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Supporting Information

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Asymmetric Bioreduction of Activated Alkenes Using Cloned 12-Oxophytodienoate Reductase Isoenzymes OPR-1 and OPR-3 from *Lycopersicon esculentum* (Tomato): A Striking Switch of Stereopreference.

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Experimental

General:

Citral, 2-methylcyclopentanone and levodione were kindly provided by BASF (Ludwigshafen), (*R*)- and (*S*)-citronellal, (*R*)-3-methylcyclopentanone, *N*-phenyl-2-methylmaleimide, (*S*)-methylsuccinic acid, NAD^+, ammonium formate and boron trifluoride-methanol solution were from Aldrich. 4-Ketoisophorone was purchased from ABCR, citraconic acid was purchased from Alfa Aesar, 2-methyl-2-cyclopentenone was from Acros, 3-methyl-2-cyclopentenone and glucose were from Fluka, NADH, NADPH and $NADP^+$ were purchased from Biocatalytics, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Biochemica, formate dehydrogenase and glucose dehydrogenase were from Jülich Chiral Solutions.

The open reading frame of *Lycopersicon esculentum* OPR1 was cloned into pET-21a and expressed as a C-terminal hexahistidine tagged protein in *E. coli* BL21 cells. The expressed recombinant protein was purified on a Ni-NTA affinity column (Invitrogen) according to the manufacturer's protocol. *Lycopersicon esculentum* OPR3 was expressed and purified as reported recently.[1]

GC-MS analyses were performed on a HP 6890 Series GC system equipped with a 5973 mass selective detector and a 7683 Series injector using a (5%-phenyl)-methylpolysiloxane capillary column (HP-5Msi, 30 m, 0.25 mm ID, 0.25 µm film). GC-FID analyses were carried

out on a Varian 3800 using H_2 as carrier gas (14.5 psi). HPLC analyses were performed using a Shimadzu system equipped with a Chiralcel OD-H column (25 cm, 0.46 cm). Circular dichroism spectra were measured on a JASCO spectropolarimeter J-715. NMR spectra were measured on a Bruker AMX spectrometer at 360 MHz.

Synthesis of substrates and reference material:

(6*R*)-Levodione (4b): Ketoisophorone was reduced to levodione using baker's yeast yielding the (6*R*)-enantiomer[2] in 62% e.e.

Methylsuccinic acid (5b): Citraconic acid (**5a**, 105 mg, 0.81 mmol) was dissolved in THF/EtOH 50:50 (10 mL) and was hydrogenated at atmospheric pressure at room temperature in presence of 10% Pd/C (5 mg) as catalyst. After 24h, the mixture was filtered through Celite and evaporated yielding 99% of *rac*-**5b** (106 mg, 0.80 mmol). ^{1}H-NMR (D_2O): δ 1.10-1.12 (d, 3H, J=7.2 Hz), 2.44-2.63 (m, 2H), 2.76-2.82 (m, 1H).

***N*-Phenyl-2-methylsuccinimide (6b)**: *N*-Phenyl-2-methylmaleimide (**6a**, 50 mg, 0.27 mmol) was dissolved in in ethyl acetate (5 mL) and was hydrogenated at atmospheric pressure at room temperature using 10% Pd/C (2.8 mg) as catalyst. After 24h, the mixture was filtered through Celite and evaporated yielding 94% of *rac*-**6b** (48 mg, 0.25 mmol). ^{1}H NMR ($CDCl_3$): δ 1.47 (d, 3H, J=7 Hz), 2.52 (dd, 1H, J=17.4 Hz, J=4 Hz), 3.01-3.10 (m, 1H), 3.11 (dd, 1H, J=17.3 Hz, J=9.2 Hz), 7.29-7.51 (m, 5H).

l-Nitro-2-phenylpropene (7a): To a stirred mixture of acetic anhydride (40 mL) and 65% nitric acid (5.28 g) was added 2-phenylpropene (3.2 mL, 12.2 mmol) at 0 °C. After 20 min, the solution was poured into water (180 mL) and stirred for additional 30 min. The organic layer was washed with sat. aq. $NaHCO_3$, water and then dried (Na_2SO_4). Removal of the solvent under reduced pressure gave an oily residue of crude 2-acetoxy-l-nitro-2-phenylpropane, which was used without purification. A solution of the nitro acetate in triethylamine (15 mL) and chloroform (30 mL) was stirred for 3 h at room temperature. After the addition of 2 N HCl (30 mL), the mixture was extracted with dichloromethane and dried (Na_2S0_4). Evaporation of the solvent followed by silica gel chromatography (eluent hexane/ethyl acetate, 20:1) afforded **7a** in 25% yield. ^{1}H NMR ($CDCl_3$): δ 2.66 (d, 3H, J=l.3 Hz), 7.32 (d, 1H, J=l.3 Hz), 7.46 (s, 5H). The (*E*)-configuration (>95%) was proven by NMR as follows: Gradient-selected quantitative J-type HMBC spectra were acquired on a Bruker Avance 500 MHz NMR spectrometer equipped with a HCN triple-resonance probe and z-axis gradients at 298K. $^3J_{HC}$ Coupling constants between H-x and C-3 / C-Ar were obtained through a series of quantitative J-type HMBC experiments. The delay for the evolution of the

