Supporting Information

Engineered Bacterial Flavin-Dependent Monooxygenases for the Regiospecific Hydroxylation of Polycyclic Phenols

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Supplementary methods

Chemicals
Cinnamic acid derivatives, naringenin, eriodictyol, esculetin, piceatannol, 2-hydroxycarbazole, p-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, FAD, NADH and the formate dehydrogenase from Candida boidinii were obtained from Sigma-Aldrich (St. Louis, USA). Resveratrol was from ABCR (Karlsruhe, Germany) and umbelliferone was obtained from Alpha Aesar (Karlsruhe, Germany). All other chemicals and solvents were of the highest purity commercially available.

Cloning and site-directed mutagenesis
Gene fragments were amplified from genomic DNA from Escherichia coli BL21(DE3) (4HPA3H, HpaC) or the Pseudomonas protegens strain CHA0 (PrnF) using the following specific primers: 4HPA3H-fw 5´-TATGGTCTCCATATGAAACCAGAAGATTTCGG-3´; 4HPA3H-rev 5´-TATGGTCTCCGAGTTTTGCAGCTTACC-3´; HpaC-fw 5´-TATGGTCTCCATATGCAATTAGAACAACGCCTG-3´; HpaC-rev 5´-TATGGTCTCCGAGCTATTGCTTGGAGGACGC-3´; PrnF-fw 5´-TATGGTCTCCATATGAATGCTGCGCCACCGAAAC-3´ and PrnF-rev 5´-TATGGTCTCCGAGCTATTGTGCGCGAGGAGGC-3´. The fragments which were generated by standard PCR methods were introduced into a modified pET28a(+) (Merck, Darmstadt, Germany) vector (sequence available on request) in frame with the NdeI and XhoI restriction sites (italic in the specified primers) by BsaI-directed ligation (BsaI sites underlined in the specific primers) according to the method of Engler et al. (2009).[1] Enzyme variants were constructed by mutagenesis of pET28a(+) 4HPA3H by the Quik Change II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, USA) according to the instructions of the manufacturer.

Enzyme production and purification
Plasmids were transformed in E. coli BL21(DE3), and a single colony was used for inoculation of pre-cultures in Luria-Bertani medium (10 g l⁻¹ tryptone, 10 g l⁻¹ NaCl, 5 g l⁻¹ yeast extract, pH 7.0) containing kanamycin (50 µg ml⁻¹). Pre-cultures grown at 37 °C were used for inoculation of 400 ml of auto-inducing medium.[2] After incubation overnight at 37 °C with shaking (220 rpm), cells were collected by centrifugation at 8,000 g for 15 min. Cell lysis and protein purification by chromatography on HiTrap Talon columns (5 ml, GE Healthcare, Chalfont St. Giles, United Kingdom) was carried out as described elsewhere.[3] The pooled protein fractions were desalted by dialysis against a 100-fold volume of 20 mM HEPES/NaOH, 100 mM NaCl, 5 % (w/v) glycerol, pH 7.5. The purified proteins were checked for homogeneity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed in polyacrylamide gels (10 or 14 % crosslinking) according to Laemmli.[4] The gels were stained with Coomassie Brilliant Blue R250, and apparent molecular masses were
estimated using molecular markers from Fermentas (Waltham, USA). The enzymes were stored at -80 °C until use.

**Protein determination**

The protein concentration was determined by the method of Bradford.[5] Protein assays in microplate scale were carried out with commercial Bradford reagent (Roth, Karlsruhe, Germany) according to manufacturer’s instructions. Protein concentrations were calculated using standard curves of bovine serum albumin.

**Whole-cell bioconversions**

Reactions were performed in 50 ml Erlenmeyer flasks. 10 ml of auto-inducing medium[2] containing hydroxylase substrate (200 µM, added from stock solutions (200 mM) in methanol) and kanamycin (50 µg ml⁻¹) were inoculated with *E. coli* BL21(DE3) harboring either the plasmids for production of 4HPA3H or its variants (hydroxylation reactions) or the empty vector (controls). Cells were grown at 37 °C with shaking (200 rpm). After 16 hours of incubation, samples (1.5 ml) were taken. For all conversions except those of 2-hydroxycarbazole, the samples were centrifuged at 14,000 g for 10 min. The supernatants (1 ml) were acidified by the addition of 10 µl of formic acid and extracted two times with 500 µl of ethyl acetate. For samples containing the poorly soluble 2-hydroxycarbazole, the centrifugation step was omitted, and the cell suspension was directly used in the extraction procedure. After extraction the organic phases were combined, the solvent was removed by vacuum centrifugation and the residue was re-dissolved in acetonitrile (200 µl). This solution was used for HPLC measurements (see Section below). The data shown in this study were generated from two independent experiments.

