HPLC-chromatogram showing furan-3-methanol and the two enantiomers of the product furoin (E1 and E2).

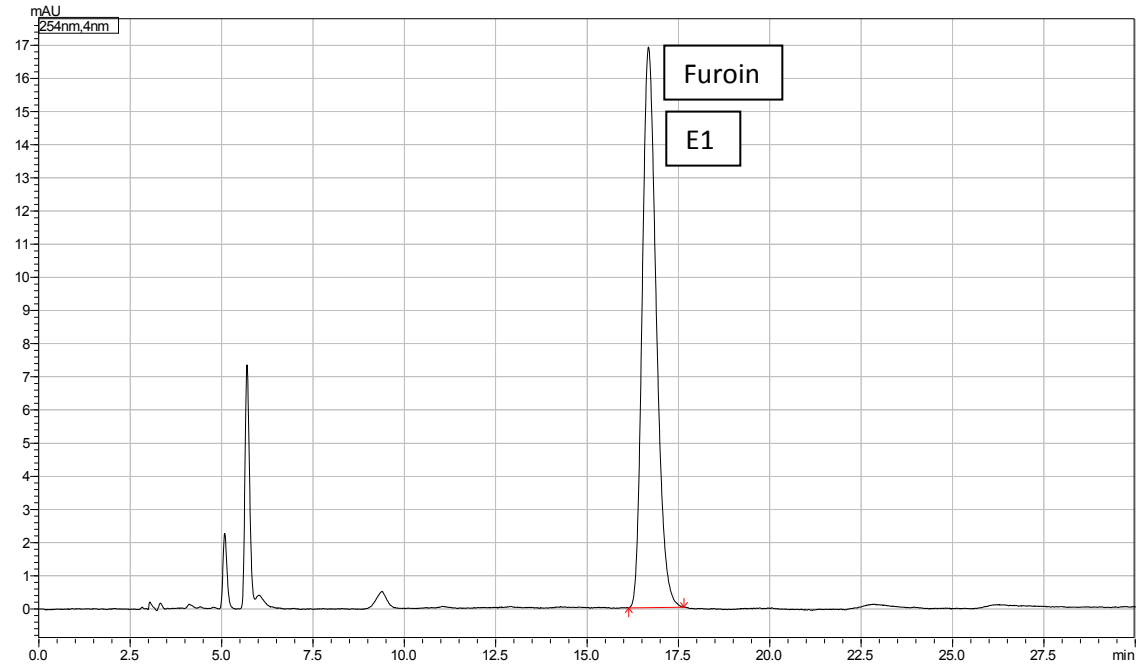


Figure S7 Representative HPLC-chromatogram for the cascade reaction starting from 20 mM furan-3-methanol showing the enantioselective formation of furoin after 30 hours. Furan-3-methanol: 4.1 min. (*R*)- or (*S*)-furoin: E1: 17.0 min, E2: 21.7 min.

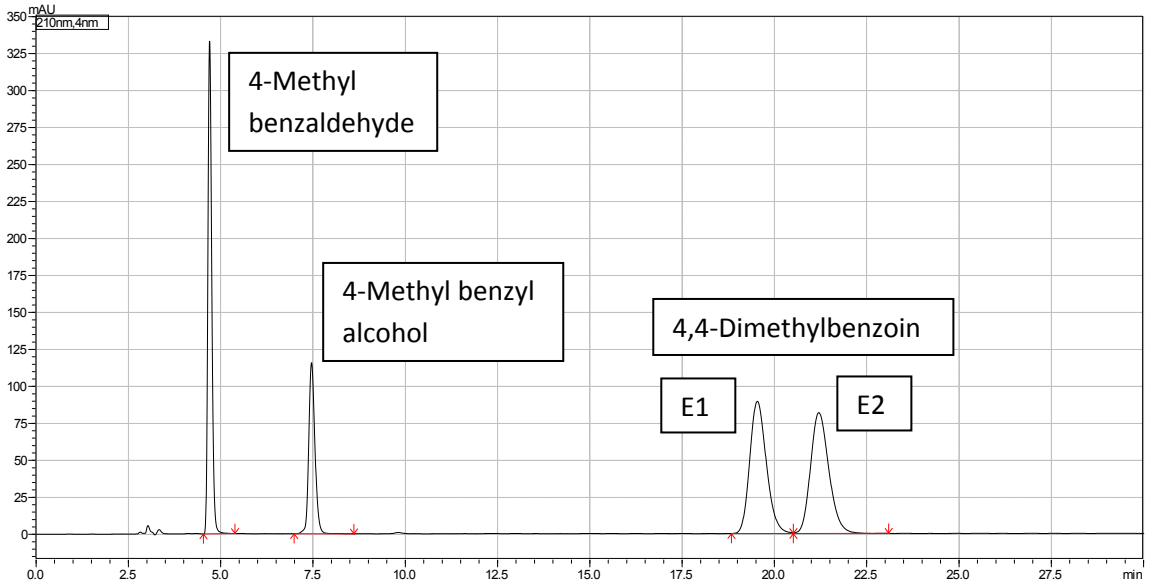


Figure S8 Representative HPLC-chromatogram showing 4-methyl benzyl alcohol and the two enantiomers of the product 4,4-dimethylbenzoin (E1 and E2).

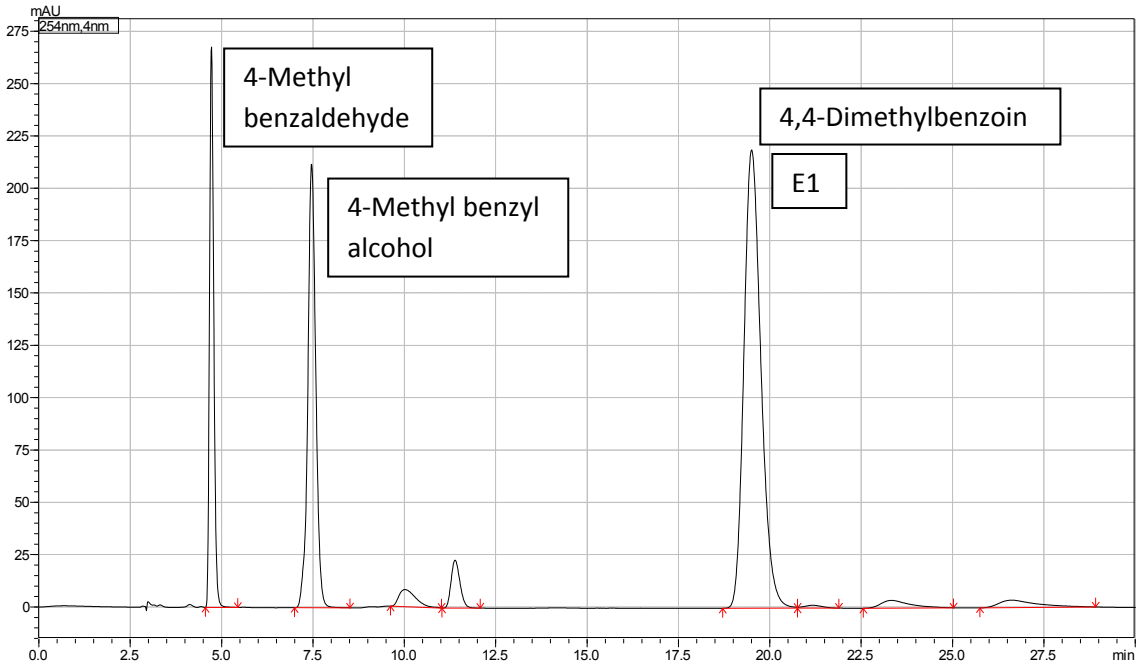


Figure S9 Representative HPLC-chromatogram for the cascade reaction starting from 20 mM 4-methyl benzyl alcohol showing the enantioselective formation of 4,4-dimethylbenzoin after 72 hours. 4-Methyl benzyl alcohol: 7.5min; 4-Methyl benzaldehyde: 4.7min; (*R*)- or (*S*)-4,4-dimethylbenzoin: E1: 19.6 min, E2: 21.2 min.