long-range couplings was varied between 30 and 250 ms in steps of 10 ms and the coupling constants were obtained by finding the minimum peak intensity in this series. $^{3}J_{H1C3}$ = 6.0 ± 0.3 Hz, $^{3}J_{H1CAr} \leq$ 5.2 Hz.

1-Nitro-2-phenylpropane (7b): Trans-β-nitrostyrene (0.45 g, 3 mmol) in 20 mL dry ether was added to methylmagnesium iodide (5 mL of a 3 M solution, 15 mmol) in 40 mL of ether at -20 °C. Within 10 min, the solution was added to ice cold 5% aqueous HCl solution and stirred for 30 min. The solution was extracted with CH_2Cl_2, dried over $MgSO_4$, filtered and the solvent was evaporated to give **7b** in 22% yield. ^{1}H NMR ($CDCl_3$): δ 1.4 (d, 3H, J=6.9 Hz), 3.62-3.72 (m, 1H), 4.51-4.60 (m, 2H), 7.24-7.38 (m, 5H).

*General procedure for the enzymatic bioreduction of substrates **1a**-**7a**:*

An aliquot of OPR1 or OPR3 (protein purity >90%, protein content 62-190 μg/mL) was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM) and the cofactor NADH or NADPH (15 mM). *N*-Phenyl-2-methylmaleimide (**6a**) was added as a 10% DMF sol. (1% final conc.) to overcome its poor solubility in water. The mixture was shaken at 30 °C and 140 rpm. After 48h, products were extracted with EtOAc (2 x 0.5 mL) containing 0.05% (v/v) of 1-octanol (for **1a**/**1b**) or (*R*)-limonene (for **2a**/**2b**-**7a**/**7b**) as internal GC standard. The combined organic phases were dried (Na_2SO_4) and the resulting samples were analyzed on achiral GC. Products were identified by comparison with authentic reference materials (which were either commercially available or were independently synthesized) via co-injection on GC-MS and achiral GC.

General procedure for cofactor recycling:

OPR1 or OPR3 were added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM), the oxidized form of the cofactor (NAD^+ or $NADP^+$, 100 μM), the cosubstrate (ammonium formate, glucose or glucose-6-phosphate, 20 mM) and the corresponding recycling enzyme (formate dehydrogenase, glucose dehydrogenase or glucose-6-phosphate dehydrogenase, 10 U). The mixture was shaken at 30 °C and 140 rpm for 24 h and worked up as described above. Cofactor-recycling of NAD^+ or $NADP^+$ using GDH or G6PDH, resp., was performed in presence of $MgCl_2$ (5mM) with substrate **5a**.

Analytical Procedures

Determination of conversion:

Citronellal (**1b**): Products were analyzed by GC-FID using a PEG-phase capillary column (Varian CP-Wax 52 CB, 30 m, 0.25 mm, 0.25 μm), detector temperature 250 °C, split ratio 20:1. Program: 100 °C, hold for 2 min, 15 °C/min to 240 °C, hold for 10 min. Retention times were as follows: citronellal 5.21 min, neral 7.10 min, geranial 7.53 min. Conversions of 2-methylcyclopentenone (**2a**), 3-methylcyclopentenone (**3a**), ketoisophorone (**4a**), citraconic acid (**5a**, analysed as dimethyl ester, see below), *N*-phenyl-2-methylmaleimide (**6a**) and 1-nitro-2-phenylpropene (**7a**) were analyzed using a 6% cyanopropyl-phenyl phase capillary column (Varian CP-1301, 30 m, 0.25 mm, 0.25 μm), detector temperature 250 °C, split ratio 30:1. Temperature program for **2a** and **3a**: 80 °C hold 10 min, 30 °C/min to 200 °C, hold 2 min. Retention times: **2b** 4.25 min, **2a** 5.82 min, **3b** 4.44 min, **3a** 8.77 min. Temperature program for ketoisophorone **4a**: 110 °C hold 5 min, 30 °C/min to 200 °C, hold 2 min. Retention times: **4a** 6.78 min and **4b** 7.28 min. Citraconic acid (**5a**) was analyzed as the corresponding dimethyl ester after derivatization using boron trifluoride-methanol solution: Boron trifluoride-methanol solution (14%, 0.5 mL) was added to the freeze-dried sample, the mixture was stired at 100 °C for 1 h and the reaction was quenched by addition of H_2O (0.5 mL). After extraction with *n*-hexane, the organic layer was dried over Na_2SO_4. Temperature program for citraconic acid (as dimethylester): 80 °C hold 2 min, 20 °C/min to 160 °C, 30 °C/min to 220 °C hold 2 min. Retention times: methylsuccinic acid dimethylester 5.82 min and citraconic acid dimethylester 6.21 min. Temperature program for *N*-phenyl-2-methylmaleimide **6a**: 110 °C hold 2 min, 30 °C/min to 210 °C, hold 6 min. Retention times: **6a** 8.78 min and **6b** 9.90 min. Temperature program for 1-nitro-2-phenylpropene (**7a**): 120 °C hold 3 min, 10 °C/min to 180 °C, 20 °C/min to 220 °C, hold 2 min. Retention times: **7b** 8.88 min and **7a** 9.56 and 10.27 min (*E/Z*-isomers).

