# Supporting Information

## Rational Active-Site Redesign Converts a Decarboxylase into a C=C Hydratase: 'Tethered Acetate' Supports Enantioselective Hydration of 4-Hydroxystyrenes

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## 1 General information

Solvents and reagents were obtained from commercial sources and were used as received unless otherwise stated. Substrate 4-vinylphenol was purchased and used as solution in propylene glycol to avoid spontaneous polymerization (actual content according to $^{1}$H NMR signals of 4-vinylphenol and propylene glycol 8.6 % w/w) (Sigma Aldrich).

Conversion of biotransformation samples was analyzed on an Agilent Infinity 1260 HPLC system equipped with a Phenomenex Luna C18(2) reversed phase column (250 mm × 4.6 mm, 5 µM) and a diode array detector (DAD). Enantiomeric excess was measured on an Agilent 7890A GC system equipped with an Agilent Cp-Chirasil DEX-CB column (25 m × 0.32 mm, 0.25µm film) and a flame ionization detector (FID).

Purification of the preparative-scale biotransformation product was afforded on Merck Silica gel 60 (0.040–0.063 mm) using freshly distilled petrolether (boiling point fraction between 40–60 °C) and ethyl acetate as eluent. Analytical TLC was carried out on Merck Silica gel 60 $F_{254}$ coated aluminium sheets using UV-light (λ = 254 nm) and/or basic potassium permanganate [$KMnO_4$ (1.5 g), $K_2CO_3$ (10 g), NaOH (aq. 10 %, 1.25 mL), $H_2O$ (200 mL)] for visualization. Melting points were determined on a Gallenkamp melting point device (UK). Optical rotation was measured at 20 °C against the sodium D-line (589 nm) on a Perkin Elmer polarimeter 341 using a 10 cm pathlength cell. $^{1}$H and $^{13}$C NMR spectra of the hydration product were acquired on a Bruker Avance 300 MHz NMR unit. Chemical shifts in ppm were referenced to the methanol solvent residual signal ($^{1}$H: 3.31 ppm, $^{13}$C: 49.00 ppm).

## 2 General method for the preparation of sodium carboxylates

1.0 equiv. of sodium hydroxide was dissolved in MeOH (5 mL) and cooled to 0 °C. 1.0 g of the free carboxylic acid was dissolved in methanol (5 mL) and added dropwise to the stirred sodium methoxide solution at 0 °C. Upon complete addition, the pH was checked (7.0) and the mixture was warmed to 21 °C. After evaporation of the solvent and drying in an evacuated desiccator overnight, the respective salts were obtained as colorless solids.

## 3 General procedure for hydration of 4-vinylphenol under optimized conditions

Lyophilized *E. coli* cells containing the heterologously expressed FDC_*Es* variant or wild type enzyme were provided in 2.0 mL reaction vessels and suspended in potassium phosphate buffer (50 mM, pH 6.0, 990 µL). Cells were rehydrated in buffer for 30 min at 25°C with shaking in an Eppendorf Thermomixer at 700 rpm in a horizontal position. Substrate 4-vinylphenol (**1**) was added as aliquot of a solution in propylene glycol (8.6% w/w, 13.44 µL) and incubation was continued for 24 h at 25°C with 700 rpm horizontal shaking.

The hydration with HisTag-purified enzyme was carried out as follows: potassium phosphate buffer (50 mM, pH 6.0, 980 µL) was provided in 2.0 mL reaction vessels; freshly thawed enzyme solution (10 µL, 3 – 4 mM) and substrate stock (13.44 µL, 8.6% w/w in propylene glycol) were added. The reaction was incubated for 24 h at 25°C with 700 rpm horizontal shaking.

## 4 Determination of conversion and enantiomeric excess (*e.e.*)

### *4.1 Sample workup and analysis*

For determination of conversion by HPLC, an aliquot of the homogeneous reaction mixture (100 µL) was withdrawn and diluted with a mixture of water and acetonitrile (1:1, 900 µL) containing trifluoroacetic acid (1 % v/v) and anisole as external standard (1.187 mM). After thorough mixing, incubation at room temperature for 30 min and centrifugation at 14000 rpm for 10 min, the clear supernatant was subjected to HPLC analysis. Analysis method: flow = 1 mL/min; mobile phase A: water + 0.1%v/v TFA, mobile phase B: acetonitrile + 0.1 %v/v TFA; 0–2 min (100% A), 2–13 min (100–0% A), 13–15 min (0% A), 15–18 min (0–100% A), 18–22 min (100% A); integration at 270 nm. Retention times: 4-vinylphenol (**1**): 13.5 min; 1-(4-hydroxyphenyl)ethanol (**2**): 10.0 min; anisole (external standard): 14.7 min.

