Eur. J. Org. Chem. **2008** · © WILEY-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2008 · ISSN 1434–193X

SUPPORTING INFORMATION

Title: Asymmetric Bioreduction of Activated C=C Bonds Using *Zymomonas mobilis* NCR Enoate Reductase and Old Yellow Enzymes OYE 1–3 from Yeasts

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Ref. No.: O200701208

Purification of "Old Yellow Enzyme" from baker's yeast

Homogenization: 600g Baker's yeast (commercial source) was dissolved in homogenisation buffer (25mM Tris/HCl, pH 8.0, 5mM EDTA, 2µM NADH/NADPH and tablets of Complete (protease inhibitor mix). The suspension was filtered (1160mL) and homogenized twice at 1500bar (Mikrofluidizer, Mikrofluidics). After washing of the equipment with 500mL buffer, 1700mL homogenate were obtained and centrifuged for 30 min at 12000 rpm (Sorvall) and 4 °C. The supernatant (1500 mL) were adjusted to pH 7.5 (conductivity 4.8 mS/cm) and stored at -20 °C in 150mL portions.

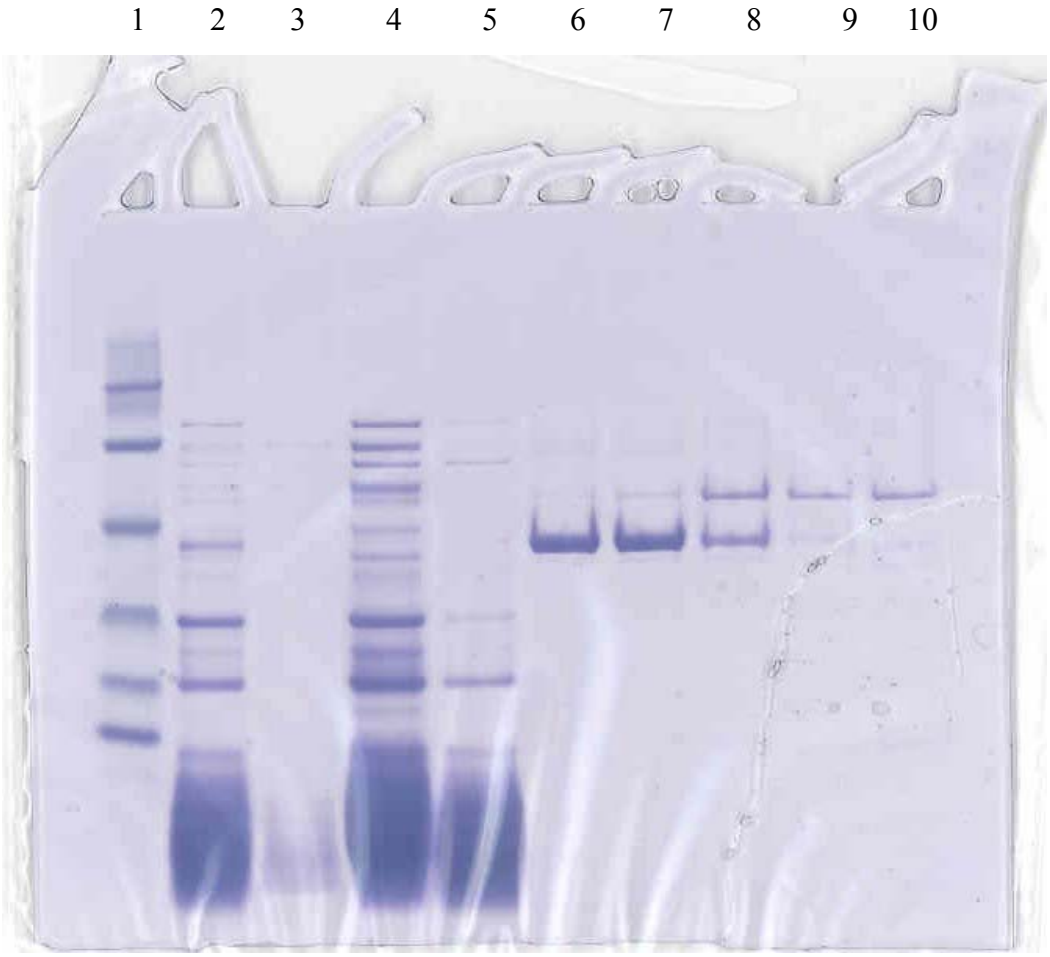
Ion exchange chromatography (Q-Sepharose FF): A Q-Sepharose FF column (Pharmaccia, 400mL volume) was washed with 25 mM Tris/HCl, pH 8.0, 2 mM EDTA, 2 µM NADP/NADPH (buffer A). A portion of the raw homogenate was diluted with water to 380 mL (conductivity 2.6 mS/cm) and applied to the column. The column was then washed with 700mL buffer A (10 mL/min) and eluted with a linear gradient to 100% buffer B (buffer A with 500 mM NaCl) during 100 min. Buffer B was applied for further 50 min. Fractions 71 to 90 were used for further purification. In total, two homogenates were processed to this step.

