Asymmetric Enzyme Catalysis

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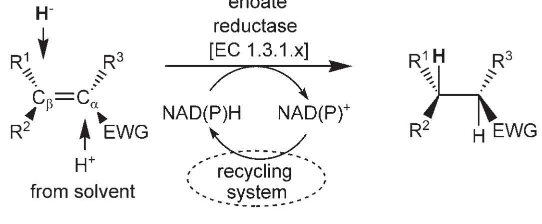
Asymmetric Bioreduction of Activated Alkenes Using Cloned 12-Oxophytodienoate Reductase Isoenzymes OPR-1 and OPR-3 from *Lycopersicon esculentum* (Tomato): A Striking Change of Stereoselectivity**

*Mélanie Hall, Clemens Stueckler, Wolfgang Kroutil, Peter Macheroux, and Kurt Faber**

The asymmetric reduction of C=C bonds goes in hand with the creation of up to two chiral centers and is thus one of the most widely employed strategies for the synthesis of chiral compounds. Whereas *cis* hydrogenation using homogeneous catalysts based on (transition) metals has been developed to a high standard,[1] stereocomplementary *trans* reduction is still at the stage of development.[2,3]

The biocatalytic equivalent of this reaction is catalyzed by enoate reductases [EC 1.3.1.x],[4,5] commonly denoted as the "old yellow enzyme" family.[6] These common enzymes act through transfer of a hydride ion, derived from a flavin cofactor ($FMNH_2$), onto the β-carbon atom of an α,β-unsaturated carbonyl compound, while a proton, derived from the solvent, adds from the opposite side onto the α-carbon atom. As a consequence of this mechanism, the hydrogenation occurs in a *trans*-specific fashion. The catalytic cycle is completed by the reduction of FMN at the expense of NAD(P)H, which is regenerated by an additional redox reaction (Scheme 1). Although the mode of action of these enzymes has been elucidated in great detail, their application in preparative-scale biotransformations has been hampered for several reasons: although the best-studied enzymes from this group, which were isolated from strictly anaerobic bacteria such as *Proteus* and *Clostridium* spp.,[7] were shown to be highly stereoselective, the sensitivity of these proteins towards traces of molecular oxygen prevented their practical application. As a consequence, whole-cell biotransformations using aerobic microorganisms, most prominently baker's yeast,[8] dominated the field. Although the stereoselectivities achieved were often excellent, the chemoselectivity of whole-cell bioreductions with respect to the reduction of C=C against C=O bonds was notoriously plagued by the competing



Scheme 1. Asymmetric bioreduction of activated alkenes bearing an activating electron-withdrawing group (EWG) by enoate reductases. EWG = ketone, aldehyde, carboxylic acid or anhydride, lactone, imide, or nitro.

reduction of the carbonyl group that was catalyzed by alcohol dehydrogenases.[9,10] Since enoate reductases and alcohol dehydrogenases depend on the same nicotinamide cofactor, redox decoupling of both enzyme activities is not possible.

Isolated and/or cloned enoate reductases are required in sufficient amounts, together with a suitable recycling system for the nicotinamide cofactor, to make this biotransformation practically feasible. Only recently, the first successful attempts in the chemo- and stereoselective reduction of conjugated enones were reported by using cloned old yellow enzymes from *Saccharomyces carlsbergensis*[11] and baker's yeast.[12]

Since enoate reductases have been shown to possess a much broader substrate spectrum than their name would suggest,[13] we initiated a search for a candidate that possessed a broad substrate specificity but showed high stereoselectivity towards a wide range of C=C bonds bearing an electron-withdrawing activating group. In this context, we came across 12-oxophytodienoate reductase (OPR) which occurs in several isoforms in plants.[14] Whereas isoenzyme OPR3 is responsible for the reduction of (9*S*,13*S*)-12-oxophytodienoate to the corresponding cyclopentanoic acid OPC-8:0 for the biosynthesis of the plant hormone jasmonic acid,[15] the role of isoenzymes OPR1 and OPR2 remains unknown. The substrate spectrum of OPR1 and OPR3 from *Lycopersicon esculentum* (tomato) was tested using a range of alkenes bearing an electron-withdrawing substituent (Table 1).[16] Although OPRs were reported to show a preference for NADPH,[17] we also tested NADH as the cofactor,[18] since its recycling is generally more facile.[19]

