DOI: 10.1002/adsc.200700458

Asymmetric Bioreduction of C=C Bonds using Enoate Reductases OPR1, OPR3 and YqjM: Enzyme-Based Stereocontrol

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Received: September 19, 2007; Published online: February 6, 2008

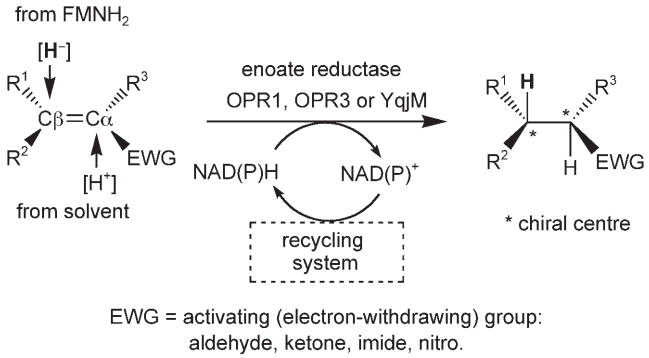
Abstract: Three cloned enoate reductases from the "old yellow enzyme" family of flavoproteins were investigated in the asymmetric bioreduction of activated alkenes. 12-Oxophytodienoate reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum* (tomato), and YqjM from *Bacillus subtilis* displayed a remarkably broad substrate spectrum by reducing α,β-unsaturated aldehydes, ketones, maleimides and nitroalkenes. The reaction proceeded with absolute chemoselectivity – only the conjugated C=C bond was reduced, while isolated olefins and carbonyl groups remained intact – with excellent stereoselectivities (*ee*s up to >99%). Upon reduction of a nitroalkene, the stereochemical outcome could be determined *via* choice of the appropriate enzyme (OPR1 *versus* OPR3 or YqjM), which furnished the corresponding enantiomeric nitroalkanes in excellent *ee*. Molecular modelling suggests that this "enzyme-based stereocontrol" is caused by subtle differences within the active site geometries.

Keywords: asymmetric bioreduction; enoate reductase; nitroalkenes; old yellow enzyme; stereocomplementary process; α,β-unsaturated carbonyl compounds

Introduction

The asymmetric reduction of C=C bonds goes hand in hand with the creation of (up to) two chiral carbon centres and is thus one of the most widely employed strategies for the production of chiral materials. Whereas *cis*-hydrogenation using transition metal-based homogeneous catalysts has been developed to an impressive standard,[1] stereocomplementary asymmetric *trans*-hydrogenation, which resembles the conjugate (Michael-type) addition of a hydride onto a C=C bond bearing an electron-withdrawing substituent is still at the stage of development.[2]

The biocatalytic counterpart for the stereoselective reduction of activated alkenes is catalysed by enoate reductases [EC 1.3.1.X],[3–5] members of the "old yellow enzyme" family.[6] These enzymes are ubiquitous in nature and their catalytic mechanism has been investigated in great detail (Scheme 1).[3,7] The reaction proceeds *via* a ping-pong bi-bi mechanism. First, the flavin cofactor is reduced at the expense of a nicotinamide cofactor NAD(P)H in the "reductive half reaction". In the subsequent "oxidative half reaction" the hydride is transferred onto Cβ of the substrate, while an essential Tyr residue (conserved along the OYE family) adds a proton onto Cα from the opposite side. As a consequence of this mechanism, the ad-



Scheme 1. Asymmetric bioreduction of activated alkenes bearing an electron-withdrawing group (EWG) by enoate reductases.

dition of [H_2] proceeds in a *trans*-fashion with absolute stereospecificity.[8] Overall, the reaction resembles an asymmetric conjugate (Michael-type) addition of a chiral hydride onto an enone and thus, non-activated C=C bonds are completely unreactive.[9,10,18] In order to avoid the requirement for external NAD(P)H recycling, the vast majority of asymmetric C=C-bioreductions were performed using whole fermenting cells, usually baker's yeast.[11] Although the stereoselectivities thus obtained were often excellent, the chemoselectivities with respect to C=C *versus* C=O reduction were severely impeded by the action of competing carbonyl reductases, which not only depleted the substrate (by forming allylic alcohols as dead-end products) but also the product (furnishing saturated alcohols due to "over-reduction"). Since enoate reductases and alcohol dehydrogenases depend on the same cofactor, redox decoupling of both enzyme activities is hardly possible. Just recently, this major drawback was circumvented by the use of cloned enoate reductases from various yeasts.[12,13]