Hydrophobic interaction chromatography (TSK-Phenyl): The collected fractions of two ion exchange chromatographs (702 mg protein) were adjusted to 30% saturation with $(NH_4)_2SO_4$ (pH 7.0, 320 mL). A TSK-Phenyl (Tosoh, 2.6cm inner diameter, total volume 200 mL, 10 mL/min) was equilibrated in 20 mM KH_2PO_4 pH 7.0, 30% saturation in $(NH_4)_2SO_4$ and 2 tablets Complete/L. The protein solution was applied to the column and then washed with further 300 mL of this buffer. The column was eluted with a linear gradient of the same buffer lacking $(NH_4)_2SO_4$ (50 min). The elution with this buffer was held for 20 min and then the same buffer with 20% isopropanol was added. Fractions were tested for activity and fractions 66-80 were collected.

Affinity chromatography (4-hydroxybenzoic acid): Synthesis of the column: 50 mL Sepharose (Pharmacia) containing an aminohexanoic acid spacer was washed with 5000 mL water. The filter cake was suspended in water (40mL). 4-Acetoxybenzoic acid (4.2 g) was added in 50 mL DMF. The pH was adjusted to 4.7 (10M NaOH). Then 3.9g EDC were added in 10mL of water during 15 min, while the pH was maintained at 4.7 by addition of HCl. A second volume of 50 mL DMF was added and the solution was agitated at room temperature for 16 h. The beads were

separated on a G2 glass funnel and washed with 1000 mL water/DMF (1:1) followed by a wash with 1 L water. Then the material was suspended in 200 mL of cold $NaHCO_3$. Acetic acid anhydride (2mL) was added. After the deprotection step, the column material was washed with water and suspended in 1000 mM imidazole, pH 7.9 for 1 h, washed again with water and used as above. A 50mL column (internal diameter 2.6 cm) was equilibrated in 100 mM Tris/HCl, pH 8, 2 tablets Complete/l. The active fractions from the preceding column were precipitated with $(NH_4)_2SO_4$ (80%). The turbidity was collected by centrifugation (12000 rpm, Sorvall, 4^{o}C). The pellet was dissolved in a small amount of the equilibration buffer and dialysed intensively (3.5 kDa size exclusion). The volume (18 mL) was applied to the affinity column (2.5m L/min). After washing of the column the elution was started in the same buffer by addition of 10 mM $Na_2S_2O_4$. The sequence of the main band was determined by Edman degradation and was found to be SFVKDFKPQALGDTNLFKPI (Old Yellow Enzyme 1).

Figure S1. SDS Page gel of protein fractions from purification of Old Yellow Enzyme (OYE-1) from baker's yeast.