For product identification, preparative transformations were performed similar to the reactions described above but in larger scale, i.e. in 2,000 ml Erlenmeyer flasks containing 500 ml of medium. After incubation at 37 °C for 16 hours, formic acid (5 ml) was added to the cell suspension (conversion of 2-hydroxycarbazole) or to the supernatant from centrifugation (8,000 g, 10 min) (all other conversions). The combined organic phases from extraction with ethyl acetate (2 x 250 ml) were dried with anhydrous Na₂SO₄, filtered and the solvent was removed under reduced pressure. The residue was re-dissolved in a minimum volume of methanol and used for preparative HPLC (see below).

**HPLC**

Samples (10 µl) from whole-cell biotransformation (see above) or from in vitro assays (see below) were applied to a C18 reversed-phase column (Pack ODS-A, 5 µm, 150 x 4.6 mm, YMC Europe Dinslaken, Germany) on a Merck Hitachi HPLC system. The compounds were separated by elution with acetonitrile-water (containing 0.2 % (v/v) formic acid). The following
gradient was used at a flow of 0.8 ml min\(^{-1}\) (percentage of acetonitrile in v/v): 5 % for 4 min, 5 to 100 % for 21 min, 100 % for 5 min, 100 to 5 % for 1 min and 5 % for 5 min.

All products that were generated in large-scale biotransformations (see above) were separated on a preparative HPLC system by using a C18 reversed-phase column (Column ODS-A, 5 µm, 150 x 20 mm, YMC Europe, Dinslaken, Germany). The following gradient was used at a flow of 15 ml min\(^{-1}\) (percentage of acetonitrile in v/v): 50 % for 4 min, 50 to 100 % for 21 min, 100 % for 5 min, 100 to 50 % for 1 min and 50 % for 5 min.

For both analytical and preparative HPLC, elution was monitored at a wavelength of 280 nm. Reaction products were identified by means of authentic standards, which were applied in different concentrations for quantification, or in case of 4-(3,4-dihydroxyphenyl)-butan-2-one (16) and 2,3-dihydroxycarbazole (12) by NMR and ESI-MS analysis of reaction products obtained from preparative whole-cell transformations (as described above).

**NMR and ESI-FTMS analysis**

The \(^1\)H NMR spectra were recorded on a 400 MHz Agilent DD2 400 NMR spectrometer at 25 °C. Substances were dissolved in CD\(_3\)OD, and chemical shifts were referenced to internal tetramethylsilane. The data (see page 10) were evaluated by Mestrelab Research S.L. MestReNova 6.0.2 software.

The negative ion high resolution ESI mass spectra (see page 10) and the collision induced dissociation (CID) MS\(^2\) measurements were obtained from a Orbitrap Elite mass spectrometer (Thermofisher Scientific, Bremen, Germany) equipped with an HESI electrospray ion source (spray voltage 4.0 kV; capillary temperature 275 °C, source heater temperature 40 °C; FTMS resolution 60.000). Nitrogen was used as sheath gas. The sample solutions were introduced continuously via a 500 µl Hamilton syringe pump with a flow rate of 5 µl min\(^{-1}\). The instrument was externally calibrated by the Pierce® LTQ Velos ESI positive ion calibration solution (product number 88323) and Pierce® ESI negative ion calibration solution (product number 88324) from Thermofisher Scientific, Rockford, IL, 61105 USA). The data were evaluated by the Xcalibur software 2.7 SP1 by averaging 25 scans.