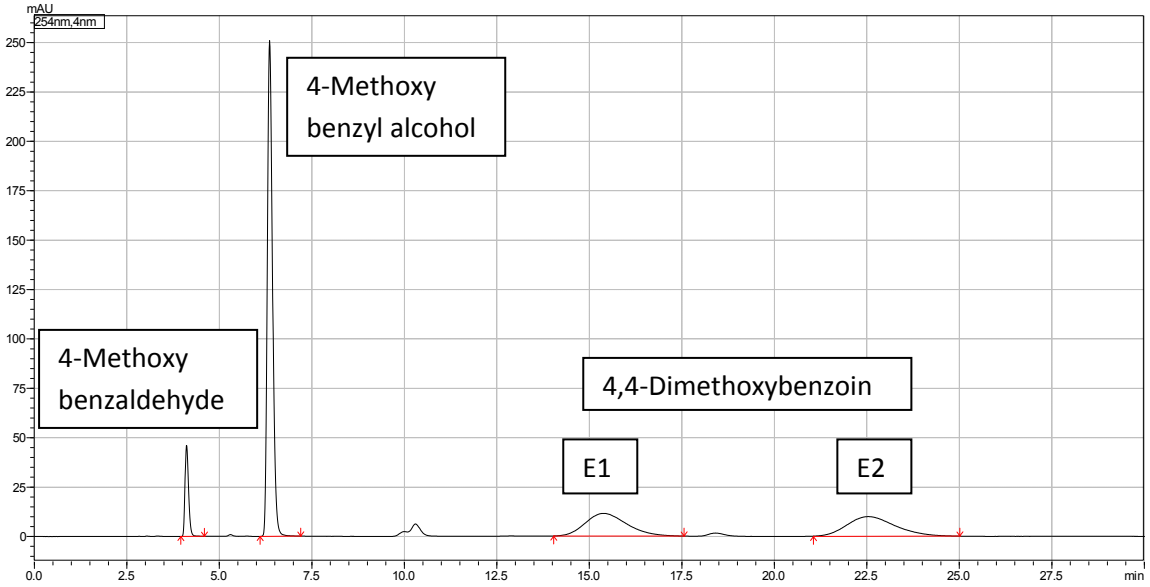


Figure S10 Representative HPLC-chromatogram showing 4-methoxy benzyl alcohol and the two enantiomers of the product 4,4-dimethoxbenzoin (E1 and E2).

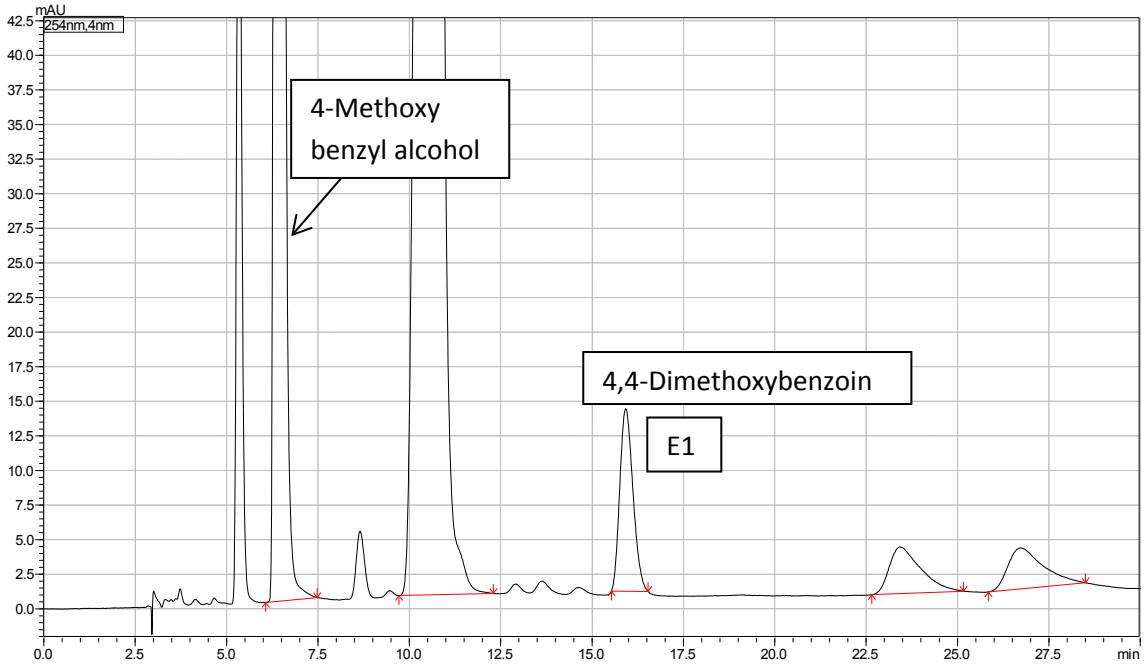


Figure S11 Representative HPLC-chromatogram for the cascade reaction starting from 20 mM 4-methoxy benzyl alcohol showing the enantioselective formation of 4,4-dimethoxy benzoin after 72 hours. 4-Methoxy benzyl alcohol: 6.4 min; 4-Methoxy benzaldehyde: 4.1 min; (*R*)- or (*S*)-4,4-Di Methoxybenzoin: E1: 15.4 min, E2: 22.6 min.

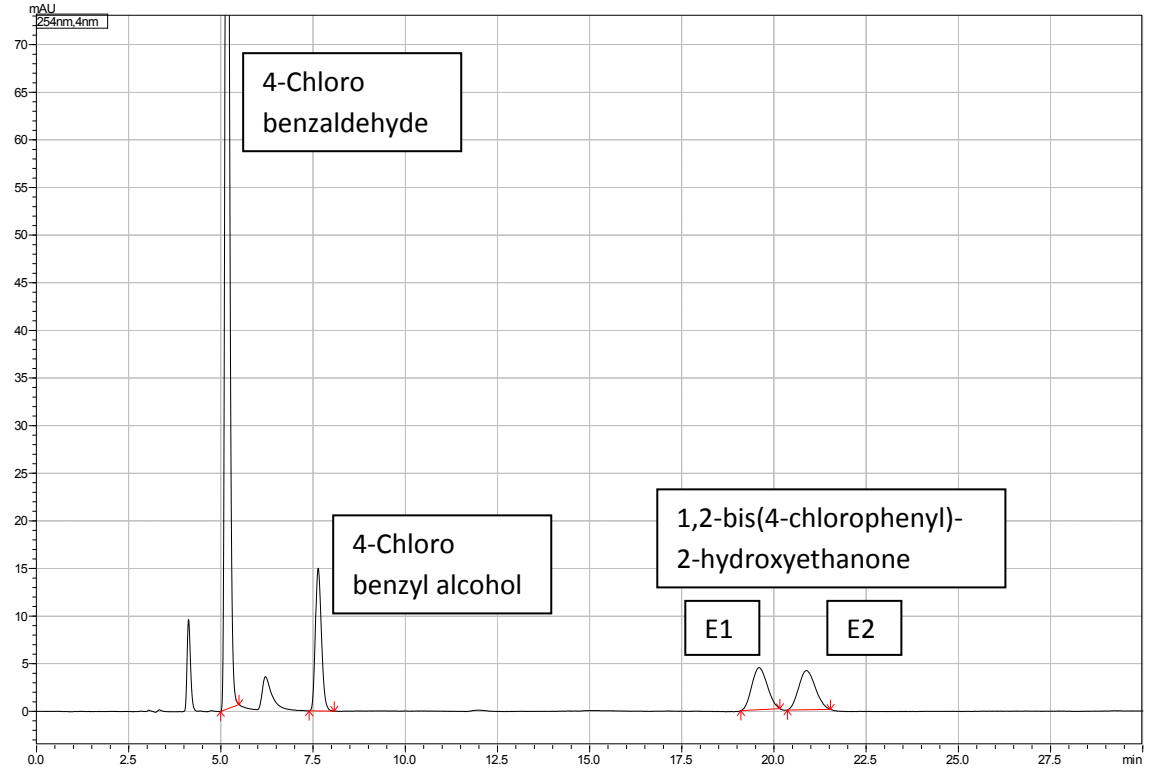


Figure S12 Representative HPLC-chromatogram showing 4-chloro benzyl alcohol and the two enantiomers of the product 1,2-bis(4-chlorophenyl)-2-hydroxyethanone (E1 and E2).