Lane 1: standard proteins (from top) 113 kDa, 92 kDa, 53 kDa, 35 kDa, 29 kDa, 21,5 kDa; lane 2: active fractions from Q-Sepharose; lane 3: fraction 3; lane 4: fraction 5; lane 5: fraction 6; lane 6: fraction 21; OYE 1 elutes at 50 kDa; lane 7: fraction 22; lane 8: fraction 30; lane 9: fraction 35; lane 10: fraction 40.

Synthetic genes for OYE1 – 3

The amino acid sequences from the three OYEs from *Saccharomyces* were backtranslated into DNA according to the *Escherichia coli* K12 codon usage. The following sequences were obtained by custom DNA-synthesis and ligated into the *p*DHE-vector (relevant restrictions sites are underlined):

OYE1 (Genbank ID Q02899) *Nde*I:

catatgagctttgtgaaagattttaaaccgcaggcgctgggcgataccaacctgtttaaaccgattaaaattggcaacaacgaactgctgcatc
gcgcggtgattccgccgctgacccgcatgcgcgcgctgcatccgggcaacattccgaaccgcgattgggcggtggaatattatacccagc
gcgcgcagcgcccgggcaccatgattattaccgaaggcgcgtttattagcccgcaggcgggcggctatgataacgcgccgggcgtgtgg
agcgaagaacagatggtggaatggaccaaaatttttaacgcgattcatgaaaaaaaaagctttgtgtgggtgcagctgtgggtgctgggctg
ggcggcgtttccggataacctggcgcgcgatggcctgcgctatgatagcgcgagcgataacgtgtttatggatgcggaacaggaagcgaa
agcgaaaaaagcgaacaacccgcagcatagcctgaccaaagatgaaattaaacagtatattaaagaatatgtgcaggcggcgaaaaacag
cattgcggcgggcgcggatggcgtggaaattcatagcgcgaacggctatctgctgaaccagtttctggatccgcatagcaacacccgcacc
gatgaatatggcggcagcattgaaaaccgcgcgcgctttaccctggaagtggtggatgcgctggtggaagcgattggccatgaaaaagtg
ggcctgcgcctgagcccgtatggcgtgtttaacagcatgagcggcggcgcggaaaccggcattgtggcgcagtatgcgtatgtggcgggc
gaactggaaaaacgcgcgaaagcgggcaaacgcctggcgtttgtgcatctggtggaaccgcgcgtgaccaacccgtttctgaccgaagg
cgaaggcgaatatgaaggcggcagcaacgattttgtgtatagcatttggaaaggcccggtgattcgcgcgggcaactttgcgctgcatccg
gaagtggtgcgcgaagaagtgaaagataaacgcaccctgattggctatggccgctttttttattagcaacccggatctggtggatcgcctgga
aaaaggcctgccgctgaacaaatatgatcgcgataccttttatcagatgagcgcgcatggctatattgattatccgacctatgaagaag

*Pst*I

cgctgaaactgggctgggataaaaaatgacttaactgcag

OYE2 (Genbank ID Q03558) *Nde*I:

catatgccgtttgtgaaagattttaaaccgcaggcgctgggcgataccaacctgtttaaaccgattaaaattggcaacaacgaactgctgcatc
gcgcggtgattccgccgctgacccgcatgcgcgcgcagcatccgggcaacattccgaaccgcgattgggcggtggaatattatgcgcagc
gcgcgcagcgcccgggcaccctgattattaccgaaggcacctttccgagcccgcagagcggcggctatgataacgcgccgggcatttgg
agcgaagaacagattaaagaatggaccaaaatttttaaagcgattcatgaaaacaaaagctttgcgtgggtgcagctgtgggtgctgggctg
ggcggcgtttccggataccctggcgcgcgatggcctgcgctatgatagcgcgagcgataacgtgtatatgaacgcggaacaggaagaaa
aagcgaaaaaagcgaacaacccgcagcatagcattaccaaagatgaaattaaacagtatgtgaaagaatatgtgcaggcggcgaaaaaca
gcattgcggcgggcgcggatggcgtggaaattcatagcgcgaacggctatctgctgaaccagtttctggatccgcatagcaacaaccgcac
cgatgaatatggcggcagcattgaaaaccgcgcgcgctttaccctggaagtggtggatgcggtggtggatgcgattggcccggaaaaagt
gggcctgcgcctgagcccgtatggcgtgtttaacagcatgagcggcggcgcggaaaccggcattgtggcgcagtatgcgtatgtgctggg
cgaactggaacgccgcgcgaaagcgggcaaacgcctggcgtttgtgcatctggtggaaccgcgcgtgaccaacccgtttctgaccgaag
gcgaaggcgaatataacggcggcagcaacaaatttgcgtatagcatttggaaaggcccgattattcgcgcgggcaactttgcgctgcatcc
ggaagtggtgcgcgaagaagtgaaagatccgcgcaccctgattggctatggccgctttttttattagcaacccggatctggtggatcgcctgg
aaaaaggcctgccgctgaacaaatatgatcgcgataccttttataaaatgagcgcggaaggctatattgattatccgacctatgaa

*Pst*I

gaagcgctgaaactgggctgggataaaaacatgacttaactgcag

OYE3 (P41816) *Nde*I:

catatgccgtttgtgaaaggctttgaaccgattagcctgcgcgataccaacctgtttgaaccgattaaaattggcaacacccagctggcgcatc
gcgcggtgatgccgccgctgacccgcatgcgcgcgacccatccgggcaacattccgaacaaagaatgggcggcggtgtattatggccag
cgcgcgcagcgcccgggcaccatgattattaccgaaggcacctttattagcccgcaggcgggcggctatgataacgcgccgggcatttgg
agcgatgaacaggtggcggaatggaaaaacatttttctggcgattcatgattgccagagctttgcgtgggtgcagctgtggagcctgggctg
ggcgagctttccggatgtgctggcgcgcgatggcctgcgctatgattgcgcgagcgatcgcgtgtatatgaacgcgaccctgcaggaaaa
agcgaaagatgcgaacaacctggaacatagcctgaccaaagatgatattaaacagtatattaaagattatattcatgcggcgaaaaacagcat
tgcggcgggcgcggatggcgtggaaattcatagcgcgaacggctatctgctgaaccagtttctggatccgcatagcaacaaacgcaccgat
gaatatggcggcaccattgaaaaccgcgcgcgctttaccctggaagtggtggatgcgctgattgaaaccattggcccggaacgcgtgggc
ctgcgcctgagcccgtatggcacctttaacagcatgagcggcggcgcggaaccgggcattattgcgcagtatagctatgtgctgggcgaac
tggaaaaacgcgcgaaagcgggcaaacgcctggcgtttgtgcatctggtggaaccgcgcgtgaccgatccgagcctggtggaaggcga
aggcgaatatagcgaaggcaccaacgattttgcgtatagcatttggaaaggcccgattattcgcgcgggcaactatgcgctgcatccggaa
gtggtgcgcgaacaggtgaaagatccgcgcaccctgattggctatggccgctttttttattagcaacccggatctggtgtatcgcctggaagaa
ggcctgccgctgaacaaatatgatcgcagcacctttataccatgagcgcggaaggctataccgattatccgacctatgaagaagcgg

*Hind*III

tggatctgggctggaacaaaaacaagctt

After transformation of *Escherichia coli* with the *p*DHE-derivatives the recombinant protein was produced as described elsewhere.[1]

Synthesis of substrates and reference materials:

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

2-Methylmaleimide (7a): Ammonium acetate (2g, 26.0 mmol) and citraconic anhydride (0.9 mL, 10 mmol) were added to acetic acid (5 mL) and heated under reflux for 2 h. The solution was cooled down to room temperature and evaporated. After the addition of ice water (20 mL) to the dark syrupy residue, the aqueous phase was extracted with EtOAc (8 x 5 ml) and CH_2Cl_2 (2 x 5 ml). The combined organic phases were evaporated to give a yellow solid that was purified by

flash chromatography on silica gel (EtOAc/light petroleum 1:1) to yield 300 mg of **7a** (27%, 300 mg) as white crystals.[3] Mp. 105 °C; ^{1}H NMR (DMSO): δ 1.93-1.94 (3H, d, J=1.8 Hz), 6.48-6.50 (1H, m), 10.74 (br. s, NH).