The electron-withdrawing substituent attached to the C=C bond (EWG, Scheme 1) usually consists of a carbonyl group, such as an aldehyde, ketone, carboxylic acid, ester, anhydride, lactone or imide.[5] Due to the electronic similarity of the nitro and the carboxy groups, it is not surprising that also nitroolefins are sufficiently activated. The remarkable catalytic promiscuity[14] of enoate reductases was recently demonstrated by the *trans*-bioreduction of ynones.[15] In search for (oxygen-)stable enoate reductases possessing a broad substrate tolerance, we recently investigated 12-oxophytodienoate reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum* (tomato).[16] Herein we report on the substrate tolerance of OPR1, OPR3 and the "old yellow enzyme" homologue YqjM from *Bacillus subtilis,* which is involved in the oxidative stress response.[17] In order to obtain reasonably broad information on the substrate selectivity pattern, substrates were selected for the (i) nature of the activating group, (ii) chain-length or ring size and (iii) relative position of substituents at the C=C bond, and (iv) the influence of the cofactor type (NADH *vs.* NADPH) on activity or selectivity.

Results and Discussion

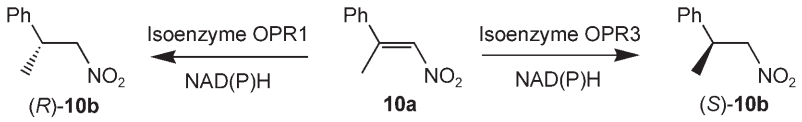
Due to the presence of multiple C=C and C=O bonds, the bioreduction of citral (**1a**) in a chemo-, regio-, and stereoselective fashion represents a challenging task. OPR1, OPR3 and YqjM reduced the α,β-unsaturated C=C-moiety in a highly selective fashion at the expense of NADH or NADPH to yield (*S*)-citronellal (**1b**) in >95% *ee*. (Table 1, entry 1). Both the non-activated C=C bond and the aldehyde moiety remained untouched, which is in contrast to whole-cell bioreduction.[10,18] However, when NADH was recycled using the FDH/formate system, the aldehyde moiety was rapidly reduced to furnish (*S*)-citronellol (3,7-dimethyloct-6-ene-1-ol, *ee* >95%) and nerol (3,7-dimethylocta-2,6-dien-1-ol) as the major products (entry 2). This drawback – presumably caused by *prim*-ADH activities present in commercial FDH preparations [crude NAD^+-specific FDH preparations from Jülich Fine Chemicals (No. 09.11 and 24.11)] – was successfully circumvented by switching to the GDH/glucose system (entry 3). Alternatively, NADPH could be recycled *via* G6PDH/glucose 6-phosphate (entry 4). The short-chain (*E*)-2-methypentenal (**2a**) was selected as an α-substituted α,β-unsaturated aldehyde analogue; this substrate was reduced with poor stereoselectivity by the three enzymes, although with opposite stereoselectivities (entry 5): whereas OPR1 furnished (*R*)-2-methylpentanal (**2b**) in moderate *ee* (47%), OPR3 produced the opposite enantiomer with poor stereoselectivity (19% *ee*). With YqjM, the saturated aldehyde (*R*)-**2b** was obtained almost racemic (10% *ee*). The apparent large selectivity difference between the β- (**1a**) and α-substituted enals (**2a**) might be due to racemisation of the latter catalysed by enoate reductases.[19] A related switch of stereopreference for citral (**1a**) *versus* 2-methylpentenal (**2a**) was recently observed with several OYEs from yeast.[13] Next, α,β-unsaturated cyclic ketones **3a**–**6a** were investigated with respect to the influence of the ring size and the position of the substituent on activity and selectivity. 2-Methylcyclopentenone (**3a**) was reduced to (*S*)-**3b** in reasonable to excellent stereoselectivity by all enzymes in up to 94% *ee* (entries 6–9). At present, the remarkable variation of stereoselectivities depending on the type of cofactor (or recycling system) used cannot be explained on a molecular basis, but similar effects have been observed with related enzymes and are presumably due to "allosteric effects".[12,20] Much to our surprise, the ring size of the substrate had a tremendous effect on the stereochemical outcome of the bioreduction. In contrast to **3a**/(*S*)-**3b**, a switch of stereopreference was observed when reducing 2-methylcyclohexenone (**4a**), where all enzymes furnished (*R*)-**4b** with up to 93% *ee* (entries 10–13). This effect was most pronounced with YqjM using the $NADP^+$/G6PDH recycling system, which furnished (*S*)-**3b** and (*R*)-**4b** in 94% and 93% *ee*, respectively. In order to verify this unexpected steric effect of an additional CH_2 moiety on the stereopreference, the absolute configuration of **3b** and **4b** was double-checked using various methods (see Experimental Section) and verified by molecular modelling (data from docking studies not shown).