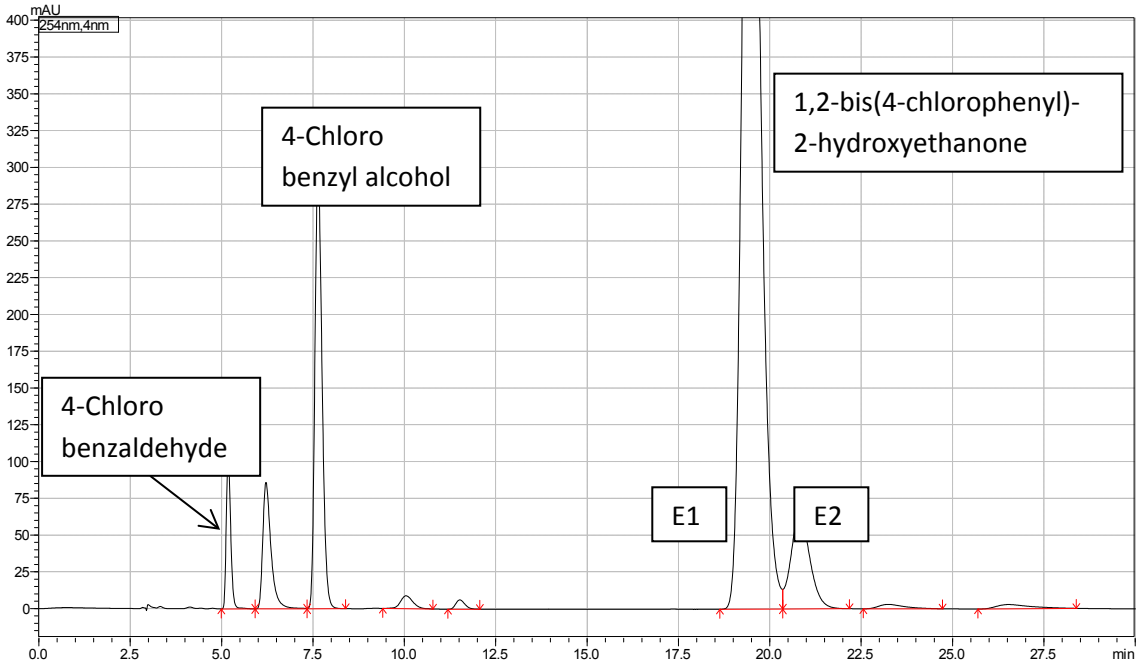


Figure S13 Representative HPLC-chromatogram for the cascade reaction starting from 20 mM 4-chloro benzyl alcohol showing the enantioselective formation of 1,2-bis(4-chlorophenyl)-2-hydroxyethanone after 72 hours. 4-Chloro benzyl alcohol: 7.5 min; 4-Chloro benzaldehyde: 4.7 min; (*R*)- or (*S*)- 1,2-bis(4-chlorophenyl)-2-hydroxyethanone: E1: 19.6 min, E2: 20.9 min.

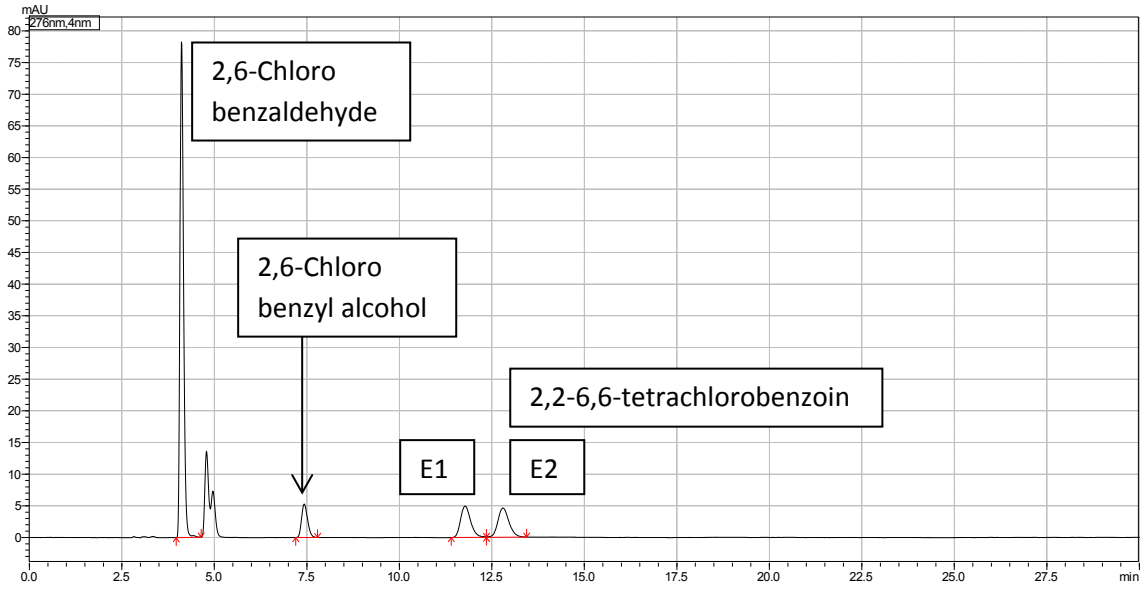


Figure S14 Representative HPLC-chromatogram showing 2,6-chloro benzyl alcohol and the two enantiomers of the product 2,2-6,6-tetrachlorobenzoin (E1 and E2).

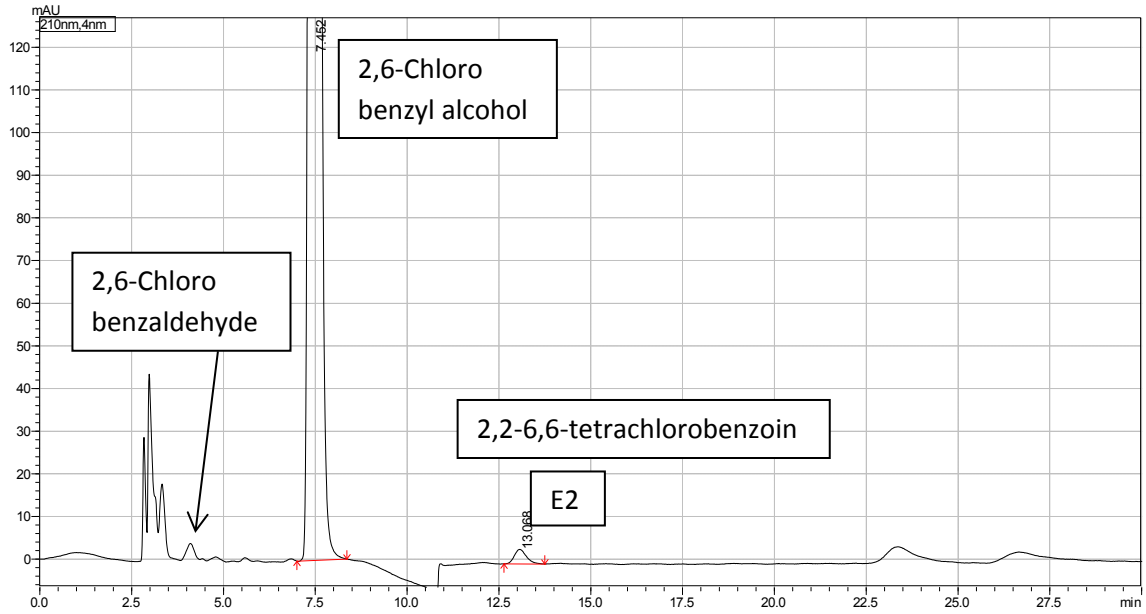


Figure S15 Representative HPLC-chromatogram for the cascade reaction starting from 20 mM 2,6-chloro benzyl alcohol showing the enantioselective formation of 2,2-6,6-tetrachlorobenzoin after 72 hours. 2,6-Chloro benzyl alcohol: 7.4 min; 2,6-Chloro benzaldehyde: 4.1 min; (*R*)- or (*S*)-2,2-6,6-tetrachlorobenzoin: E1: 11.8 min, E2: 13.0 min.