**Homology modeling**

The three-dimensional structures of 4HPA3H from *E. coli* and *P. aeruginosa* were modeled by means of the online protein fold recognition server Phyre\(^2\) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, accession at 01.08.2013).[6] The model with the highest score (confidence: 100 %, coverage: 93 %), which is based on crystallographic data of the 4HPA3H enzyme from *Thermus thermophilus* (PDB accession 2YYJ) was used for alignments and active site studies. The determination of RMSD values was conducted using the alignment tool integrated in the software Molecular Operating Environment (MOE, Chemical Computing Group ULC 2019, version 2019.0101).
Activity assay for PrnF

The activity of the flavin reductase component PrnF was measured by the decrease of the absorbance at 339 nm ($\varepsilon_{340} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1}$) due to the oxidation of NADH. The reaction was performed in a cuvette with a light path of 1 cm that contained reaction buffer (100 mM Tris/HCl, pH 7.5), NADH (200 µM) and FAD (30 µM) in a total volume of 1 ml. The reactions were started by the addition of 12.5 µg ml$^{-1}$ PrnF to the reaction mixture. An assay mixture without enzyme was used as a blank. The decrease in absorbance was recorded continuously at intervals of 40 s for a total reaction time of 10 min. One unit of enzyme activity represents the amount of enzyme converting 1 µmol of NADH in one minute of reaction time. The data shown in this study are the means of three independent measurements.

Spectrophotometric activity assay for 4HPA3H

The assay was performed in a microplate scale. Reactions (80 µl) contained reaction buffer (100 mM Tris/HCl, pH 7.5), substrate (2 mM $p$-coumaric acid or ferulic acid which were added from a stock solution (200 mM) in methanol), FAD (10 – 1000 µM), NADH (25 – 200 µM), sodium formate (300 mM) and formate dehydrogenase from Candida boidinii (100 mU ml$^{-1}$). Reactions were started by addition of PrnF (566 mU ml$^{-1}$) and hydroxylase (1 mg ml$^{-1}$). The reactions were stopped after distinct times of incubation (0 – 240 min) at 25 °C by mixing with 40 µl of catechol reagent (2 mM FeCl$_3$ in 10 mM HCl, 1 mM Triton X-100) to generate a colored Fe$^{3+}$/product complex. From the absorbance at 595 nm, measured after 5 min of equilibration at room temperature, the concentration of formed product was calculated by means of a standard curve taken with mixtures of the substrate (x mM $p$-coumaric acid or ferulic acid) and the corresponding product (2-x mM caffeic acid or 5-hydroxy-ferulic acid). One unit of enzyme activity represents the amount of hydroxylase converting 1 µmol of $p$-coumaric acid in one minute of reaction time. The data shown in this study are the means of two independent experiments.

determination of shunt H$_2$O$_2$ produced by uncoupled NADH consumption

Microplate reactions (80 µl) were performed as described in the Section “Spectrophotometric activity assay for 4HPA3H”, except that the substrate $p$-coumaric acid was added from an aqueous stock solution of its sodium salt and that the reactions contained less enzyme (0.25 mg ml$^{-1}$ wildtype enzyme or 4HPA3H Y301F/S462A) and an optimized amount of FAD (10 µM) and NADH (200 µM). Twelve independent reactions were set up per enzyme. After 60 min of incubation at 25 °C, four of the replicates were treated with catechol reagent (see Section above) to determine the formed caffeic acid. Four other replicates were used for determination of H$_2$O$_2$ using an adaptation of the method described by Dippe and Ulbrich-Hofmann. One unit of enzyme activity represents the amount of hydroxylase converting 1 µmol of $p$-coumaric acid in one minute of reaction time. The data shown in this study are the means of two independent experiments.
20 µl of horseradish peroxidase (50 µg ml⁻¹ in 100 mM Tris/HCl, pH 7.5). The absorbance at 490 nm caused by the formation of the quinoneimine dye was measure after 10 min of incubation at 25 °C. For the calculation of the H₂O₂ concentration the difference in absorbance to the remaining four independent reactions, which were treated with water instead of peroxidase, was used. The calculation was based on a standard curve (0 – 500 µM H₂O₂ in 100 mM Tris/HCl, pH 7.5) taken under similar conditions.