The position of the methyl substituent at the C=C bond turned out to be crucial: Whereas both α-substituted cycloalkenones (**3a**, **4a**) were quickly converted with good stereoselectivities (entries 6–13), the corre-

Table 1. Asymmetric bioreduction of activated alkenes **1a–10a** by enoate reductases OPR1, OPR3 and YqjM.

Entry	Substrate	Product	Cofactor	OPR1		OPR3		YqjM	
				Conv.%	ee%	Conv.%	ee%	Conv.%	ee%
1			NADH or NADPH	>99	(*S*)>95	90	(*S*)>95	70	(*S*)>95
2			NAD$^+$/FDH[a]	<5	-	<5	-	-	
3	**1a**	(*S*)-**1b**	NAD$^+$/GDH	79	(*S*)>95	96	(*S*)>95	59	(*S*)>95
4			NADP$^+$/G6PDH	15	(*S*)>95	95	(*S*)>95	57	(*S*)>95
5	**2a**	(*R*)- or (*S*)-**2b**	NADH[b]	96	(*R*) 47	70	(*S*) 19	78	(*R*) 10
6			NADH	58	(*S*) 61	27	(*S*) 45	50	(*S*) 55
7			NADPH	45	(*S*) 64	19	(*S*) 45	>99	(*S*) 66
8	**3a**	(*S*)-**3b**	NAD$^+$/GDH	82	(*S*) 63	38	(*S*) 64	>99	(*S*) 92
9			NADP$^+$/G6PDH	14	(*S*) 61	10	(*S*) 58	72	(*S*) 94
10			NADH	79	(*R*) 77	92	(*R*) 62	61	(*R*) 77
11			NADPH	78	(*R*) 70	87	(*R*) 61	51	(*R*) 71
12	**4a**	(*R*)-**4b**	NAD$^+$/FDH	93	(*R*) 75	95	(*R*) 68	90	(*R*) 84
13			NADP$^+$/G6PDH	82	(*R*) 66	92	(*R*) 71	95	(*R*) 93
14			NADH	n.c.	-	1	(*S*)>99	0.5	(*S*) 59
15			NADPH	n.c.	-	2	(*S*)>99	n.c.	-
16	**5a**	(*S*)-**5b**	NAD$^+$/FDH	n.d.	-	5	(*S*)>99	1	(*S*) 64
17			NADP$^+$/G6PDH	n.d.	-	1	(*S*)>99	n.d.	-
18			NADH	n.c.	-	3	(*S*)>99	n.c.	-
19			NADPH	n.c.	-	2	(*S*)>99	n.c.	-
20	**6a**	(*S*)-**6b**	NAD$^+$/FDH	n.d.	-	11	(*S*)>99	n.d.	-
21			NADP$^+$/G6PDH	n.d.	-	1	(*S*)>99	n.d.	-
22			NADH	>98	(*R*) 51	85	(*R*) 43	95	(*R*) 42
23			NADPH	>95	(*R*) 52	77	(*R*) 33	86	(*R*) 37
24	**7a**	(*R*)-**7b**	NAD$^+$/FDH	>95	(*R*) 51	>95	(*R*) 52	96	(*R*) 47
25			NADP$^+$/G6PDH	>95	(*R*) 91	>95	(*R*) 99	91	(*R*) 99
26			NADH	>99	(*R*) 99	>99	(*R*) 99	>99	(*R*) 99
27			NADPH	>99	(*R*) 99	>99	(*R*) 99	>99	(*R*) 99
28	**8a**	(*R*)-**8b**	NAD$^+$/FDH	>99	(*R*) 99	>99	(*R*) 99	>99	(*R*) 99
29			NADP$^+$/G6PDH	>99	(*R*) 99	>99	(*R*) 99	>99	(*R*) 99
30			NADH	99	(*R*)>99	99	(*R*)>99	99	(*R*)>99
31			NADPH	99	(*R*)>99	99	(*R*)>99	98	(*R*)>99
32	**9a**	(*R*)-**9b**	NAD$^+$/FDH	99	(*R*) 97	99	(*R*) 92	99	(*R*) 92
33			NADP$^+$/G6PDH	99	(*R*) 96	99	(*R*) 97	99	(*R*) 96
34			NADH	>99	(*R*) 97	69	(*S*) 82	94	(*S*) 92
35			NADPH	>99	(*R*) 96	72	(*S*) 87	85	(*S*) 70
36	**10a**	(*R*)- or (*S*)-**10b**	NAD$^+$/FDH	>90	(*R*) 95	40	(*S*) 75	50	(*S*) 85
37			NADP$^+$/G6PDH	90	(*R*) 98	75	(*S*) 93	47	(S) 84