For analysis of the enantiomeric purity, the remaining reaction mixture was extracted with ethyl acetate (2 × 500 µL) and the extract was dried over anhydrous potassium carbonate. Acetic anhydride (5 eq, 50 mM, 4,7 µL) was added to the extract in presence of solid $K_2CO_3$ and the suspension was shaken for 30 min at 40°C and 900 rpm in an Eppendorf Thermomixer. Excess anhydride was quenched by adding water (200 µL) and shaking was continued for 10 min. After the phases were separated by centrifugation, the organic phase was dried over anhydrous $MgSO_4$ and the sample was subjected to GC-FID using the following temperature program: 100 °C (1 min hold) – [10 °C/min] – 160 °C (6 min hold) – [20 °C/min] – 180 °C (1min hold) with $H_2$ (1.3 mL/min) as carrier gas. Retention times: (*R*)-1-(4-hydroxyphenyl)ethanol [(*R*)-**2**]: 11.4 min; (*S*)-1-(4-hydroxyphenyl)ethanol [(*S*)-**2**]: 11.6 min.

### *4.2 HPLC calibration curves*

Calibration of the HPLC method was performed by dilution of a stock solution of authentic reference material with solvent (water/acetonitrile 1:1 containing TFA and 1 mM anisole as internal standard) and integration of HPLC peaks at 270 nm.

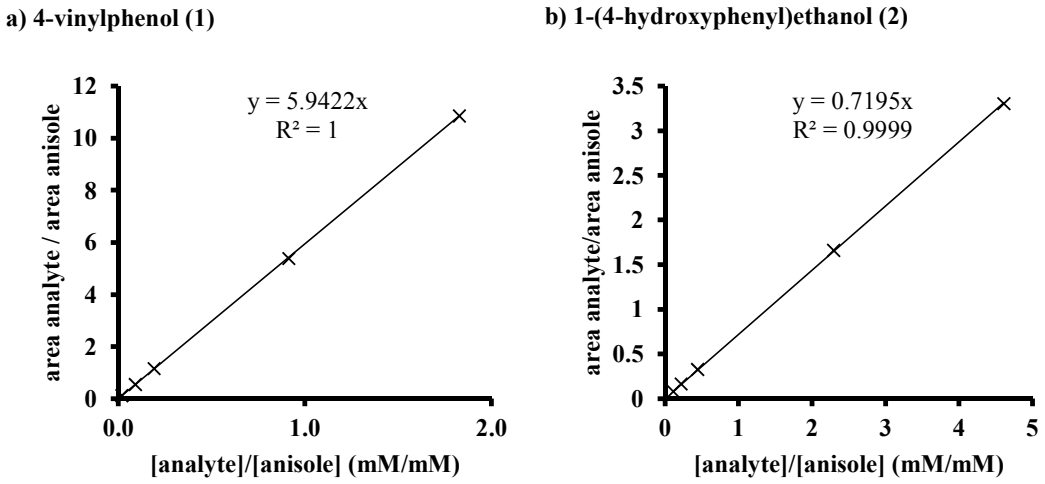


**Figure S1** HPLC Calibration curves for a) substrate 4-vinylphenol (**1**) and b) hydration product 1-(4-hydroxyphenyl)ethanol (**2**).