Determination of enantiomeric excess and absolute configuration:

Citronellal (**1b**): The enantiomeric excess was determined using a modified β-cyclodextrin capillary column (Hydrodex-β-TBDAc, 25 m, 0.25 mm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 20:1. Temperature program for **1b**: 40 °C hold 2 min, 4 °C/min to 120 °C, hold 1 min, 20 °C/min to 180 °C, hold 3 min. Retention times: (*S*)-**1b** and (*R*)-**1b** 19.84 and 19.97 min, resp. Enantiomeric excess of **2b**, **3b** and **5b** was determined using a modified β-cyclodextrin capillary column (Chiraldex B-TA, 40 m, 0.25 mm). Detector temperature 200 °C, injector temperature 180 °C, split ratio 25:1. Temperature

program for **2b**: 70 °C hold 8 min, 10 °C/min to 80 °C, hold 2 min, 30 °C/min to 180 °C, hold 2 min. Retention times: (*R*)-**2b** and (*S*)-**2b** 10.35 and 10.62 min, resp. Temperature program for **3b**: 70 °C hold 10 min, 30 °C/min to 180 °C, hold 4 min. Retention times: (*S*)-**3b** and (*R*)-**3b** 11.19 and 11.27 min, resp. Temperature program for **5b** (as dimethylester): 90 °C hold 4 min, 3 °C/min to 115 °C, 30 °C/min to 180 °C. Retention times: (*S*)- and (*R*)-methylsuccinic acid dimethylester 8.51 and 8.63 min, resp. Enantiomeric excess of **4b** and **7b** was determined using a β-cyclodextrin capillary column (CP-Chirasil-DEX CB, 25 m, 0.32 mm, 0.25 μm film). Temperature program for **4b**: 90 °C hold 2 min, 4 °C/min to 115 °C, 20 °C/min to 180 °C, hold 2 min. Retention times: (*R*)-**4b** and (*S*)-**4b** 6.42 and 6.74 min, resp. Temperature program for **7b**: 105 °C hold 5 min, 1 °C/min to 115 °C, hold 1 min, 20 °C/min to 180 °C, hold 2 min. Retention times: (*S*)-**7b** and (*R*)-**7b** 7.90 and 8.08 min, resp. The absolute configuration was determined by co-injection with reference materials of known absolute configuration.[3] The enantiomeric excess of **6b** was determined on HPLC using *n*-heptane/ EtOH 95:5 (isocratic) at 18 °C. Retention times: (*R*)-**6b** and (*S*)-**6b** 27.15 min and 29.10 min, resp. The absolute configuration of **6b** was determined via comparison of the CD spectra using independently synthesised reference material. Enantio-enriched **6b** (16 mg) obtained by reduction of **6a** using OPR1 was dissolved in $CHCl_3$ (3 mL) and the solution was analysed on a spectropolarimeter in a 5mm plexiglas cuvette. The scan was performed between 300 and 230 nm. CD $[\theta]_{272}$ –14, the (*R*) configuration[4] was attributed to the pure enantiomer (verified by HPLC), the racemate gave a flat baseline.

References

[1] C. Breithaupt, R. Kurzbauer, H. Lilie, A. Schaller, J. Strassner, R. Huber, P. Macheroux, T. Clausen, *Proc. Natl. Acad. Sci. USA* **2006** *103*, 14337-14342.

[2] H. G. W. Leuenberger, W. Boguth, E. Widmer, R. Zell, *Helv. Chim. Acta* **1976**, *59*, 1832-1849.

[3] V. Bertolasi, O. Bortolini, M. Fogagnolo, G. Fantin, P. Pedrini, *Tetrahedron: Asymmetry* **2001**, *12*, 1479-1483.

[4] T. Hirata, A. Takarada, M. E. F. Hegazy, Y. Sato, A. Matsushima, Y. Kondo, A. Matsuki, H. Hamada, *J. Mol. Catal. B: Enzym.* **2005**, *32*, 131-134.