***rac*-2-Methylsuccinimide (7b)**: 2-Methylmaleimide (**7a**, 107 mg, 0.96 mmol) was dissolved in THF (10 mL) and was hydrogenated at atmospheric pressure at room temperature in presence of 10% Pd/C (5 mg) as catalyst. After 20h, the mixture was filtered through Celite and evaporated yielding 89% of *rac*-**7b** (97 mg, 0.86 mmol). Mp. 65 °C; ^{1}H NMR (360, DMSO): δ 1.35-1.37 (3H, d, J=7.2 Hz); 2.35-2.42 (1H, m); 2.89-3.00 (2H, m); 8.80 (br. s, NH).

***rac*-*N*-Phenyl-2-methylsuccinimide (8b)**: *N*-Phenyl-2-methylmaleimide (**8a**, 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*-**8b** (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 (9a): 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 nitroacetate 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_2SO_4). Evaporation of the solvent followed by silica gel chromatography (eluent hexane/ethyl acetate, 20:1) afforded *E*-**9a** 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). J-type HMBC: $^3J_{H1C3} = 6.0 \pm 0.3$ Hz, $^3J_{H1CAr} \leq 5.2$ Hz.

1-Nitro-2-phenylpropane (9b): β-Nitrostyrene (0.45 g, 3 mmol) in 20 mL dry ether was added to a solution of 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 **9b** 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).

***rac*-2-Methylsuccinic acid (10b)**: Citraconic acid (**10a**, 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*-methylsuccinic acid **10b** (106 mg, 0.80 mmol). m.p.=110-115 °C. ^{1}H-NMR (D_2O): δ 3.62-3.64 (d, 3H, J=7.2 Hz), 4.96-5.15 (m, 2H), 5.29-5.34 (m, 1H). ^{13}C-NMR (D_2O): δ 18.7, 38.2, 39.6, 179.0, 182.8.[4]

***rac*-Dimethyl 2-methylsuccinate (13b)**: A solution of *rac*-2-methylsuccinic acid (**10b**, 32 mg, 0.24 mmol) in BF_3/MeOH (0.5 mL, 14%) was stirred at 100 °C for 1h. H_2O (0.5mL) was added and the reaction mixture was extracted with *n*-hexane (3 x 1 mL).[5] The combined organic layers were dried over Na_2SO_4, filtered and evaporated, yielding *rac*-**13b** (17 mg, 46%, 0.11 mmol). ^{1}H-NMR ($CDCl_3$): δ 1.22-1.24 (d, 3H, J=7.2 Hz), 2.39-2.45 (dd, 1H, J=6.06 Hz, J=16.52 Hz), 2.72-2.79 (dd, 1H, J=8.15 Hz, J=16.51 Hz), 2.91-2.94 (m, 1H), 3.69 (s, 3H), 3.71 (s, 3H). ^{13}C-NMR ($CDCl_3$): δ 17.0, 35.7, 37.4, 51.7, 51.9, 172.3, 175.7.[6]

Dimethyl esters 13a-15a were synthesized *via* esterification of diacids **10a-12a** using BF_3/MeOH according to the procedure described above. Thus were obtained:

Citraconic acid dimethylester (13a): ^{1}H-NMR ($CDCl_3$): δ 2.06-2.07 (d, 3H, J=1.6 Hz), 3.73 (s, 3H), 3.83 (s, 3H), 5.86-5.87 (d, 1H, J=1.6 Hz). ^{13}C-NMR ($CDCl_3$): δ 20.5, 51.8, 52.4, 120.6, 145.7, 165.4, 169.4.[7]