*4.3 Example chromatograms*

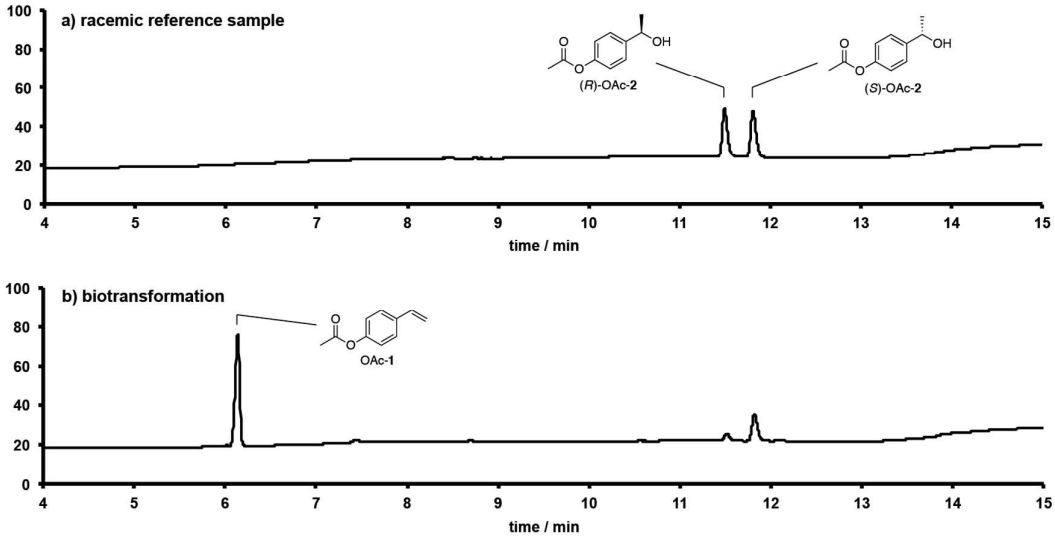


**Figure S2** example chromatogram for the separation of acetylated (*R*) and (*S*)-**2** on a chiral DEX-CB-column.

## 5 Identification of compounds and determination of absolute configuration

Racemic and (*S*)-1-(4-hydroxyphenyl)ethanol were prepared as described previously.[1] Compounds were identified by co-injection of authenthic reference material on HPLC. Absolute configuration was determined by comparison of the elution order from a chiral phase GC column with enantiomerically enriched reference material and literature values.[2]

## 6 Docking of 4-vinylphenol substrate

In silico docking of the quinone-methide form of 4-vinylphenol into chain A of the crystal structure of FDC_*E*s wt (homodimer, PDB-ID 4UU3) and models of FDC_*E*s Val46Glu and Val46Asp (generated with the SwissModel server) was performed with the Autodock Vina plugin of the UCSF Chimera program. Ligand energy minimization was done with the Avogadro program using a Ghemical force field following the steepest descent ($E_{min}$ = 6.67 kJ/mol).

The receptor protein was prepared for docking with the UCSF chimera program while keeping solvent molecules and ions. Sidechains were completed using the Dunbrack rotamer library[3] and hydrogens were added considering hydrogen bonds. Protonation states were deduced from residue names and additional hydrogen geometry info for Thr and Ile (Atom H, #1) were assigned manually to a single substituent geometry with one substituent each. Charges were assigned with AMBER ff 14SB force field for standard residues and AM1-BCC for other residues.

Docking was performed within a 20 × 20 × 20 Å search box centered at coordinates 38/110/155 (carboxylate oxygen of Glu46) using the Opal web service as executable location. Docking results were finally evaluated with PyMol and superimposed with a wild-type structure containing structural water to estimate the location of the latter in the mutant structure.

## 7 Molecular Biology

### *7.1 DNA sequence*

Ferulic acid decarboxylase from *Enterobacter* sp. Px6-4 gene sequence (in pET28a vector: pEG188):

ATGAACACCTTCGATAAACATGATCTGAGCGGTTTTGTTGGTAAACATCTGGTGTATAC
CTATGATAATGGCTGGGAGTATGAGATCTATGTGAAAAATGAAAACACCCTGGATTAT
CGCATTCATAGCGGTCTGGTTGGTAATCGTTGGGTTAAAGATCAGCAGGCATATATTGT
TCGTGTGGGTGAAAGCATCTATAAAATCAGCTGGACCGAACCGACCGGCACCGATGTT
AGCCTGATTGTTAATCTGGGTGATAGCCTGTTTCATGGCACCATCTTTTTTCCGCGTTGG
GTGATGAATAATCCGGAAAAAACCGTTTGCTTTCAGAACGATCATATTCCGCTGATGA
ATAGCTATCGTGATGCAGGTCCGGCATATCCGACCGAAGTTATTGATGAATTTGCCACC
ATTACCTTTGTTCGTGATTGTGGTGCAAATAACGAAAGCGTTATTGCATGTGCAGCAAG
CGAACTGCCGAAAAACTTTCCGGATAATCTGAAATAA

*7.2 Site directed mutagenesis and primer sequences*

Site directed mutagenesis was performed with an Agilent QuikChange II XL site directed mutagenesis kit using the primers below (fw: forward primer, rv: reverse primer, altered codons are underlined; plasmid numbers are given in parentheses).