In vitro conversion of monophenols
The reactions (200 µl) were performed under optimized conditions in 1.5 ml reaction tubes. Reaction buffer (100 mM Tris/HCl, pH 7.5), monophenol substrate (1 mM from a stock (10 mM) in methanol), FAD (10 µM), NADH (25 µM), sodium formate (300 mM) and formate dehydrogenase from Candida boidinii (100 mU ml⁻¹) were mixed with PrnF (566 mU ml⁻¹) and hydroxylase (1 mg ml⁻¹ 4HPA3H or variants (Y301I, Y301F/S462A)). After 16 hours of incubation, the samples were centrifuged at 14,000 rpm for 10 min. The supernatant was acidified by the addition of formic acid (2 µl) and extracted two times with 200 µl of ethyl acetate. The combined organic phase was evaporated and the residual was absorbed in 200 µl of acetonitrile. The samples were used for HPLC analysis (see product analysis). The data shown in this study are the means of three independent experiments.

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Supplementary figures and tables

**Supplementary Figure S1.** SDS-PAGE of cells expressing 4HPA3H (lane 1), the variants Y301F (lane 2), S462A (lane 3), M293P (lane 4), I157V (lane 5), Y301L (lane 6), Y301I (lane 7), Y301F/S462A (lane 8), Y301L/S462A (lane 9), Y301I/S462A (lane 10), Y301F/I157V (lane 11) and I157V/S462A (lane 12), or harboring the empty vector pET28a(+) (lane 13). Lysates from equal amounts of cells were applied to polyacrylamide gels with 10 % (w/v) crosslinking as specified in the Supplementary methods. Expression of recombinant HPA3H proteins is indicated by bands with an apparent molecular weight of 56 kDa. M: molecular markers.

![Supplementary Figure S1](image1)

**Supplementary Figure S2.** SDS-PAGE of purified enzymes used in in vitro hydroxylation. Equal amounts of protein (5 µg) were used for separation in polyacrylamide gels with 10 % (w/v) crosslinking as specified in the Supplementary methods. Lane 1: wild-type 4HPA3H, lane 2: 4HPA3H-Y301I, lane 3: 4HPA3H-Y301F/S462A, lane 4: PrnF, M: molecular marker.

![Supplementary Figure S2](image2)
Supplementary Figure S3. Catechol complex formation assay for determination of 4HPA3H activity. (A) Standard curve for the determination of caffeic acid (2). Mixtures of caffeic acid and p-coumaric acid (1) were incubated with FeCl₃ and analyzed at 595 nm as described in the Experimental Section. (B) Time course of the oxidation of p-coumaric acid (1) by wild-type 4HPA3H. The enzymatic reaction was performed under optimized conditions, and produced caffeic acid (2) was detected as Fe³⁺ complex as described in the Supplementary methods. Data are the means ± standard deviations obtained from three measurements.

Supplementary Figure S4. Dependence of the in vitro conversion of p-coumaric acid on the FAD (filled circles) and NADH concentration (open circles). The reactions were performed using the 4HPA3H/PrnF/FDH system as specified in the Supplementary methods.
**Supplementary Table S1.** In vivo conversion data corresponding to Fig. 2. The conversion of substrates (200 µM) was performed in duplicates using life whole-cell biocatalysts was specified in the Supporting methods. n.d., not detectable; STD, standard deviation of the mean.