Itaconic acid dimethylester (14a): ^{1}H-NMR ($CDCl_3$): δ 3.35 (s, 3H), 3.70 (s, 3H), 3.77 (s, 3H), 5.73 (s, 1H), 6.34 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ 37.5, 52.1, 52.2, 128.6, 133.6, 166.6, 171.2.[8]

Mesaconic acid dimethylester (15a): ^{1}H-NMR ($CDCl_3$): δ 2.30 (d, 3H, J=1.4 Hz), 3.78 (s, 3H), 3.81 (s, 3H), 6.79 (d, 1H, J=1.5). ^{13}C-NMR ($CDCl_3$): δ 14.3, 51.7, 52.6, 126.5, 143.7, 166.3, 167.6.[7]

Analytical Procedures for compounds 1a-9a

GC- and HPLC-Analysis

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.

Determination of conversion:

Citronellal (**1b**) and 2-methylcyclohexanone (**3b**) 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 for **1b** and **3b**: 100 °C, hold for 2 min, 15 °C/min to 210 °C, hold for 2.5 min. Retention times were as follows: citronellal (**1b**) 5.21 min, neral (*Z*-**1a**) 7.10 min, geranial (*E*-**1a**) 7.53 min, **3b** 5.21 min and **3a** 7.10 min. Conversions of 2-methylcyclopentenone (**2a**), 3-methylcyclopentenone (**4a**), 3-methylcyclohexenone (**5a**), ketoisophorone (**6a**), *N*-phenyl-2-methylmaleimide (**8a**) and 1-nitro-2-phenylpropene (**9a**) were determined 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**, **4a** and **5a**: 80 °C hold 10 min, 30 °C/min to 200 °C, hold 2 min. Retention times: **2b** 4.25 min, **2a** 5.82 min, **4b** 4.44 min, **4a** 8.77 min, , **5b** 7.45 min, **5a** 10.84 min. Temperature program for ketoisophorone **6a**: 110 °C hold 5 min, 30 °C/min to 200 °C, hold 2 min. Retention times: **6a** 6.78 min and **6b** 7.28 min. Temperature program for *N*-phenyl-2-methylmaleimide **8a**: 110 °C hold 2 min, 30 °C/min to 210 °C, hold 6 min. Retention times: **8a** 8.78 min and **8b** 9.90 min. Temperature program for 1-nitro-2-phenylpropene (**9a**): 120 °C hold 3 min, 10 °C/min to 180 °C, 20 °C/min to 220 °C, hold 2 min. Retention times: **9b** 8.88 min and **9a** 9.56 and 10.27 min (*E/Z*-isomers). Conversion of 2-methylmaleimide **7a** was determined using a modified β-cyclodextrin capillary column (Chiraldex B-TA, 40 m, 0.25 mm), detector temperature 200 °C, split ratio 10:1. Program: 130 °C hold 2 min, 10 °C/min to 160 °C, hold 6 min. Retention times: **7a** 6.15 min, (*S*)-**7b** and (*R*)-**7b** 8.74 and 8.89 min, respectively.