| | |
|---|---|
| FDC_*Es* Val46Glu (pEG 397) | fw: 5'–C CCA ACG ATT ACC TTC CAG ACC GCT ATG A–3'<br>rv: 5'–T CAT AGC GGT CTG GAA GGT AAT CGT TGG G–3' |
| FDC_*Es* Val46Gln (pEG 400) | fw: 5'–C TTT AAC CCA ACG ATT ACC CTG CAG ACC GCT ATG AAT GC–3'<br>rv: 5'–GC ATT CAT AGC GGT CTG CAG GGT AAT CGT TGG GTT AAA G–3' |
| FDC_*Es* Val46Asp (pEG 396) | fw: 5'–T AAC CCA ACG ATT ACC ATC CAG ACC GCT ATG AAT G–3'<br>rv: 5'–C ATT CAT AGC GGT CTG GAT GGT AAT CGT TGG GTT A–3' |
| FDC_*Es* Val46His (pEG 398) | fw: 5'–TTT AAC CCA ACG ATT ACC ATG CAG ACC GCT ATG AAT GCG A–3'<br>rv: 5'–T CGC ATT CAT AGC GGT CTG CAT GGT AAT CGT TGG GTT AAA–3' |
| FDC_*Es* Val46Arg (pEG 399) | fw: 5'–TTT AAC CCA ACG ATT ACC ACG CAG ACC GCT ATG AAT GCG–3'<br>rv: 5'–CGC ATT CAT AGC GGT CTG CGT GGT AAT CGT TGG GTT AAA–3' |
| FDC_*Es* Val46Glu/Arg49Met (pEG 402) | fw: 5'–GC CTG CTG ATC TTT AAC CCA CAT ATT ACC CTC CAG ACC GCT ATG AAT GCG–3'<br>rv: 5'–CGC ATT CAT AGC GGT CTG GAG GGT AAT ATG TGG GTT AAA GAT CAG CAG GC–3' |
| FDC_*Es* Arg49Glu (pEG 401) | fw: 5'–CTG CTG ATC TTT AAC CCA CTC ATT ACC AAC CAG ACC GC–3'<br>rv: 5'–GC GGT CTG GTT GGT AAT GAG TGG GTT AAA GAT CAG CAG–3' |

*7.3 Protein expression and biocatalyst preparation*

A pET28a(+) vector harboring the gene encoding for FDC_*Es* or one of its variants was transformed into competent *E. coli* BL21(DE3) cells and heterologously expressed with a *N*-terminal $His_6$ Tag as described previously.[2] The biocatalyst was either used as lyophilized *E. coli* whole cell preparation (stored at +4 °C until use) or was purified from *E. coli* using Ni-affinity chromatography (GE healthcare), and stored at –80 °C as solution in potassium phosphate buffer (50 mM, pH 7.0, 4.55 mM enzyme concentration) until further use.

*7.4 Determination of specific activity*

The specific hydration activity of lyophilized *E. coli* whole cells containing the heterologously expressed FDC_*E*s variant was determined with 4-vinylphenol (**1**) as substrate. The assay mixture consisted of a lyophilized biocatalyst preparation (20 mg) suspended in potassium phosphate buffer (50 mM, pH 6.0) to which substrate 4-vinylphenol (13.44 µL of a 8.6 % w/w solution in propylene glycol, 10 mM) was added to start the reaction (700 rpm, 30 °C). Sample aliquots (100 µL) were withdrawn between 1 and 15 min and worked up as described in section 4.1 followed analysis of the conversion by HPLC. One unit was defined as the amount of biocatalyst required for hydration of 1 µmol 4-vinylphenol to give 1-(4-hydroxyphenyl)ethanol per minute. The specific activity was determined to 5.8 mU/mg for FDC_*E*s Val46Glu and 6.4 mU/mg for FDC_*E*s Val46Asp.

The specific decarboxylation activity of lyophilized *E. coli* whole cells containing the heterologously expressed FDC_*E*s wild-type enzyme was determined with 4-hydroxycinnamic acid as substrate. The assay mixture consisted of a lyophilized biocatalyst preparation (1 mg) to which substrate (10 mM, 1 mL dissolved in potassium phosphate buffer, 50 mM, pH 8.0) was added to start the reaction (850 rpm, 30 °C). Sample aliquots (100 µL) were withdrawn between 0.5 and 10 min and worked up as described in section 4.1, followed by determination of substrate consumption with HPLC. One unit was defined as the amount of biocatalyst required for decarboxylation of 1 µmol 4-hydroxycinnamic acid to 4-vinylphenol (**1**) per minute. The specific activity was 1.6 U/mg.