2) ^{1}H-NMR spectroscopy

All measurements were recorded on a Bruker NMR unit (Bruker, Karlsruhe, Germany) at 400 (^{1}H) MHz. The ^{1}H NMR spectra were recorded either in $CDCl_3$ or deuterated DMSO. The impurities observed in the spectra were identified according to Gottlieb et al.[2]

Representative NMR spectra

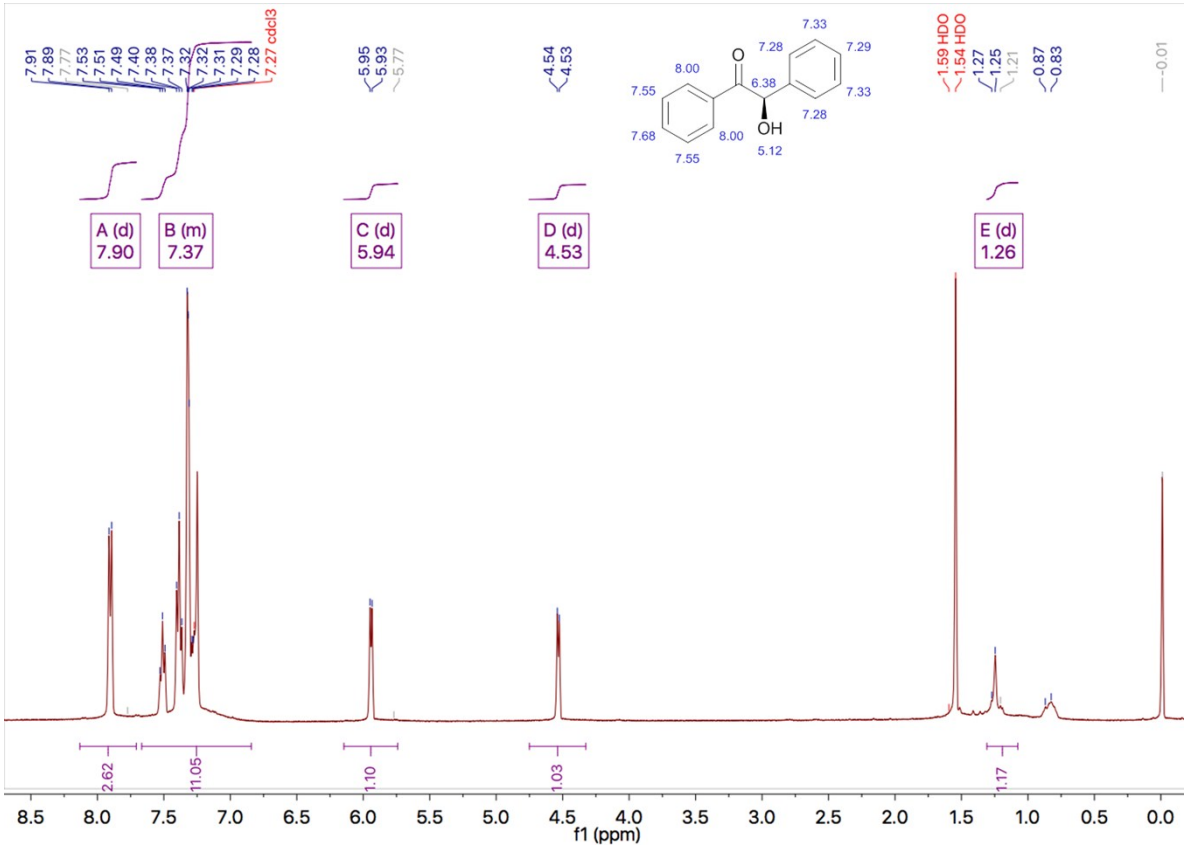


Figure S16 ^{1}H NMR in $CDCl_3$ from the crude (*R*)-benzoin product filtered from the preparative scale synthesis after 96 hours.

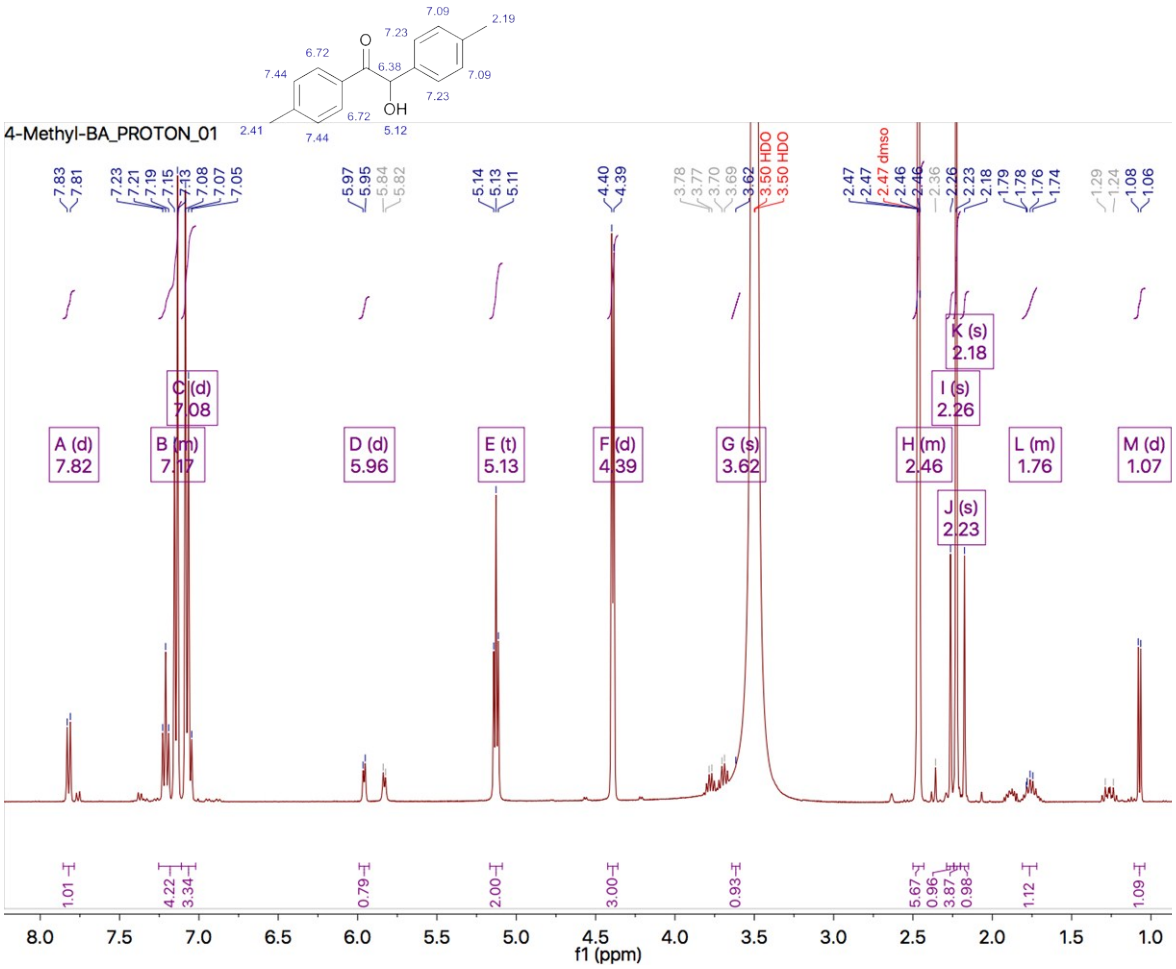


Figure S17 ^{1}H NMR in DMSO from the crude 4,4-dimethylbenzoin product filtered from a biotransformation after 96 hours.