[a] (*S*)-Citronellol (3,7-dimethyloct-6-ene-1-ol, *ee*>95%) and nerol (3,7-dimethylocta-2,6-dien-1-ol) were the major products as a result of competing carbonyl-reduction.
[b] Due to the poor stereoselectivity, additional cofactor systems were not investigated.



Scheme 2. Enzyme-based stereocontrol.

sponding β-analogues (**5a**, **6a**) turned out to be "difficult" substrates (entries 14–21). They were not (or only marginally) transformed by OPR1 or YqjM, also OPR3 showed low activities, albeit with excellent stereoselectivity (*ee* > 99%).

The reduction of ketoisophorone (**7a**) to levodione (**7b**) was of special interest, since the latter is an important building block for the synthesis of zeaxanthin on an industrial scale (entries 22–25).[21] With all three enzymes, **7a** was chemoselectively reduced to (*R*)-**7b** in up to > 99% *ee*, again, the type of cofactor or recycling system seems to play an important role on the stereoselectivity.

α-Methylmaleimides **8a** and **9a** turned out to be excellent substrates, which were readily reduced to the saturated counterparts (**8b**, **9b**) by all three enzymes (entries 26–33). However, our expectation that the stereoselectivity could be altered (or possibly even switched) *via* substrate engineering[22] through variation of the size of the N substituent, i.e., N–H *versus* N–Ph, failed: both products showed the same (*R*)-configuration and were formed in 92 to > 99% *ee*.

Nitroalkenes are readily reduced by enoate reductases forming chiral nitroalkanes, which can be easily converted to useful intermediates.[23] Due to the acidity of the Cα-proton, α-substituted nitroalkanes racemise spontaneously,[24] thus 1-nitro-2-phenylpropene (**10a**) was chosen as model substrate (entries 34–37). Much to our surprise, a striking switch of stereopreference was observed (Scheme 2): while OPR1 reduced the substrate to the corresponding (*R*)-product **10b** with excellent stereoselectivity (*ee* up to 98%), OPR3 and YqjM delivered the opposite enantiomer in up to 93% *ee*. This reversal of stereoselectivity is remarkable bearing in mind that the active site architectures of isozymes OPR1 and OPR3 are highly conserved (55% sequence identity, 70% homology). Although similar "enzyme-based stereocontrol" has been reported before,[25] this complete reversal of stereoselectivity is remarkable in its magnitude. The difference in the $\Delta\Delta G^{\#}$ values[26] accounting for the formation of opposite enantiomers in 99 and 93% *ee*, respectively, amounts to 5.1 kcal M^{-1}, which is derived from the "destruction" of the stereoselectivity for (*R*)-**10b** (99% *ee*, 3.14 kcal M^{-1}) and the "generation" of stereoselectivity in the opposite sense to (*S*)-**10b** (97% *ee*, 1.96 kcal M^{-1}). Furthermore, YqjM surprisingly displayed an enhanced stereoselectivity (along with higher conversion) using NADH as cofactor (*ee* 92%, entry 34), while only 70% *ee* were reached with NADPH (entry 35).