## 8 Supporting results

### *8.1 Dependence of hydration on bicarbonate / acetate concentration*

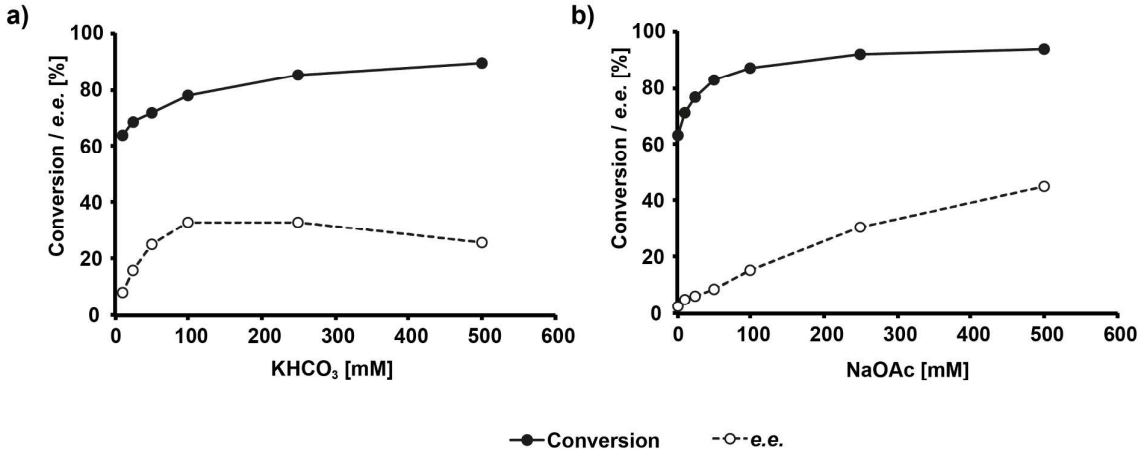


**Figure S3** Enzymatic hydration in presence of bicarbonate (a) and acetate (b) depending on anion concentration. Conditions: substrate 4-vinylphenol (10 mM, from 8.6% w/w propylene glycol solution) in potassium phosphate buffer (50 mM, pH 8.0), lyophilized *E. coli* cells containing heterologously expressed wild type FDC_*Es* (20 mg/mL, 32 U), sodium acetate or potassium bicarbonate, respectively (0.5 M).

### *8.2 Temperature effects*

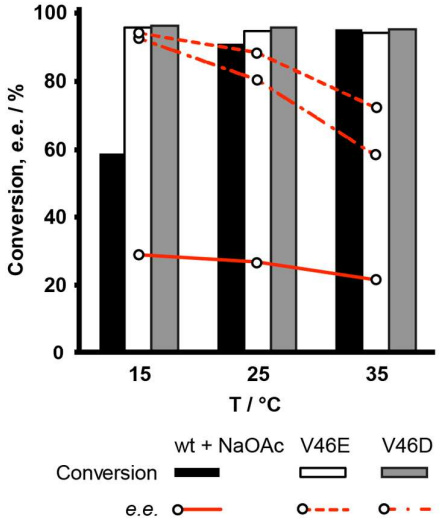


**Figure S4** Temperature study with FDC_*Es* (wild type), FDC_*Es* mutants Val46Glu and Val46Asp in presence of sodium acetate (500 mM). Conditions: purified FDC_*Es* wt or Val46Glu or Val46Asp variant (300 µM) in 1 mL potassium phosphate buffer (50 mM, pH 6.00, supplemented with NaOAc 0.5 M for the wild-type enzyme), substrate **1** (10 mM, as propylene glycol solution) incubated for 24 h at 700 rpm and the respective temperature.

### *8.3 Optimization of pH*

Initial experiments were performed at pH 8.0 to ensure constant bicarbonate concentrations (which would decompose into $CO_2$ at a more acidic pH). This was changed to a standard pH 7 later on, when bicarbonate was no longer included in the screenings. A pH profile of the reaction with purified enzyme shows that the conversion is virtually independent of the pH within the tested range (5.0–9.0; 90% conv.) but the product *e.e.* shows a shallow maximum at pH 6 (83% *e.e.*) (Figure S5a). A control experiment in the absence of enzyme revealed a slow spontaneous background hydration in acidic milieu (9% at pH 5). Correcting the measured *e.e.* by subtracting the racemic product originating from the spontaneous event yields an actual 'enzymatic' *e.e.* of 95% at optimal pH. Practically, these stereoselectivities could be obtained by increasing the amount of biocatalyst and optimization of the reaction time to suppress the spontaneous hydration.