| 4HPA3H enzyme | Substrate identifier (see Fig. 1) | Relative conversion (%) replicate I | Relative conversion (%) replicate II | Mean of relative conversion (%) | STD (%) |
|---------------|----------------------------------|-------------------------------------|-------------------------------------|--------------------------------|---------|
| wild-type 4HPA3H | 1 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 3 | n.d. | n.d. | n.d. | n.d. |
| | 5 | 77.53 | 78.55 | 78.04 | 0.51 |
| | 7 | n.d. | n.d. | n.d. | n.d. |
| | 9 | 41.23 | 36.15 | 38.69 | 2.54 |
| | 11 | 8.45 | 9.09 | 8.77 | 0.32 |
| | 13 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 15 | 100.00 | 100.00 | 100.00 | 0.00 |
| 4HPA3H-Y301F | 1 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 3 | 1.19 | 0.00 | 1.19 | 1.19 |
| | 5 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 7 | 12.17 | 13.81 | 12.99 | 0.82 |
| | 9 | 53.00 | 48.44 | 50.72 | 2.28 |
| | 11 | 26.43 | 19.97 | 23.20 | 3.23 |
| | 13 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 15 | 91.12 | 100.00 | 95.56 | 4.44 |
| 4HPA3H-S462A | 1 | 100.00 | 100.00 | 100.00 | 0.00 |
| | 3 | 0.38 | 0.38 | 0.38 | 0.00 |
| | 5 | 98.34 | 94.66 | 96.50 | 1.84 |
| | 7 | 26.36 | 27.2 | 26.78 | 0.42 |
| | 9 | 25.82 | 26.7 | 26.26 | 0.44 |
| | 11 | n.d. | n.d. | n.d. | n.d. |
| | 13 | 100.00 | 93.92 | 94.96 | 3.04 |
| | 15 | 80.98 | 93.48 | 87.23 | 6.25 |
| 4HPA3H-M293P | 1 | 81.25 | 81.25 | 76.43 | 4.82 |
| | 3 | 0.77 | 1.19 | 0.98 | 0.21 |
| | 5 | 71.88 | 70.62 | 71.25 | 0.63 |
| | 7 | 10.65 | 18.33 | 14.49 | 3.84 |
| | 9 | 7.24 | 7.22 | 7.23 | 0.01 |
| | 11 | 7.18 | 17.44 | 12.31 | 5.13 |
| | 13 | 78.08 | 71.00 | 74.54 | 3.54 |
| | 15 | 80.82 | 86.10 | 83.46 | 2.64 |
| 4HPA3H-I157V | 1 | 26.52 | 30.48 | 28.50 | 1.98 |
| | 3 | n.d. | n.d. | n.d. | n.d. |
| | 5 | 17.23 | 17.07 | 17.15 | 0.08 |
| | 7 | 5.15 | 5.19 | 5.17 | 0.02 |
| | 9 | 5.37 | 5.41 | 5.39 | 0.02 |
| | 11 | 6.39 | 5.93 | 6.16 | 0.23 |
| | 13 | 10.88 | 9.42 | 10.15 | 0.73 |
| | 15 | 43.68 | 29.82 | 36.75 | 6.93 |
**Supplementary Table S1.** continued from page 9.

| 4HPA3H enzyme | Substrate identifier (see Fig. 1) | Relative conversion (%) replicate I | Relative conversion (%) replicate II | Mean of relative conversion (%) | STD (%) |
|----------------|----------------------------------|-------------------------------------|-------------------------------------|---------------------------------|---------|
| 4HPA3H Y301L   | 1                                | 14.36                               | 13.68                               | 14.02                           | 0.34    |
|                | 3                                | 27.78                               | 29.06                               | 28.42                           | 0.64    |
|                | 5                                | 7.50                                | 7.46                                | 7.48                            | 0.02    |
|                | 7                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 9                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 11                               | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 13                               | 1.60                                | 2.04                                | 1.82                            | 0.22    |
|                | 15                               | n.d.                                | n.d.                                | n.d.                            | n.d.    |
| 4HPA3H- Y301I  | 1                                | 45.50                               | 45.80                               | 45.65                           | 0.15    |
|                | 3                                | 45.81                               | 44.07                               | 44.94                           | 0.87    |
|                | 5                                | 17.79                               | 22.53                               | 20.16                           | 2.37    |
|                | 7                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 9                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 11                               | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 13                               | 25.94                               | 24.34                               | 25.14                           | 0.80    |
|                | 15                               | n.d.                                | n.d.                                | n.d.                            | n.d.    |
| 4HPA3H- Y301F/S462A | 3                          | 5.34                                | 4.88                                | 5.11                            | 0.23    |
|                | 7                                | 28.93                               | 30.53                               | 29.73                           | 0.80    |
|                | 9                                | 24.19                               | 22.05                               | 23.12                           | 1.07    |
|                | 11                               | 18.60                               | 22.40                               | 20.50                           | 1.90    |
| 4HPA3H- Y301F/I157V | 3                          | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 7                                | 2.83                                | 1.11                                | 1.97                            | 0.86    |
|                | 9                                | 8.23                                | 7.72                                | 8.02                            | 0.30    |
|                | 11                               | 16.44                               | 36.34                               | 26.39                           | 9.95    |
| 4HPA3H- I157V/S462A | 3                          | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 7                                | 2.14                                | 1.62                                | 1.88                            | 0.26    |
|                | 9                                | 3.69                                | 3.09                                | 3.39                            | 0.300   |
|                | 11                               | 34.74                               | 39.44                               | 37.09                           | 2.35    |
| 4HPA3H- Y301L/S462A | 3                          | 39.41                               | 40.19                               | 39.80                           | 0.39    |
|                | 7                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 9                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 11                               | n.d.                                | n.d.                                | n.d.                            | n.d.    |
| 4HPA3H- Y301I/S462A | 3                          | 14.98                               | 17.86                               | 16.42                           | 1.44    |
|                | 7                                | 5.54                                | 6.68                                | 6.11                            | 0.57    |
|                | 9                                | n.d.                                | n.d.                                | n.d.                            | n.d.    |
|                | 11                               | 31.40                               | 28.86                               | 30.13                           | 1.27    |
**Supplementary Table S2.** Amount of hydrogen peroxide produced during hydroxylation of \( p \)-coumaric acid 1 under in vitro conditions. Purified hydroxylase enzymes (0.25 mg ml\(^{-1}\)) were incubated with \( p \)-coumaric acid (2 mM, sodium salt added from aqueous stock) under optimized conditions (10 \( \mu \)M FAD, 200 \( \mu \)M NADH, cofactor regeneration by the PrnF/FDH system). The conversion was determined after 60 min of incubation via the \( \text{Fe}^{3+} \) complexation method (formed caffeic acid) or by a peroxidase-mediated colorimetric assay (formed \( \text{H}_2\text{O}_2 \)) as specified in the Supplementary methods.