In order to rationalise this switch of stereopreference in the reduction of 1-nitro-2-phenylpropene (**10a**) by isoenzymes OPR1 and OPR3, modelling studies were pereformed based on the crystal structures of both proteins.[27,29] Despite the 55% sequence identity and 70% similarity, they show some subtle, but crucial differences in active site architecture. In both cases, docking calculations with Autodock 3.0[28] yielded clear binding modes for **10a** which differ mainly in the position and orientation of the phenyl group (Figure 1). Whereas the catalytically active resi-

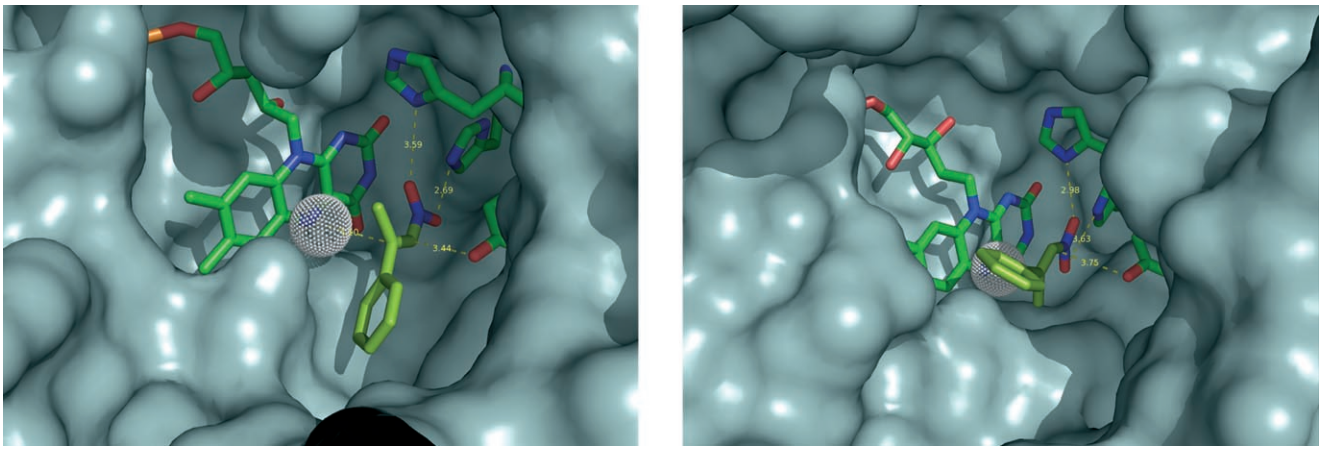


Figure 1. Docking of 1-nitro-2-phenylpropene (**10a**) within the active site of OPR1 (*left*) and OPR3 (*right*). The sphere represents the hydride being added from the flavin onto Cβ of the substrate.

dues, such as the flavin and the O binding site of the substrate are highly conserved throughout the OYE family, loop regions, especially β-strands β3 and β6,[27] which create the specificity site for substrate recognition, show almost no conservation. In OPR1 (Figure 1, *left*), Tyr-78, Lys-79 and Tyr-358 build up the entrance of the active site cavity forming a deep pocket on the bottom that accommodates the phenyl ring of **10a**. In OPR3, however, Lys-79 is altered to Pro-75, which results in a conformational change of this loop and forces Phe-74 (equivalent to Tyr-78 in OPR1) to adopt a different orientation. This conformational change reduces the distance between the two residues (Phe-74 and Tyr-370) flanking the substrate's β-substituent, leading to a pocket with a flat bottom, thus forcing the substrate to turn around and to bind in the opposite orientation: the smaller pocket on the bottom now accommodates the methyl group with the phenyl moiety pointing upwards, so that the hydride attacks the Cβ atom of **10a** from the opposite side of the C=C-bond to furnish the (*S*)-enantiomer (Figure 1, *right*).