### *8.4 Effect of water-miscible co-solvents*

Water miscible organic co-solvents are well accepted by carboxylases up to 20% v/v.[4] A correlation between decreasing solvent polarity and decreasing hydration performance could be noted, with a sharp drop in the presence of 10 %v/v DMF (relative polarity: 0.386). Values for relative polarity are normalized from measurements of solvent shifts of absorption spectra[5] (Figure S5b).

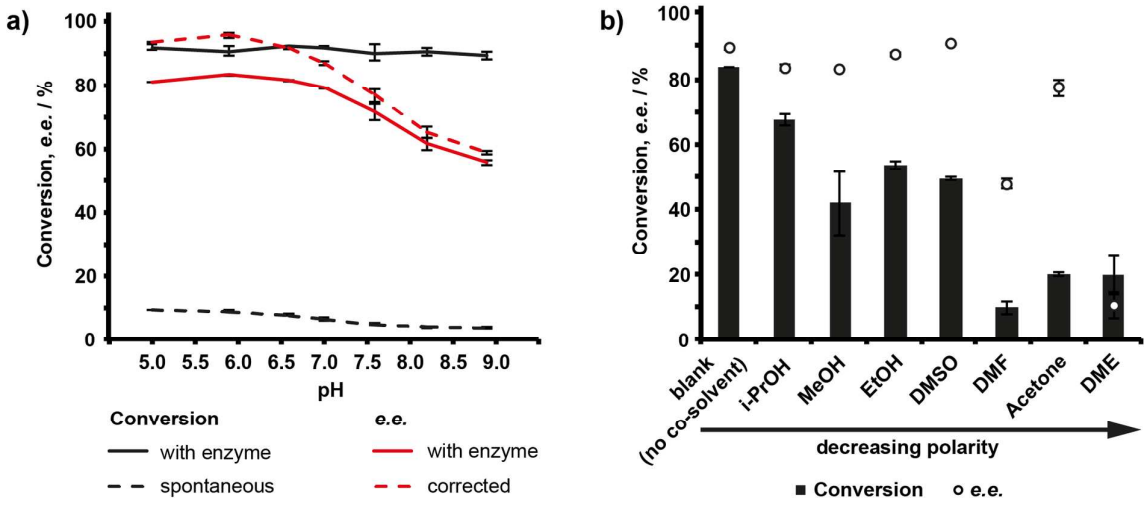


**Figure S5** a) pH study with FDC_*E*s Val46Glu. Conditions: purified FDC_*E*s Val46Glu variant (10 µL of a 3 mM stock) in 980 µL potassium phosphate buffer (50 mM, pH 5–9), substrate 4-vinylphenol (10 µL of a 8.6% w/w propylene glycol solution) incubated for 24 h at 700 rpm and 30 °C b) Effect of water-miscible co-solvents on hydration. Conditions: purified FDC_*E*s Val46Glu variant (10 µL of a 3 mM stock) in 880 µL potassium phosphate buffer (50 mM, pH 6.0), co-solvent (100 µL, 10% v/v) substrate 4-vinylphenol (10 µL of a 8.6% w/w propylene glycol solution) incubated for 24 h at 700 rpm and 25 °C.

Stereoselectivity relative to the control without co-solvent (84 % conv, 89% *e.e.*) was slightly improved only by addition of DMSO, however at the cost of conversion (49% conv. 91% *e.e.*). 2-Propanol did not show considerable adverse effects on conversion and *e.e.* (68% conv. 84% *e.e.*) and hence could be considered as 'innocent' co-solvent for dissolving non-polar substrates.

*8.5 Substrate loading*

**Table S1** Productivity of FDC_*Es* Val46Glu

| **Concentration of 1 (mM)** | **Conversion (%)** | **TON ($mol_{prod}$ $mol_{enz}^{-1}$)** | ***e.e.* (%)** |
|---|---|---|---|
| 10 | 95 | 46 | 88 |
| 25 | 95 | 113 | 91 |
| 50 | 93 | 206 | 92 |
| 75 | 13 | 55 | 67 |

Conditions: purified FDC_*Es* Val46Glu variant (227 µM) in potassium phosphate buffer (50 mM, pH 6.0), substrate 4-vinylphenol **1** (13.4, 33.6, 67.2 and 100.8 µL of a 8.6% w/w propylene glycol solution; 10, 25, 50 and 75 mM, respectively) incubated for 24 h at 700 rpm and 25 °C.