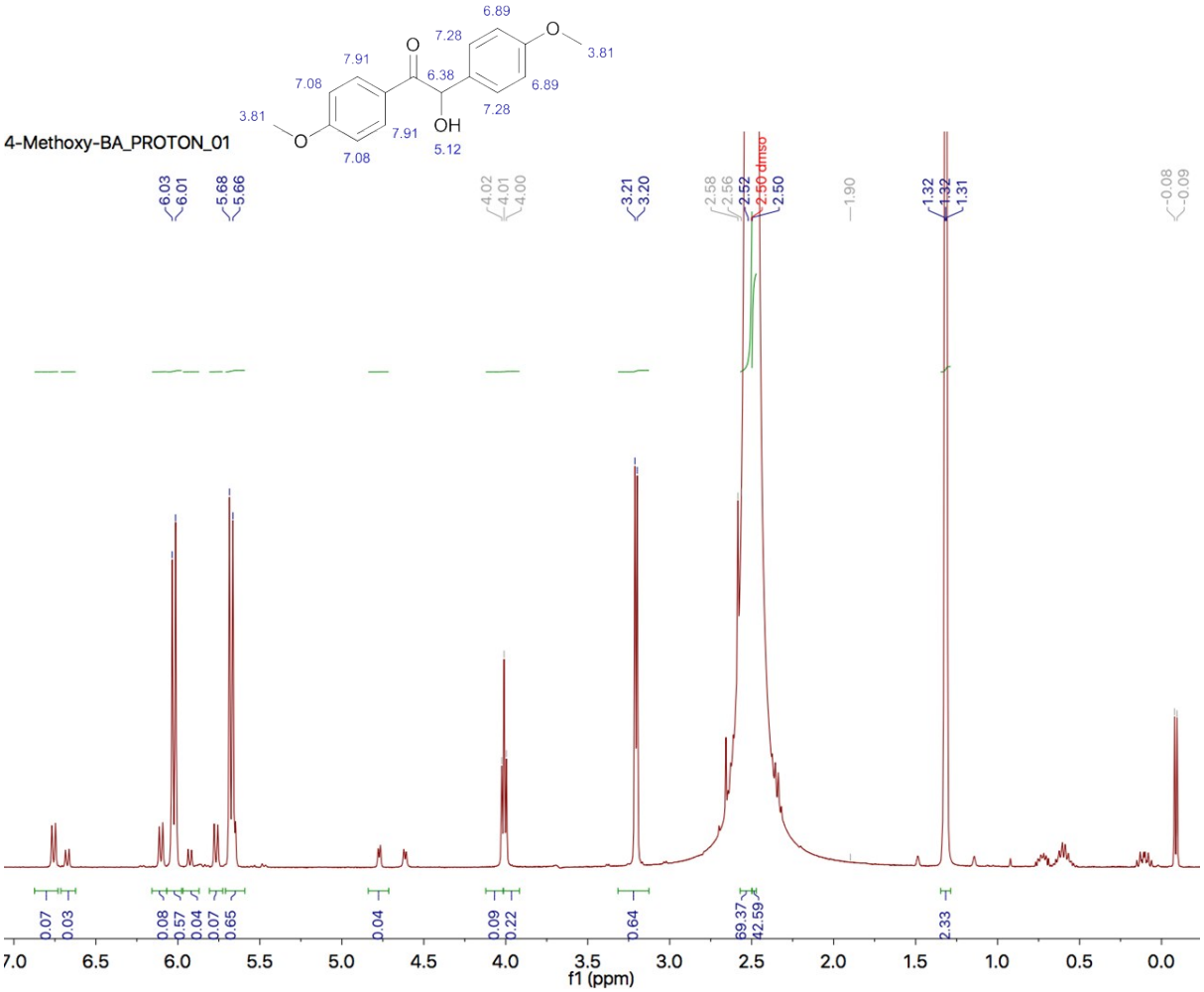


Figure S18 ^{1}H NMR in DMSO from the crude 4,4-dimethoxybenzoin product filtered from a biotransformation after 96 hours.

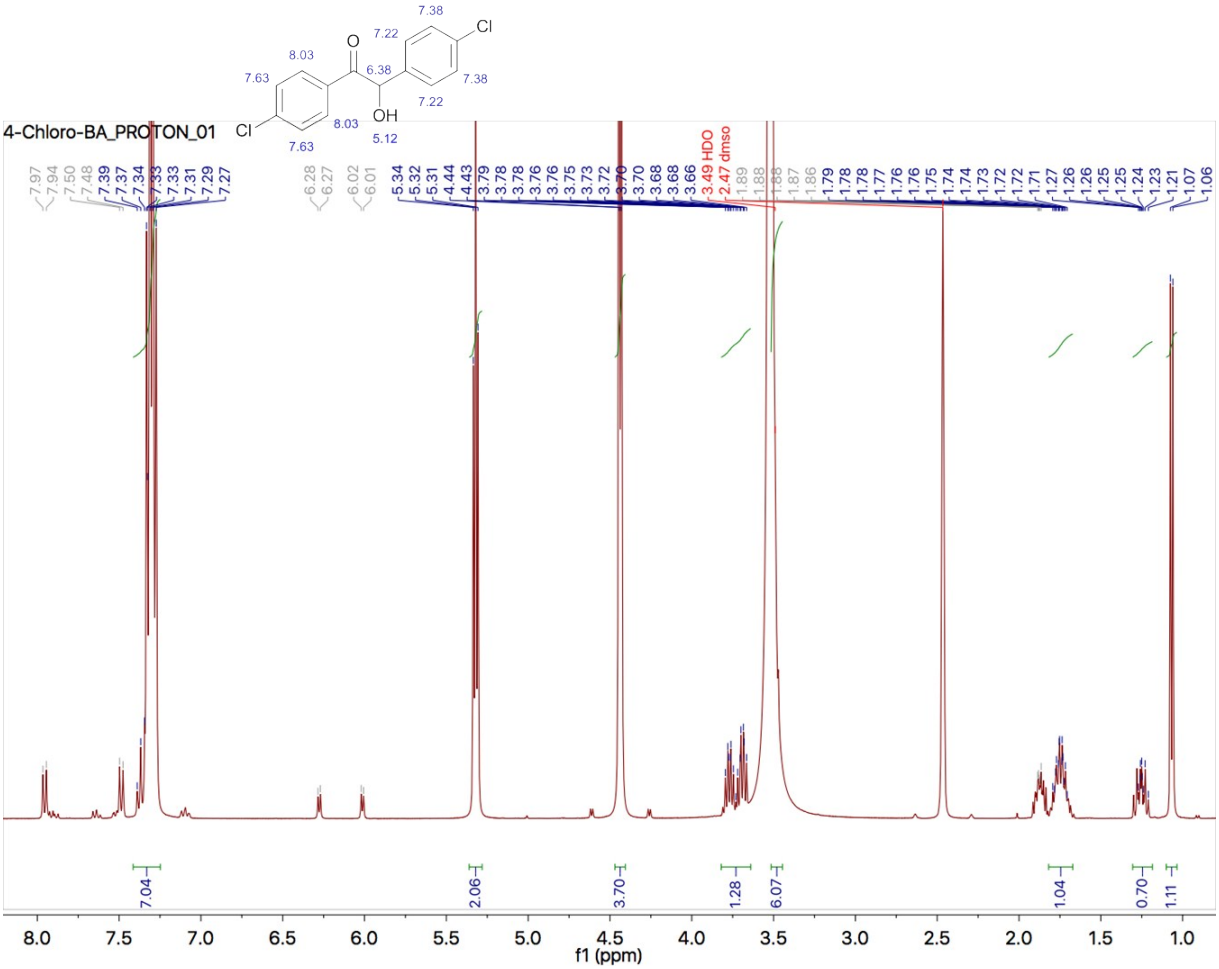


Figure S19 ^{1}H NMR in DMSO from the crude 1,2-bis(4-chlorophenyl)-2-hydroxyethanone product filtered from a biotransformation after 96 hours.