Conclusions

Isoenzymes OPR1 and OPR3 of 12-oxophytodienoate reductase from *Lycopersicon esculentum* (tomato), and the "old yellow enzyme" homologue YqjM from *Bacillus subtilis* displayed a remarkably broad substrate spectrum in the asymmetric bioreduction of olefins bearing a carbonyl or nitro group as activating substituent. Thus, enals, enones, maleimides and nitroolefins were reduced with excellent stereoselectivities to the corresponding saturated products at the expense of NADH or NADPH, which were equally well accepted. Cofactor recycling using NADH/glucose/GDH or NADPH/glucose 6-phosphate/G6PDH was successful, but the NADH/formate/FDH system turned out to be plagued by competing carbonyl reduction presumably caused by alcohol dehydrogenases in commercial FDH preparations. In contrast to whole-cell transformations, the reaction showed high chemoselectivity for the conjugated C=C bond, while isolated olefins and carbonyl groups remained untouched. With 1-nitro-2-phenylpropene, isoenzymes OPR1 and OPR3 behaved in a sharp stereocomplementary fashion by producing opposite enantiomeric products.

Experimental Section

General Remarks

Citral (**1a**), 2-methylpentenal (**2a**), 2-methylpentanal (**2b**), 2-methylpentanol, 2-methylcyclopentanone (**3b**), 2-methylcyclohexenone (**4a**) and 2-methylcyclohexanone (**4b**) were provided by BASF (Ludwigshafen), (*R*)- and (*S*)-citronellal (**1b**), (*R*)-3-methylcyclopentanone (**5b**), *N*-phenyl-2-methylmaleimide (**9a**), NAD^+ and ammonium formate were from Aldrich. 4-Ketoisophorone (**7a**) was purchased from ABCR, 2-methyl-2-cyclopentenone (**3a**) was from Acros, citraconic anhydride was purchased from Alfa Aesar, 3-methyl-2-cyclopentenone (**5a**), ammonium acetate 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 an Ni-NTA affinity column (Invitrogen) according to the manufacturer's protocol. *Lycopersicon esculentum* OPR3 and YqjM from *Bacillus subtilis* were expressed and purified as recently reported.[17,29]

GC-MS analyses were performed on an 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 (7b): Ketoisophorone was reduced to levodione using baker's yeast yielding the (6*R*)-enantiomer[21] in 62% *ee*.

2-Methylmaleimide (8a): Ammonium acetate (2 g, 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 (8×5 mL) and CH_2Cl_2 (2×5 mL). The combined organic phases were evaporated to give a yellow solid that was purified by silica flash chromatography (EtOAc/light petroleum, 1:1) to afford **8a** as white crystals; yield: 300 mg (27%);[30] mp 105 °C; ^{1}H NMR (DMSO): $\delta = 1.93$–1.94 (3H, d, $J = 1.8$ Hz), 6.48–6.50 (1H, m), 10.74 (br. s, NH).

***rac*-2-Methylsuccinimide (8b):** 2-Methylmaleimide (**8a**, 107 mg, 0.96 mmol) was dissolved in THF (10 mL) and was hydrogenated at atmospheric pressure at room temperature in the presence of 10% Pd/C (5 mg) as catalyst. After 20 h, the mixture was filtered through Celite and evaporated to afford of *rac*-**8b**; yield: 97 mg (0.86 mmol, 89%); mp 65 °C; ^{1}H NMR (360 MHz, DMSO): $\delta = 1.35$–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 (9b):** *N*-Phenyl-2-methylmaleimide (**9a**, 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 24 h, the mixture was filtered through Celite and evaporated to afford *rac*-**9b**; yield: 48 mg (0.25 mmol, 94%); ^{1}H NMR ($CDCl_3$): $\delta = 1.47$ (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 (10a): 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 saturated aqueous $NaHCO_3$, 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 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 (hexane/ethyl acetate, 20:1) afforded **10a**; yield: 25%; ^{1}H NMR ($CDCl_3$): $\delta = 2.66$ (d, 3H, $J = 1.3$ Hz), 7.32 (d, 1H, $J = 1.3$ Hz), 7.46 (s, 5H). *J*-type HMBC: $^3J_{\text{H-1,C-3}} = 6.0 \pm 0.3$ Hz, $^3J_{\text{H-1,C-Ar}} \leq 5.2$ Hz.