**Table S2** Productivity of FDC_*Es* Val46Asp

| **Concentration of 1 (mM)** | **Conversion (%)** | **TON ($mol_{prod}$ $mol_{enz}^{-1}$)** | ***e.e.* (%)** |
|---|---|---|---|
| 10 | 95 | 42 | 85 |
| 25 | 95 | 100 | 86 |
| 50 | 94 | 197 | 89 |
| 75 | 12 | 41 | 45 |

Conditions: purified FDC_*Es* Val46Asp variant (227 µM) in potassium phosphate buffer (50 mM, pH 6.0), substrate 4-vinylphenol **1** (13.4, 33.6, 67.2 and 100.8 µL of a 8.6% w/w propylene glycol solution; 10, 25, 50 and 75 mM, respectively) incubated for 24 h at 700 rpm and 25 °C.

*8.6 Preparative scale biotransformation*

4-Vinylphenol (**1**, 1.163 g of a 8.6 % w/w solution in propylene glycol, 100 mg, 0.83 mmol, 50 mM) was incubated with purified FDC_*Es* Val46Glu mutant (3.2 µmol, second portion added after 24 h) in potassium phosphate buffer (16 mL, 50 mM, pH 6.0) at 25°C. Upon full conversion as judged by tlc (petrolether/ethylacetae 7:3, product-$R_f$ = 0.35) after 48 h, the aqueous phase was saturated with sodium chloride and extracted with ethyl acetate (3 × 15 mL), the organic phase was dried over $Na_2SO_4$ and evaporated under reduced pressure. Column chromatography on silica gel afforded 68.2 mg of (*S*)-**2** as colorless solid (60%) with petrolether/ethylacetate 7:3 as eluent and 96% *e.e* after recrystallization from cyclohexane/ethylacetate 1:8. $^1$H NMR (300 MHz, MeOH-$d_4$) δ [ppm] = 7.18–7.14 (m, 2H, $C_{ar}$–H), 6.74–6.70 (m, 2H, $C_{ar}$–H), 4.72 (q, $J$ = 6.3 Hz, 1H, $C_\alpha$–H), 1.38 (d, $J$=6.6 Hz, 3H, $C_\beta H_3$); $^{13}$C NMR (75 MHz, MeOH-d4) δ [ppm] = 175.6, 138.3, 127.8, 115.9, 70.6, 25.4; $[a]_D^{20}$ = –29.2° (c = 1, MeOH, *e.e*. = 84%), m.p. = 154 °C (lit. 139 °C), in agreement with literature data.[2]

*8.7 Addition of $MeONH_2$ and HCN across 4-vinylphenol with FDC_Es Val46Glu*

**Table S3** Hydration *versus* addition of other nucleophiles (NuH) with FDC_*Es* wild type and Val46Glu.

| **NuH** | **FDC_*Es* variant** | **Hydration (%)** | ***e.e.* (%)** | **NuH addition (%)** | ***e.e.* (%)** |
|---|---|---|---|---|---|
| $MeONH_2$ | wt[1] | 5 | 39 | 95 | 17 |
| | Val46Glu | 31 | 94 | 68 | 6 |
| HCN | wt[1] | < 1 | n.d. | 82 | 64 |
| | Val46Glu | 55 | 38 | 43 | 88 |

Conditions: lyophilized *E. coli* cells (20 mg/mL, 32 U) containing the heterologously expressed FDC variant or wild-type enzyme, substrate **1** (10 mM), methoxyamine or sodium cyanide (100 mM) in $KP_i$ buffer (50 mM, pH 7.0); incubation for 24 h at 30°C and 700 rpm; n.d. = not determined due to low conversion.