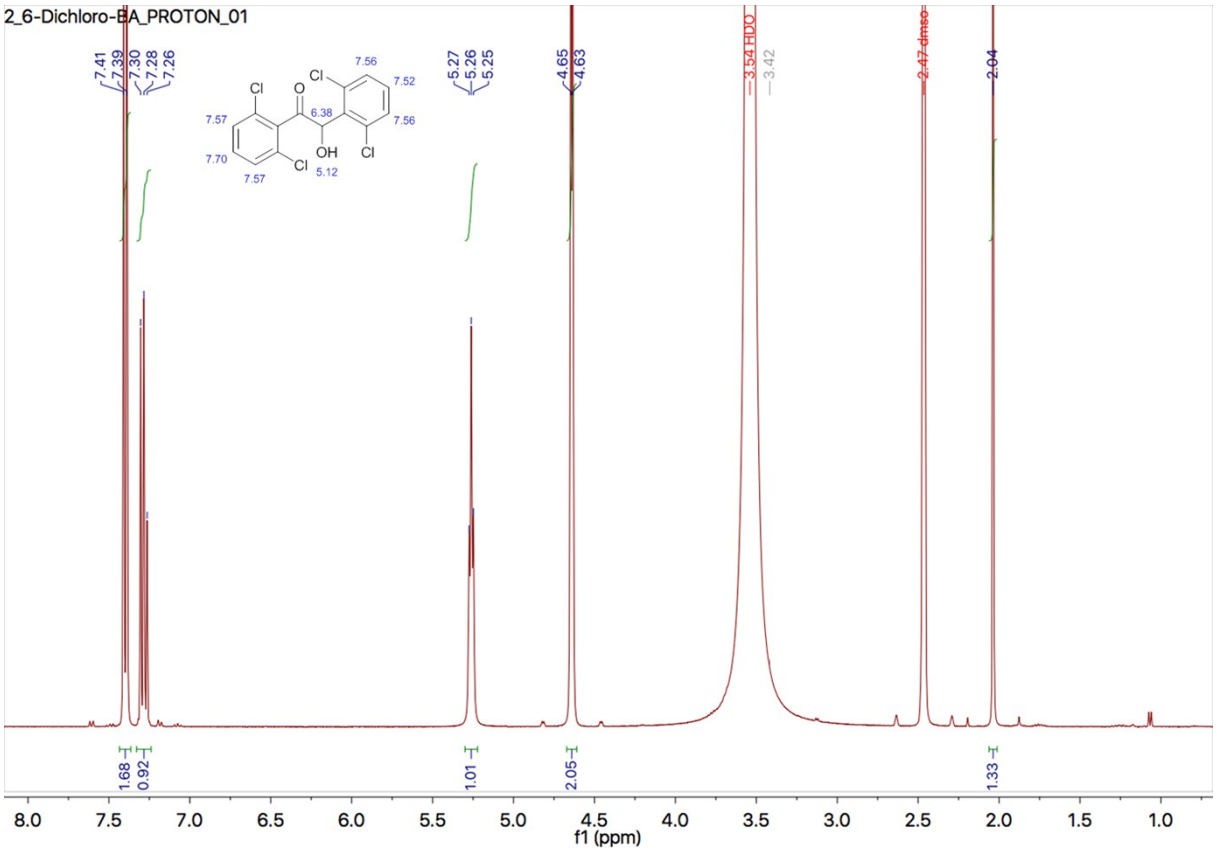


Figure S20 ^{1}H NMR in DMSO from the crude 2,2-6,6-tetrachlorobenzoin product filtered from a biotransformation after 96 hours.

Supporting results

Characterization of PpAOX

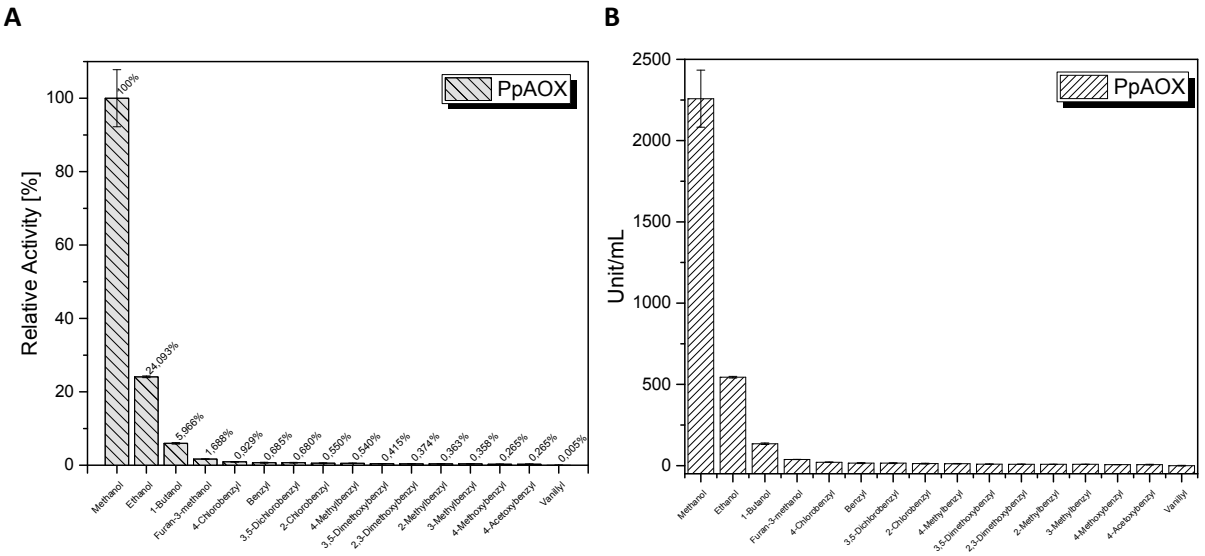


Figure S21 Substrate screening of PpAOX against various aliphatic as well as aromatic alcohols. A) Residual activities. B) Units per mL. Reaction conditions: alcohol substrate (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 25°C. Activities were determined spectrophotometrically using the ABTS assay.

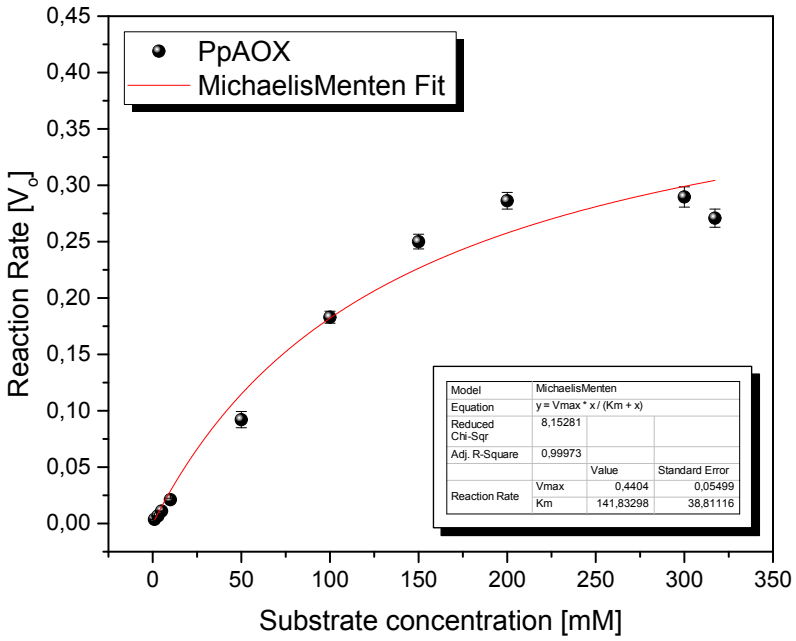


Figure S22 Michaelis-Menten kinetics for PpAOX determined against benzyl alcohol using the ABTS assay. Reaction conditions: benzyl alcohol (0-325 mM), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 25°C. Activities were determined spectrophotometrically using the ABTS assay.

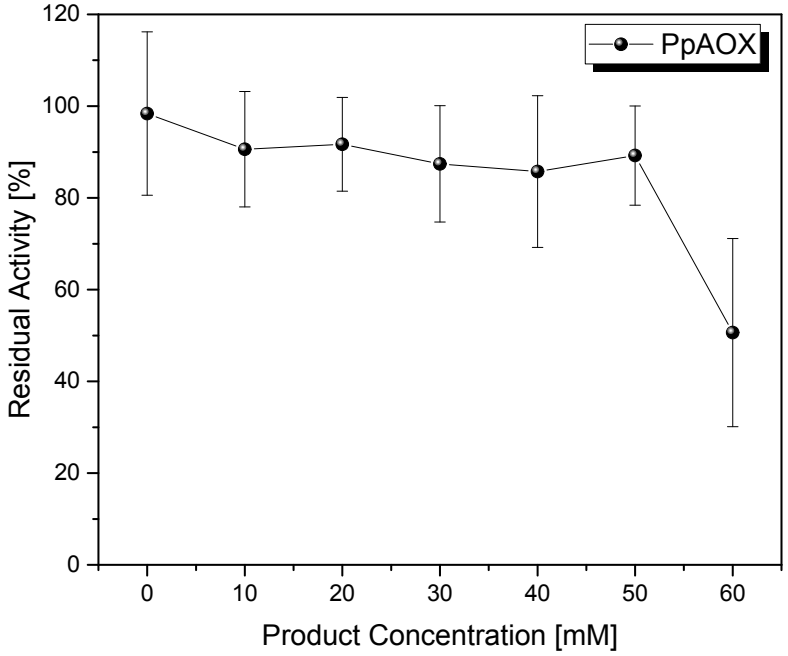


Figure S23 Inhibition of benzaldehyde on PpAOX determined against different benzaldehyde concentrations using the ABTS assay. Reaction conditions: benzaldehyde (0-60 mM), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 25°C. Activities were determined spectrophotometrically using the ABTS assay.