1-Nitro-2-phenylpropane *rac*-(10b): *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 *rac*-**10b**; yield: 22%; ^{1}H NMR ($CDCl_3$): $\delta =$ 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–10a

An aliquot of OPR1, OPR3 or YqjM (protein purity > 90%, protein content 75–275 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 (**8a**) was added as a 0.5 M DMF solution (1% final DMF concentration) to overcome its poor solubility in water. The mixture was shaken at 30 °C and 140 rpm. After 48 h, products were extracted with EtOAc (2 × 0.5 mL) containing 0.05% (v/v) of 1-octanol (for **1a**/**1b**) or (*R*)-limonene (for **3a**/**3b**-**10a**/**10b**) as internal GC standard. The combined organic phases were dried (Na_2SO_4) and the resulting samples were analysed on achiral GC. Products were identified by comparison with authentic reference materials (which were either commercially available or were independently synthesised) *via* co-injection on GC-MS and achiral GC.

General Procedure for Cofactor Recycling

OPR1, OPR3 or YqjM was added to a Tris-HCl buffer solution (0.8 mL, 50 mM, pH 7.5) containing the substrate (5 mM), the oxidised 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.

Analytical Procedures

Determination of conversion: Conversions for citronellal (**1b**) and 2-methylcyclohexanone (**4b**) were analysed 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. Programme: 100 °C, hold for 2 min, 15 °C min^{-1} 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, **4b** 5.21 min and **4a** 7.10 min. Conversions of 2-methylpentenal (**2a**), 2-methylcyclopentenone (**3a**), 3-methylcyclopentenone (**5a**), ketoisophorone (**7a**), *N*-phenyl-2-methylmaleimide (**9a**) and 1-nitro-2-phenylpropene (**10a**) 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 programme for **2a**: 40 °C hold 2 min, 20 °C min^{-1} to 180 °C, hold 1.5 min. Retention times: **2b** 4.47 min and **2a** 5.44 min. Temperature programme for **3a** and **5a**: 80 °C hold 10 min, 30 °C min^{-1} to 200 °C, hold 2 min. Retention times: **3b** 4.25 min, **3a** 5.82 min, **5b** 4.44 min, **5a** 8.77 min. Temperature programme for ketoisophorone **7a**: 110 °C hold 5 min, 30 °C min^{-1} to 200 °C, hold 2 min. Retention times: **7a** 6.78 min and **7b** 7.28 min. Temperature programme for *N*-phenyl-2-methylmaleimide **9a**: 110 °C hold 2 min, 30 °C min^{-1} to 210 °C, hold 6 min. Retention times: **9a** 8.78 min and **9b** 9.90 min. Temperature programme for 1-nitro-2-phenylpropene (**10a**): 120 °C hold 3 min, 10 °C min^{-1} to 180 °C, 20 °C min^{-1} to 220 °C, hold 2 min. Retention times: **10b** 8.88 min and **10a** 9.56 and 10.27 min (*E*/*Z*-isomers).

Conversion of 2-methylmaleimide (**8a**) 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. Programme: 130 °C hold 2 min, 10 °C min^{-1} to 160 °C, hold 6 min. Retention times: **8a** 6.15 min, (*S*)-**8b** and (*R*)-**8b** 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 programme for **1b**: 40 °C hold 2 min, 4 °C min^{-1} to 120 °C, hold 1 min, 20 °C min^{-1} to 180 °C, hold 3 min. Retention times: (*S*)-**1b** and (*R*)-**1b** 19.84 and 19.97 min, respectively.

2-Methylpentanal (**2b**) was analysed as the corresponding *prim*-alcohol (2-methylpentanol) after reduction with $NaBH_4$ in MeOH. The enantiomeric excess was determined using a modified β-cyclodextrin column (Hydrodex-β-6-TBDM, 25 m, 0.25 mm), split ratio 100:1. Temperature programme for 2-methylpentanol: 70 °C hold 9 min, 10 °C min^{-1} to 160 °C, hold 10 min. Retention times: (*R*)- and (*S*)-2-methylpentanol 10.42 and 11.05 min, respectively.