| 4HPA3H enzyme             | formed caffeic acid (\( \mu \)M) | produced \( \text{H}_2\text{O}_2 \) (\( \mu \)M) | Ratio hydroxylation to total NADH consumption (%) |
|---------------------------|----------------------------------|----------------------------------|--------------------------------------------------|
| wild-type 4HPA3H          | 1049 ± 29                        | 440 ± 8                          | 70.4                                             |
| 4HPA3H Y301F/S462A       | 1045 ± 2                         | 404 ± 4                          | 72.1                                             |

**Supplementary Table S3.** *In vitro* hydroxylation of 2-hydroxycarbazole 11 at different substrate concentrations by 4HPA3H variants. Reactions were carried out in duplicates under optimized conditions (10 \( \mu \)M FAD, 200 \( \mu \)M NADH, cofactor regeneration by the PrnF/FDH system) in the presence of the specified concentration of substrate and hydroxylase enzyme (1 mg ml\(^{-1}\)). The conversion was assayed via HPLC as specified in Supplementary methods.

| 4HPA3H variant         | Substrate concentration (mM) | Conversion into 2,3-dihydroxycarbazole (%) |
|------------------------|------------------------------|------------------------------------------|
| Y301I                  | 0.2                          | 44.5 ± 1.6                               |
|                        | 0.5                          | 1.9 ± 0.3                                |
|                        | 1.0                          | 0.7 ± 0.2                                |
| Y301F/S462A            | 0.2                          | 94.1 ± 1.6                               |
|                        | 0.5                          | 40.3 ± 10.0                              |
|                        | 1.0                          | 2.3 ± 0.4                                |
Supplementary Table S4. In vitro conversion data corresponding to Fig. 5. The conversion of substrates (1 mM) was performed in duplicates using purified enzymes was specified in the Supporting methods. n.d., not detectable; STD, standard deviation of the mean.