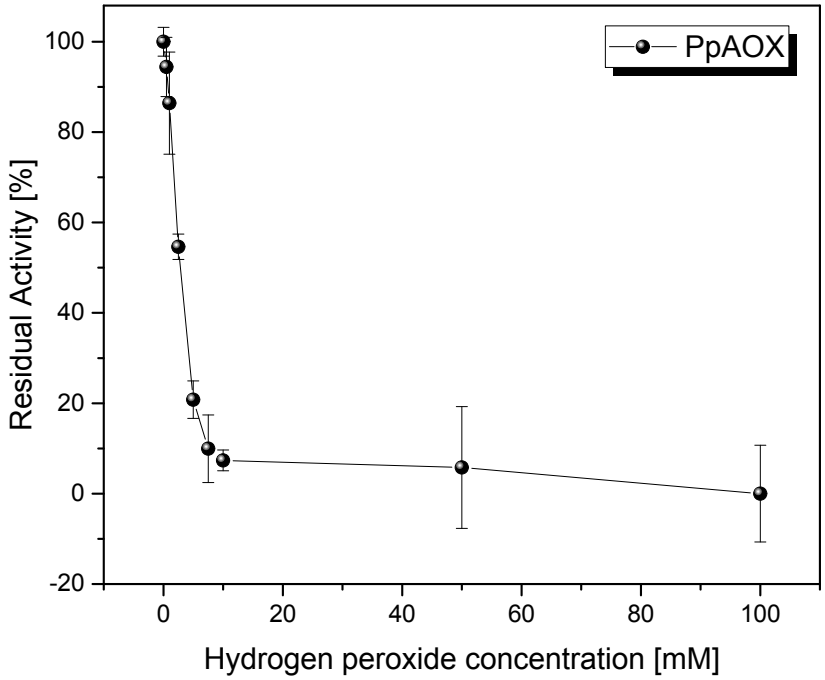


Figure S24 Oxidative stability of PpAOX determined against different hydrogen peroxide concentrations using the ABTS assay. Reaction conditions: benzyl alcohol (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 25°C, 0-100 mM hydrogen peroxide for 10 min. Activities were determined spectrophotometrically using the ABTS assay.

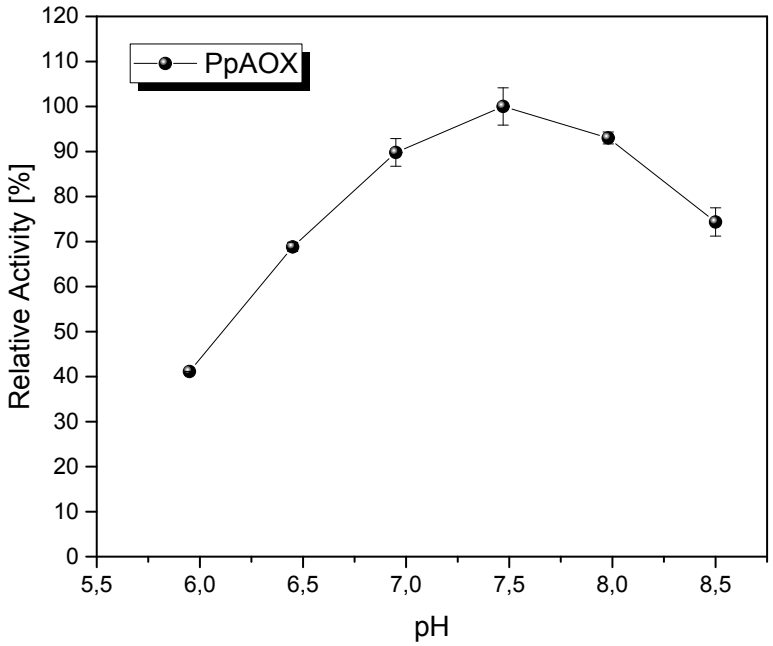


Figure S25 pH profile of PpAOX determined with benzyl alcohol as substrate using the ABTS assay. Reaction conditions: benzyl alcohol (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 25°C. Activities were determined spectrophotometrically using the ABTS assay.

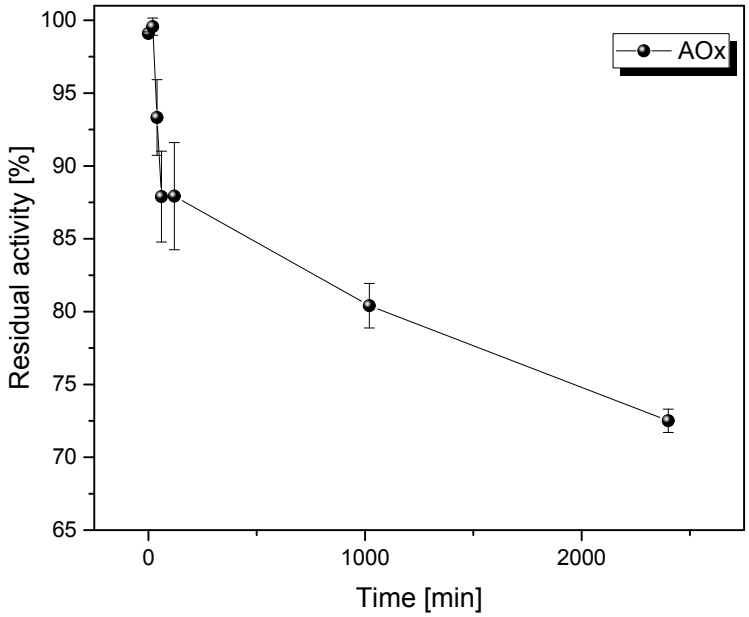


Figure S26 Inactivation of PpAOX during incubation at 30˚C. Activity was measured using the ABTS assay after defined time intervals against benzyl alcohol. Reaction conditions: benzyl alcohol (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 30°C for 0-2700 min. Activities were determined spectrophotometrically using the ABTS assay.

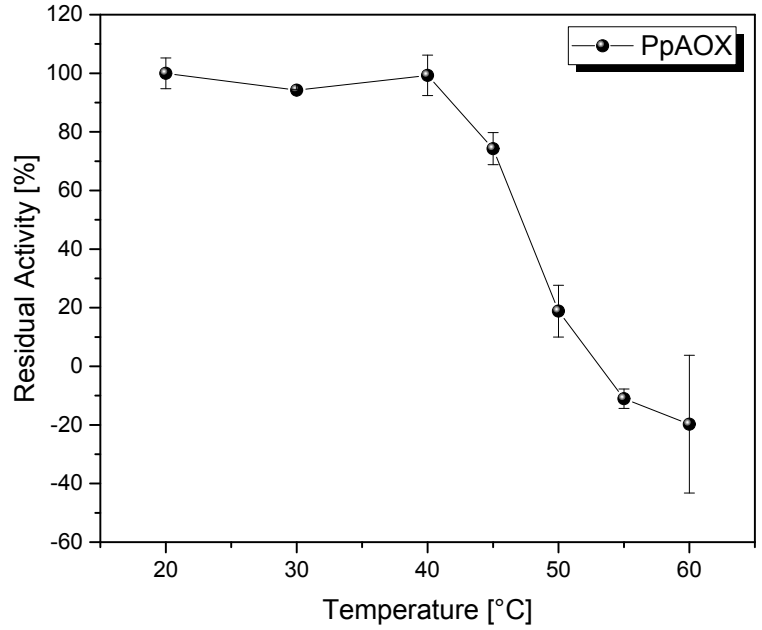


Figure S27 Temperature stability of PpAOX during incubation at various temperatures. Activity was measured using the ABTS assay after incubation of the enzyme for 10 min against benzyl alcohol. Reaction conditions: benzyl alcohol (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 20-60°C for 10 min. Activities were determined spectrophotometrically using the ABTS assay.