Enantiomeric excesses of **3b**, **4b**, **5b** and **8b** 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 programme for **3b**: 70 °C hold 8 min, 10 °C min^{-1} to

80 °C, hold 2 min, 30 °C min^{-1} to 180 °C, hold 2 min. Retention times: (*R*)-**3b** and (*S*)-**3b** 10.35 and 10.62 min, respectively. Temperature programme for **4b**: 80 °C hold 2 min, 5 °C min^{-1} to 105 °C, 10 °C min^{-1}, hold 4 min. Retention times: (*R*)-**4b** and (*S*)-**4b** 6.34 and 6.47 min, respectively. Temperature programme for **5b**: 70 °C hold 10 min, 30 °C min^{-1} to 180 °C, hold 4 min. Retention times: (*S*)-**5b** and (*R*)-**5b** 11.19 and 11.27 min, respectively. Temperature programme for **8b**: 130 °C hold 2 min, 10 °C min^{-1} to 160 °C hold 6 min. Retention times: (*S*)-**8b** and (*R*)-**8b** 8.74 and 8.89 min, respectively.

The enantiomeric excess of **6b** and **10b** was determined using a β-cyclodextrin capillary column (CP-Chirasil-DEX CB, 25 m, 0.32 mm, 0.25 μm film). Temperature programme for **6b**: 90 °C hold 2 min, 4 °C min^{-1} to 115 °C, 20 °C min^{-1} to 180 °C, hold 2 min. Retention times: (*R*)-**6b** and (*S*)-**6b** 6.42 and 6.74 min, respectively. Temperature programme for **10b**: 105 °C hold 5 min, 1 °C min^{-1} to 115 °C, hold 1 min, 20 °C min^{-1} to 180 °C, hold 2 min. Retention times: (*S*)-**10b** and (*R*)-**10b** 7.90 and 8.08 min, respectively.

The absolute configurations of **1b–7b** were determined by co-injection with reference materials of known absolute configuration.[31–33] The absolute configurations of **3b** and **4b** were additionally confirmed *via* comparison of the CD spectra using independently synthesised reference material. Enantioenriched **3b** or **4b** obtained by reduction of **3a** or **4a** (25 mg), respectively, using YqjM was dissolved in dioxane (1 mL) and the solution was analysed on a spectropolarimeter in a 1 mm plexiglas cuvette. The scan was performed between 330 and 250 nm. **3b** showed CD $[\theta]_{296} = +3.2$ and **4b** displayed CD $[\theta]_{291} = -39.3$, which are in agreement with literature data: (*S*)-**3b** $[\theta]_{298} = +4143$;[34] (*R*)-**4b** $[\theta]_{286} = +24.7$;[35] the corresponding racemates gave a flat baseline.

The enantiomeric excess of **9b** was determined on HPLC using *n*-heptane/EtOH, 95:5 (isocratic) at 18 °C. Retention times: (*R*)-**9b** and (*S*)-**9b** 27.15 min and 29.10 min, respectively. The absolute configurations of **8b** and **9b** were determined *via* comparison of the CD spectra using independently synthesised reference material. Enantioenriched **8b** (13 mg) or **9b** (16 mg) obtained by reduction of **8a** or **9a**, respectively, using OPR1 was dissolved in cyclohexane or, respectively, $CHCl_3$ (3 mL) and the solution was analysed on a spectropolarimeter in a 5 mm plexiglas cuvette. The scan was performed between 300 and 230 nm. **8b** showed CD $[\theta]_{251} = -5.3$ and **9b** showed CD $[\theta]_{272} = -14$, which are in agreement with literature data: (*R*)-**8b** CD $[\theta]_{253} = -840$, (*R*)-**9b** $[\theta]_{272} = -76.9$;[36,37] the corresponding racemates gave a flat baseline.

Acknowledgements

We would like to thank Nicole Müller, Tea Pavkov and Klaus Zangger for their excellent technical assistance.

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