As a result of the presence of multiple C=C and C=O bonds, the asymmetric reduction of citral (**1a**, Table 1) in a chemo-, regio-, and stereoselective fashion is a challenging task.[2,20] Both OPR1 and OPR3 quickly reduced the conjugated C=C-bond in a highly chemo-, regio-, and stereose-

[*] M. Hall, C. Stueckler, Prof. W. Kroutil, Prof. K. Faber
Department of Chemistry, Organic and Bioorganic Chemistry
University of Graz
Heinrichstrasse 28, 8010 Graz (Austria)
Fax: (+43) 316-380-9840
E-mail: kurt.faber@uni-graz.at
Prof. P. Macheroux
Institute of Biochemistry
Graz University of Technology
Petersgasse 12, 8010 Graz (Austria)

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Table 1: Asymmetric bioreduction of activated alkenes using OPR1 and OPR3.

Entry	Substrate	Product	Cofactor[a]	OPR1		OPR3	
				Conv. [%][b]	*ee* [%]	Conv. [%][b]	*ee* [%]
1	**1a**	(*S*)-**1b**	NADH	> 99	(*S*) > 95	90	(*S*) > 95
2			NADPH	> 99	(*S*) > 95	90	(*S*) > 95
3			NAD^+/FDH	< 5[c]	–	< 5[c]	–
4			NAD^+/GDH	20	(*S*) > 95	90	(*S*) > 95
5			$NADP^+$/G6PDH	15	(*S*) > 95	95	(*S*) > 95
6	**2a**	(*S*)-**2b**	NADH	58	(*S*) 61	27	(*S*) 45
7			NADPH	45	(*S*) 64	19	(*S*) 45
8			NAD^+/FDH	88	< 3	65	< 3
9			NAD^+/GDH	69	(*S*) 72	45	(*S*) 67
10			$NADP^+$/G6PDH	14	(*S*) 61	10	(*S*) 58
11	**3a**	(*S*)-**3b**	NADH	nr	–	1	(*S*) > 99
12			NADPH	nr	–	2	(*S*) > 99
13			NAD^+/FDH	nd	–	6	(*S*) > 99
14			$NADP^+$/G6PDH	nd	–	1	(*S*) > 99
15	**4a**	(*R*)-**4b**	NADH	> 98	(*R*) 51	78	(*R*) 55
16			NADPH	> 95	(*R*) 52	77	(*R*) 33
17			NAD^+/FDH	> 95	(*R*) 51	> 95	(*R*) 52
18			$NADP^+$/G6PDH	> 95	(*R*) 91	> 95	(*R*) 99
19	**5a**	(*R*)-**5b**	NADH	> 99	(*R*) > 99	nr	–
20			NADPH	> 99	(*R*) > 99	nr	–
21			NAD^+/GDH[d]	> 99	(*R*) > 99	nd	–
22			$NADP^+$/G6PDH[d]	96	(*R*) > 99	nd	–
23	**6a**	(*R*)-**6b**	NADH	99	(*R*) > 99	99	(*R*) > 99
24			NADPH	99	(*R*) > 99	99	(*R*) > 99
25			NAD^+/FDH	99	(*R*) 97	99	(*R*) 92
26			$NADP^+$/G6PDH	99	(*R*) 96	99	(*R*) 97
27	**7a**	(*R*)- or (*S*)-**7b**	NADH	> 99	(*R*) 97	69	(*S*) 82
28			NADPH	> 99	(*R*) 96	72	(*S*) 87
29			NAD^+/FDH	> 90	(*R*) 95	40	(*S*) 75
30			$NADP^+$/G6PDH	90	(*R*) 98	75	(*S*) 93

[a] NADH/NADPH = stoichiometric amounts of cofactors; NAD^+/FDH = NADH was recycled using formate dehydrogenase/formate; $NADP^+$/G6PDH = NADPH was recycled using glucose 6-phosphate/glucose 6-phosphate dehydrogenase; NAD^+/GDH = NADH was recycled using glucose/glucose dehydrogenase; [b] determined after 48 h by GC or HPLC analysis using an internal standard; nr = no reaction, nd = not determined; [c] carbonyl reduction that gave the unsaturated allylic alcohol prevailed; [d] in presence of Mg^{2+}.