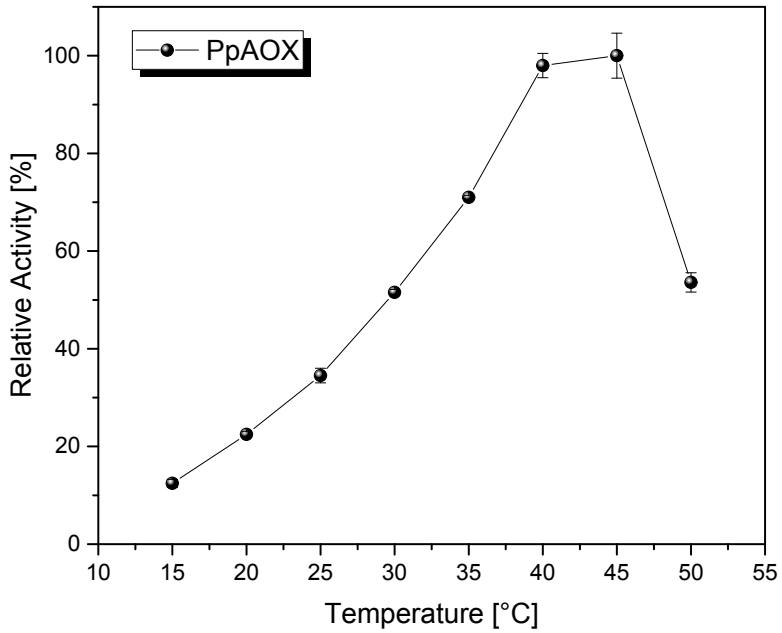


Figure S28 Temperature optimum of PpAOX during incubation at various temperatures. Activity was measured at the respective temperature using the ABTS assay after against benzyl alcohol. Reaction conditions: benzyl alcohol (0.00033% [v/v]), PpAOX (0.5 U, 1.5 mL), horseradish peroxidase (1.25 U, 1.5 mL), 1.86 mM ABTS, sodium phosphate buffer (100 mM, pH 7.5), 15-50°C for 10 min. Activities were determined spectrophotometrically using the ABTS assay.

Characterization of the cascade reaction

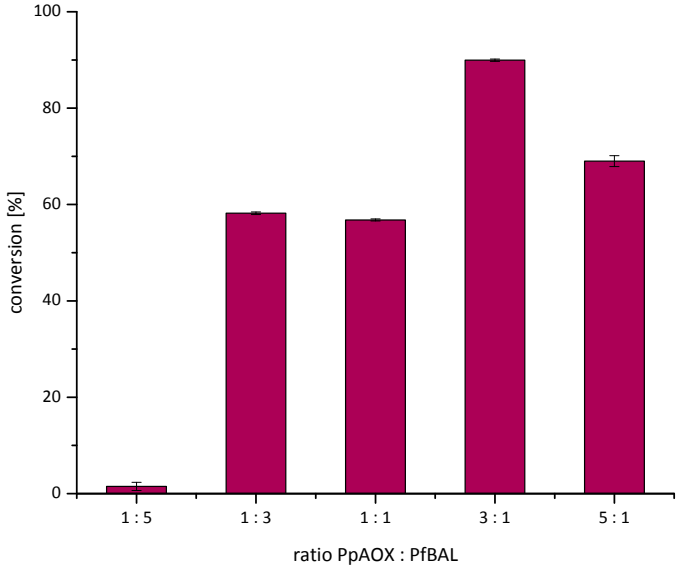
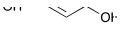
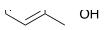


Figure S29 Conversion of benzyl alcohol to (*R*)-benzoin using different PpAOX : PfBAL ratios. Reaction conditions: alcohol substrate (20 mM), PpAOX (5-25 U), PfBAL (5-25 U), $MgCl_2$ (2.5 mM), ThDP (0.15 mM), Catalase from bovine liver (2.5 mg/mL), phosphate buffer (50 mM, pH 8.5), 30°C.

Product yields and enantioselectivities obtained in the cascade reaction

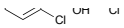
Table S2 and S3 show the obtained results for the synthetic cascade reactions with *Pp*AOX and *Pf*BAL using the soluble alcohol substrates as well as the slurry-to-slurry reactions. For these substrates, low optical purities were obtained which cannot attributed to the stereoselectivity of *Pf*BAL as this enzyme usually shows excellent enantioselectivity. At the moment, we assume that these low *ee*-values are the result of a racemization reaction during the actual reaction. These findings are currently under deeper investigations.

Table S2 Product yields and enantioselectivities obtained in the synthetic cascade reactions with *Pp*AOX and *Pf*BAL using the soluble alcohol substrates.

Substrate[a]	Product	Aldehyde [%][b]	Yield [%][b]	*ee* [%][c]
		0	1.2	>99
		2.9	82.5	89
		0	1.4	95

Reaction conditions: The reaction mix contained the liquid alcohol substrate (350 mM), phosphate buffer (50 mM, pH 8.5) containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and catalase from bovine liver (2.5 mg/mL). Crude cell extract containing PfBAL (around 5 mg, 62.72 µM, 8 U), and PpAOX (17.75 µL, 788.89 nM, 25 U), oxygen-supply, 800 rpm, 30°C, 24 hours. [a] Non-soluble alcohols formed a two-liquid phase system; [b] Determined from ^{1}H NMR spectra; [c] Enantioselectivities were determined after 18 hours of reaction by HPLC using enantiopure product standards or, if not available, literature data was used.

Table S3 Conversions and enantioselectivities obtained in the synthetic cascade reactions with *Pp*AOX and BAL in a slurry-to- slurry.

Substrate[a]	Product	Aldehyde [%][b]	Yield [%][b]	*ee* [%][c]
		0	50.1	99
		6.5	3.2	96
		7.4	88.1	93
		0.99	3.4	93

Reaction conditions: The reaction mix contained the solid alcohol substrate (350 mM), phosphate buffer (50 mM, pH 8.5) containing $MgSO_4$ (2.5 mM), ThDP (0.15 mM) and catalase from bovine liver (2.5 mg/mL). Crude cell extract containing *Pf*BAL (5 mg, 62.72 µM, 8 U), and *Pp*AOX (17.75 µL, 788.89 nM, 25 U), oxygen-supply, 800 rpm, 30°C, 24 hours; [a] Alcohols were directly used in their solid form due to their low solubility; [b] Determined from ^{1}H NMR spectra; [c] Enantioselectivities were determined after 18 hours of reaction by HPLC using enantiopure product standards or, if not available, literature data was used.

E-Factor calculation

Table S4 Estimation of the generated waste in the production process of 1 g PpAOX and PfBAL, respectively.

Contribution	PpAOX[3,4] Waste [g]	PfBAL Waste [g]
Water in media	103	34
BMMY media	5[a]	
Cultivation media (yeast extract, peptone, glycerol, salts)		2.8[b]
Antibiotic	≅ 0	≅ 0
Inducer	1.0	≅ 0
Lysozyme/DNase/RNase	-	≅ 0
Sum	109	36.8

[a] Data for a secreted expression of PpAOX.

[b] Data for an intracellular expression of PfBAL, which makes a cell disruption necessary.

References

1. C. T. Chung, S. L. Niemela and R. H. Miller, *P Natl Acad Sci USA*, 1989, **86**, 2172-2175.
2. H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J Org Chem*, 1997, **62**, 7512-7515.
3. S. J. B. Duff and W. D. Murray, *Biotechnol Bioeng*, 1988, **31**, 44-49.
4. https://tools.thermofisher.com/content/sfs/manuals/easyselect_man.pdf (accessed on Dec. 19th 2016)