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, respectively. Enantiomeric excesses of **2b**, **3b**, **4b**, **5b** and **7b** were 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, respectively. Temperature program for **3b**: 80 °C hold 2 min, 5 °C/min to 105 °C, 10 °C/min, hold 4 min. Retention times: (*R*)-**3b** and (*S*)-**3b** 6.34 and 6.47 min, respectively. Temperature program for **4b**: 70 °C hold 10 min, 30 °C/min to 180 °C, hold 4 min. Retention times: (*S*)-**4b** and (*R*)-**4b** 11.19 and 11.27 min, respeectively. Temperature program for **5b**: 80 °C hold 17 min, 15 °C/min to 180 °C, hold 2 min. Retention times: (*R*)-**5b** and (*S*)-**5b** 13.61 and 14.83 min, respectively. Temperature program for **7b**: 130 °C hold 2 min, 10 °C/min to 160 °C hold 6 min. Retention times: (*S*)-**7b** and (*R*)-**7b** 8.74 and 8.89 min, respectively. Enantiomeric excess of **6b** and **9b** was determined using a β-cyclodextrin capillary column (CP-Chirasil-DEX CB, 25 m, 0.32 mm, 0.25 μm film). Temperature program for **6b**: 90 °C hold 2 min, 4 °C/min to 115 °C, 20 °C/min to 180 °C, hold 2 min. Retention times: (*R*)-**6b** and (*S*)-**6b** 6.42 and 6.74 min, respectively. Temperature program for **9b**: 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*)-**9b** and (*R*)-**9b** 7.90 and 8.08 min, respectively. The absolute configuration was determined by co-injection with reference materials of known absolute configuration.[9-11] The absolute configuration of **2b** and **3b** was additionally confirmed by comparison of the CD spectra using independently synthesised reference material. Enantioenriched **2b** respectively **3b** were obtained by enzymatic reduction of **2a** respectively **3a** (25 mg) using YqjM.[12] Samples of **2b** and **3b** (20 mg) were dissolved in dioxane (1 mL) and the solution was analysed on a spectropolarimeter in a 1mm plexiglas cuvette. The scan was performed between 330 and 250 nm. **2b** displayed CD $[\theta]_{296}$ +3.2 and was assigned to be (*S*)-configurated [(*S*)-**2b** = $[\theta]_{298}$ +4143].[13] **3b** displayed CD $[\theta]_{291}$ –39.3 and was assigned to be (*R*)-configurated [(*R*)-**3b** = $[\theta]_{286}$ +24.7].[14] The corresponding racemates gave a flat baseline. The enantiomeric excess of **8b** was determined on HPLC using *n*-heptane/EtOH 95:5 (isocratic) at 18 °C. Retention times: (*R*)-**8b** and (*S*)-**8b** 27.15 min and 29.10 min, respectively. The absolute configuration of **7b** and **8b** was determined *via* comparison of the CD spectra using independently synthesised reference material. Enantioenriched **7b** (13 mg) respectively **8b** (16 mg) obtained by reduction of **7a** respectively **8a** using OPR1[12] was dissolved in cyclohexane, respectively $CHCl_3$ (3 mL) and the solution was analysed on a spectropolarimeter in a 5mm plexiglass cuvette. The scan was performed between

300 and 230 nm. **7b** displayed CD $[\theta]_{251}$ –5.3, **8b** displayed CD $[\theta]_{272}$ –14, which both were assigned to be (*R*)[15,16] (verified by GC and HPLC, respectively).

Analytical Procedures for compounds 13a-15a

Determination of conversion:

Conversion of citraconic acid dimethylester (**13a**), itaconic acid dimethylester (**14a**) and mesaconic acid dimethylester (**15a**) were determined 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: 80 °C hold 2 min, 20 °C/min to 160 °C, 30 °C/min to 220 °C hold 2 min. Retention times: cf. Table S3.

Determination of enantiomeric excess and absolute configuration:

The enantiomeric excess of **13b** 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 **13b**: 90 °C hold 4 min, 3 °C/min to 115 °C, 30 °C/min to 180 °C. The absolute configuration was determined by co-injection with reference materials of known absolute configuration. Retention times: cf. Table S3.

Table S3. Retention times of substrates and products **13a-15a** and **13b** on GC-columns.

Compounds	Retention times [min] CP-1301 column	Retention times [min] B-TA column
rac-Dimethyl-2-methylsuccinate (**13b**)	5.82	-
(*S*)-**13b**	-	7.33
(*R*)-**13b**	-	7.45
Citraconic acid dimethylester (**13a**)	6.21	11.71
Itaconic acid dimethylester (**14a**)	6.18	10.12
Mesaconic acid dimethylester (**15a**)	6.23	9.79

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