| 4HPA3H enzyme | Substrate identifier (see Fig. 1) | Relative conversion (%) replicate I | Relative conversion (%) replicate II | Mean of relative conversion (%) | STD (%) |
|---------------|-----------------------------------|------------------------------------|------------------------------------|-------------------------------|---------|
| wild-type 4HPA3H | 1                                 | 97.27                              | 96.08                              | 96.68                         | 0.59    |
|               | 3                                 | n.d.                               | n.d.                               | n.d.                          | n.d.    |
|               | 5                                 | 94.60                              | 74.72                              | 84.66                         | 9.94    |
|               | 7                                 | 25.26                              | 25.30                              | 25.28                         | 0.02    |
|               | 9                                 | 18.71                              | 14.84                              | 16.78                         | 1.94    |
|               | 11                                | 4.96                               | 0.00                               | 2.48                          | 2.48    |
|               | 13                                | 98.31                              | 98.29                              | 98.30                         | 0.01    |
|               | 15                                | 100.00                             | 100.00                             | 100.00                        | 0       |
| 4HPA3H-Y301I | 3                                 | 18.88                              | 7.92                               | 13.40                         | 5.48    |
|               | 7                                 | 4.56                               | 8.22                               | 6.39                          | 1.83    |
|               | 9                                 | n.d.                               | n.d.                               | n.d.                          | n.d.    |
|               | 11                                | n.d.                               | n.d.                               | n.d.                          | n.d.    |
| 4HPA3H-Y301F/S462A | 3                               | 4.32                               | 5.42                               | 4.87                          | 0.55    |
|               | 7                                 | 58.77                              | 41.11                              | 49.94                         | 8.83    |
|               | 9                                 | 15.84                              | 10.34                              | 13.09                         | 2.75    |
|               | 11                                | n.d.                               | n.d.                               | n.d.                          | n.d.    |
Analytical data

Caffeic acid (2)

$^1$H NMR: $\delta = 7.53$ (d, $^3\!J(\text{H}, \text{H}) = 15.79$ Hz, 1H; CH), $\delta = 7.04$ (s, 1H; CH), $\delta = 6.93$ (d, $^3\!J(\text{H}, \text{H}) = 7.89$ Hz, 1H; CH), $\delta = 6.78$ (d, $^3\!J(\text{H}, \text{H}) = 7.89$ Hz, 1H; CH), $\delta = 6.21$ ppm (d, $^3\!J(\text{H}, \text{H}) = 15.79$ Hz, 1H; CH).

ESI-FTMS: $m/z$ 179.0351 [M-H]; (-)-ESI-MS$^2$ (25 eV): $m/z$ (%) 179.0352 (32) [M-H], 135.0454 (100) [M-H-CO$_2$].
5-Hydroxyferulic acid (4)

$^1H$ NMR: δ = 7.52 (d, $^3J$(H, H) = 15.79 Hz, 1H; CH), δ = 7.26 (s, 1H; CH), δ = 7.24 (s, 1H; CH), δ = 6.26 (d, $^3J$(H, H) = 15.78 Hz, 1H; CH), δ = 3.29 ppm (s, 3H; CH$_3$).

ESI-FTMS: $m/z$: 209.0457 [M-H]; (-)-ESI-MS$^2$ (25 eV): $m/z$ (%): 209.0455 (46) [M-H], 194.0221 (100) [M-H-CH$_3$], 165.0558 (44) [M-H-CO$_2$], 150.0324 (14) [M-H-C$_2$H$_3$O$_2$].
Piceatannol (6)

$^1$H NMR: $\delta = 6.97$ (s, 1H; CH), $\delta = 6.89$ (d, $^3$J(H, H) = 16.22 Hz, 1H; CH), $\delta = 6.82$ (d, $^3$J(H, H) = 7.89 Hz, 1H; CH), $\delta = 6.73$ (d, $^3$J(H, H) = 7.9 Hz, 1H; CH), $\delta = 6.74$ (d, $^3$J(H, H) = 16.22 Hz, 1H; CH), $\delta = 6.43$ (2 x s, 2H; 2 x CH), $\delta = 6.16$ ppm (s, 1H; CH).

ESI-FTMS: $m/z$: 243.0664 [M-H]; (-)-ESI-MS$^2$ (35 eV): $m/z$ (%): 243.0665 (4) [M-H]$^-$, 225.0559 (100) [M-H-H$_2$O], 215.0715 (14) [M-H-CO], 201.0559 (64) [M-H-C$_2$H$_2$O], 181.0661 (8) [M-H-CH$_2$O$_3$], 175.0767 (36) [M-H-C$_3$O$_2$], 159.0454 (15) [M-H-C$_4$H$_4$O$_2$].
Eriodictyol (8)

$^1\text{H NMR}$: $\delta = 6.91$ (s, 1H; CH), $\delta = 6.79$ (s, 1H; CH), $\delta = 5.29$ (dd, $^3J(\text{H}, \text{H}) = 13.0$, $^2J(\text{H}, \text{H}) = 3.07$ Hz, 1H, CH), $\delta = 3.06$ (dd, $^3J(\text{H}, \text{H}) = 13.0$, $^3J(\text{H}, \text{H}) = 17.1$, 1H; CH), $\delta = 2.69$ ppm (dd, $^3J(\text{H}, \text{H}) = 17.1$, $^2J(\text{H}, \text{H}) = 3.0$ Hz, 1H; CH).