## 9 Spectroscopic data

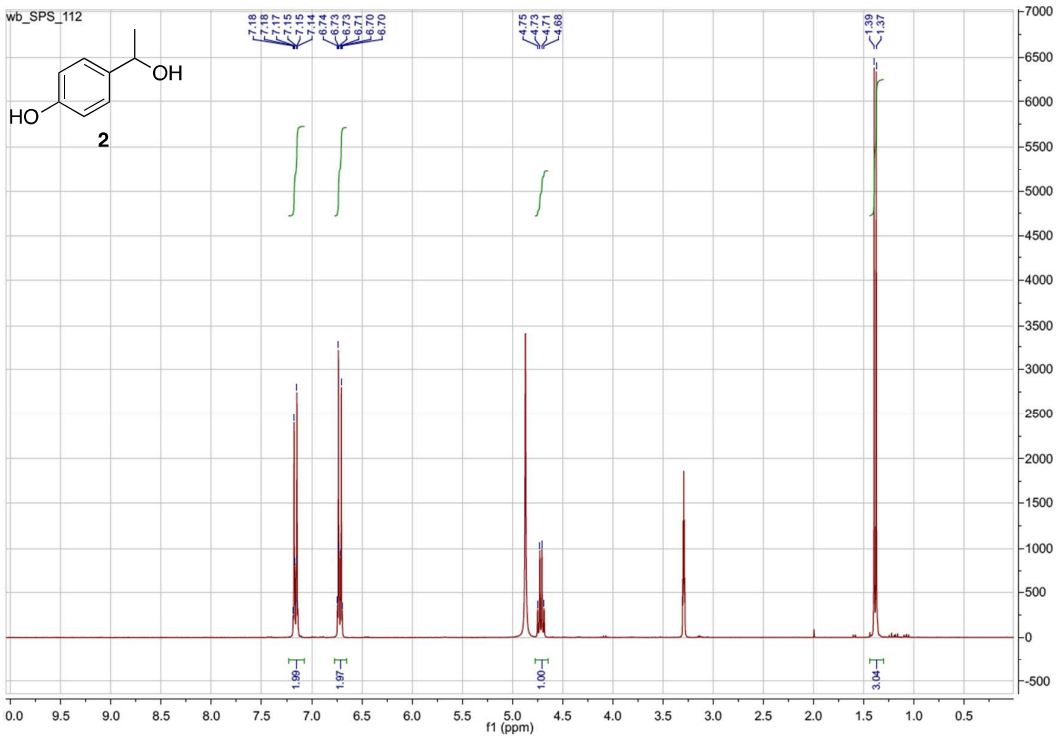


**Figure S6** $^{1}$H NMR of biotransformation product **2**

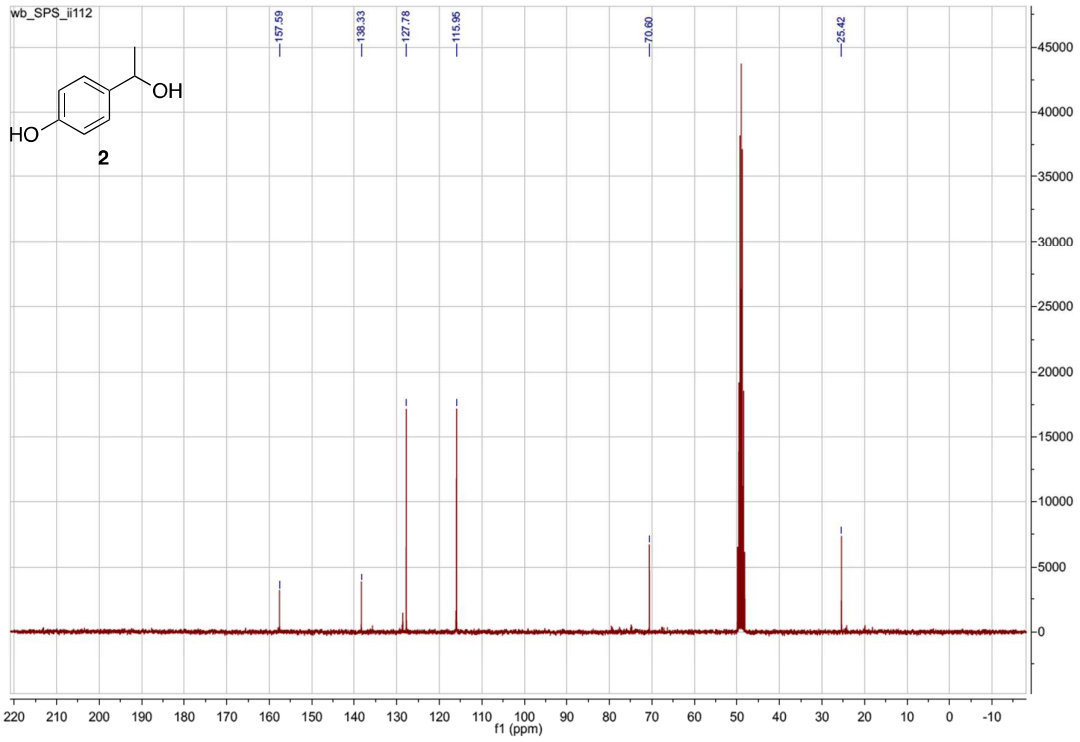


**Figure S7** $^{13}$C NMR of biotransformation product **2**

## 10 References


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