lective fashion to give (*S*)-**1b** in greater than 95% *ee* using either NADH or NADPH as cofactor (Table 1, entries 1 and 2). The nonconjugated olefin and the aldehyde moiety, which is quickly reduced in whole-cell bioreductions,[9] remained untouched. Attempts to recycle NADH using formate dehydrogenase/formate (FDH)[19] failed because of the predominant reduction of the carbonyl group that furnished the corresponding allylic alcohol (3,7-dimethylocta-2,6-dien-1-ol) in greater than 95% yield (Table 1, entry 3). This side reaction was presumably caused by primary-alcohol dehydrogenases, which are present as impurities in commercially supplied FDH preparations. However, the recycling of NADH or NADPH was achieved by using the glucose dehydrogenase/glucose (GDH) and glucose-6-phosphate dehydrogenase/glucose-6-phosphate (G6PDH) systems, respectively (Table 1, entries 4 and 5). Significant differences in the activities and stereoselectivities of OPR1 and OPR3 were observed using the α- and β-substituted 2-cyclopentenones **2a** and **3a**. Substrate **2a** was converted in reasonable rates by both enzymes in up to 72% *ee*, whereas **3a** was not reduced by OPR1 and was only slowly reduced by OPR3, albeit with excellent stereoselectivity (Table 1, entries 11–14). Again, recycling of the NADH cofactor with the FDH system failed because of racemization of the product α-methylcyclopentanone formed ((*S*)-**2b;** Table 1, entry 8); fortunately, the GDH system succeeded again. Encouraged by these results, we tested the reduction of ketoisophorone (**4a**) to the levodione (**4b**), which is an important building block for the synthesis of zeaxanthin on an industrial scale.[10] Both isoenzymes converted **4a** into (*R*)-**4b** smoothly in up to

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99% *ee* in the absence of the competing carbonyl reductions[21] (Table 1, entries 15–18).

The stereoselective reduction of α-methylmaleic acid (citraconic acid, **5a**) failed with OPR3 but succeeded with isoenzyme OPR1, and (*R*)-α-methylsuccinate was obtained with excellent reaction rates and absolute stereoselectivity (>99% *ee*; Table 1, entries 19 and 20), which proved that the carboxylic acid moiety serves as an excellent activating group. The *Z* configuration of **5a** appears to play a crucial role, since its counterpart with the *E* configuration (mesaconic acid) and the *exo*-methylene analogue (itaconic acid) proved to be completely unreactive. With the dicarboxylic acid **5a**, recycling of the cofactor using GDH or G6PDH initially failed completely under standard conditions, which presumably is due to removal of essential metal ions (such as Ca^{2+} or Mg^{2+}) from GDH and G6PDH, respectively, by complexation caused by the dicarboxylic acid. This drawback was efficiently circumvented by addition of Mg^{2+} ions (equimolar to substrate **5a**) to the GDH or G6PDH system (Table 1, entries 21 and 22). An α-substituted maleimide **6a** was investigated to extend the substrate tolerance of OPRs on carboxylic acid derivatives. Again, excellent reaction rates and stereoselectivities were obtained using both enzymes (Table 1, entries 23–26).

As a result of the electronic similarity of the carboxy and nitro groups, nitroalkenes also can be reduced by enoate reductases to furnish the corresponding nitroalkanes.[22] From the two possible chiral centers that are created, only the distant one is configurationally stable, whereas the carbon atom which bears the nitro group undergoes spontaneous epimerization.[23] Bioreduction of 2-phenyl-1-nitropropene (**7a**) gave surprising results: although **7a** was reduced using OPR1 with excellent rates to yield (*R*)-**7b** in greater than 99% *ee*, a complete reversal of stereoselectivity was observed using isoenzyme OPR3, which furnished (*S*)-**7b** in up to 93% *ee*. This rare case of stereocomplementary behavior is particularly remarkable in view of the fact that the isoenzymes OPR1 and OPR3 are structural homologues with active-site architectures that are highly conserved (overall sequence identity 53%). This conservation not only encompasses the carbonyl-binding motif H-X-X-H(N)-X-Y in the active site, but also extends to the amino acid side chain interactions with FMN.[24] Preliminary data from modeling studies based on the crystal structures of OPR1 and OPR3 suggest that this stereochemical reversal is caused by subtle differences in the shape of the active sites.[25] Stereocomplementary behavior of enzymes has been observed in a number of cases;[26] however, the magnitude of the "stereochemical switch" was usually moderate and generally *ee* values no greater than around 80% were achieved that was caused by the logarithmic dependence of the difference in transition energies of stereoisomers on the stereochemical outcome.[27] The difference in the $\Delta\Delta G^{\#}$ values for the transformation of **7a** into (*R*)-**7b** or (*S*)-**7b** in 99 and 93% *ee*, respectively, is remarkably 5.1 kcal M^{-1}.[28]