ESI-FTMS: $m/z$: 287.0565 [M-H]; (-)-ESI-MS$^2$ (25 eV): $m/z$ (%): 287.0565 (6) [M-H], 181.0145 (4) [M-H-C$_7$H$_2$O], 151.0040 (100) [M-H-C$_8$H$_6$O$_2$], 125.0247 (4) [M-H-C$_6$H$_6$O$_3$].
3,4-Dihydroxybenzoic acid (10)

$^1H\text{ NMR}$: $\delta = 7.41$ (s, 1H; CH), $\delta = 7.03$ (d, $^3J(H, H) = 8.34$ Hz, 1H; CH), $\delta = 6.70$ ppm (d, $^3J(H, H) = 8.33$ Hz, 1H; CH).

ESI-FTMS: $m/z$: 153.0195 [M-H]; (-)-ESI-MS$^2$ (25 eV): $m/z$ (%): 153.0196 (6) [M-H], 109.0298 (100) [M-H-CO$_2$].
2,3-Dihydroxycarbazole (12)

$^1$H NMR: δ = 7.80 (d, $^3$J(H, H) = 7.46 Hz, 1H; CH), δ = 7.39 (s, 1H; CH), δ = 7.30 (d, $^3$J(H, H) = 7.89 Hz, 1H; CH), δ = 7.19 (dd, $^3$J(H, H) = 7.01 Hz, $^3$J(H, H) = 7.89 Hz, 1H; CH), δ = 7.02 ppm (dd, $^3$J(H, H) = 7.01 Hz, $^3$J(H, H) = 7.45 Hz, 1H; CH).

ESI-FTMS: $m/z$: 198.0560 [M-H]; (-)-ESI-MS$^2$ (35 eV): $m/z$ (%): 198.0562 (2) [M-H], 180.0456 (100) [M-H-H$_2$O], 170.0613 (40) [M-H-CO], 154.0664 (18) [M-H-CO$_2$], 130.0664 (35) [M-H-C$_3$O$_2$].
Esculetin (14)

\(^1\)H NMR: \(\delta = 7.77\) (d, \(J(H, H) = 9.21\) Hz, 1H; CH), \(\delta = 6.94\) (s, 1H; CH), \(\delta = 6.76\) (s, 1H; CH), \(\delta = 6.18\) ppm (d, \(J(H, H) = 9.21\) Hz, 1H; CH).

ESI-FTMS: \(m/z\): 177.0191 [M-H]; (−)-ESI-MS\(^2\) (25 eV); \(m/z\) (%): 177.0193 (14) [M-H], 149.0244 (7) [M-H-CO], 133.0296 (100) [M-H-CO\(_2\)], 105.0347 (8) [M-H-C\(_2\)O], 89.0398 (3) [M-H-C\(_2\)O\(_3\)].
4-(3,4-Dihydroxyphenyl)-butan-2-one (16)

$^1H$ NMR: δ = 6.65 (d, $^3J(H, H) = 7.89$ Hz, 1H; CH), δ = 6.61 (s, 1H; CH), δ = 6.48 (d, $^3J(H, H) = 7.9$ Hz, 1H; CH), δ = 3.31 (t, $^3J(H, H) = 6.58$ Hz, 2H; CH$_2$), δ = 2.71 (t, $^3J(H, H) = 6.58$ Hz, 2H; CH$_2$), δ = 2.10 ppm (s, 3H, CH$_3$).

ESI-FTMS: $m/z$: 179.0714 [M-H]; (−)-ESI-MS$^2$ (25 eV): $m/z$ (%): 179.0715 (42) [M-H], 121.0297 (100) [M-H-C$_3$H$_6$O], 109.0297 (14) [M-H-C$_4$H$_5$O].