In contrast to the enoate reductases reported so far that showed a preference for a specific substrate-type,[5] the 12-oxophytodienoate reductase isoenzymes OPR1 and OPR3 from tomato displayed a remarkably broad substrate range for the stereoselective asymmetric bioreduction of α,β-unsaturated enals, enones, dicarboxylic acids, N-substituted maleimides, and nitroalkenes. Although both isoenzymes are structurally closely related, they reduced a nitroalkene in a strict stereocomplementary fashion. The scope of these enzymes for the asymmetric bioreduction of activated alkenes in a stereocomplementary fashion on a preparative scale is currently under investigation.

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[1] a) R. Noyori, *Angew. Chem.* **2002**, *114*, 2108–2123; *Angew. Chem. Int. Ed.* **2002**, *41*, 2008–2022; b) W. S. Knowles, *Angew. Chem.* **2002**, *114*, 2096–2107; *Angew. Chem. Int. Ed.* **2002**, *41*, 1998–2007.

[2] a) J. W. Yang, M. T. Hechavarria Fonseca, N. Vignola, B. List, *Angew. Chem.* **2005**, *117*, 110–112; *Angew. Chem. Int. Ed.* **2005**, *44*, 108–110; b) J. W. Yang, M. T. Hechavarria Fonseca, B. List, *Angew. Chem.* **2004**, *116*, 6829–6832; *Angew. Chem. Int. Ed.* **2004**, *43*, 6660–6662.

[3] N. J. A. Martin, B. List, *J. Am. Chem. Soc.* **2006**, *128*, 13368–13369.

[4] a) R. E. Williams, N. C. Bruce, *Microbiology* **2002**, *148*, 1607–1614; b) S. Steinbacher, M. Stumpf, S. Weinkauf, F. Rohdich, A. Bacher, H. Simon in *Flavins and flavoproteins* (Eds.: S. K. Chapman, R. N. Perham, N. S. Scrutton), Weber, Berlin, **2002**, pp. 941–949.

[5] R. Stuermer, B. Hauer, M. Hall, K. Faber, *Curr. Opin. Chem. Biol.* **2007**, in press.

[6] O. Warburg, W. Christian, *Biochem. Z.* **1933**, *266*, 377–411.

[7] a) B. Rambeck, H. Simon, *Angew. Chem.* **1974**, *86*, 675–676; *Angew. Chem. Int. Ed. Engl.* **1974**, *13*, 609; b) I. Thanos, H. Simon, *Angew. Chem.* **1986**, *98*, 455–456; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 462–463; c) F. Rohdich, A. Wiese, R. Feicht, H. Simon, A. Bacher, *J. Biol. Chem.* **2001**, *276*, 5779–5787.

[8] a) S. Servi, *Synthesis* **1990**, 1–25; b) R. Csuk, B. I. Glänzer, *Chem. Rev.* **1991**, *91*, 49–97.

[9] a) A. Müller, B. Hauer, B. Rosche, *J. Mol. Catal. B* **2006**, *38*, 126–130; b) M. Hall, B. Hauer, R. Stuermer, W. Kroutil, K. Faber, *Tetrahedron: Asymmetry* **2006**, *17*, 3058–3062.

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

[11] M. A. Swiderska, J. D. Stewart, *J. Mol. Catal. B* **2006**, *42*, 52–54.

[12] M. Wada, A. Yoshizumi, Y. Noda, M. Kataoka, S. Shimizu, H. Takagi, S. Nakamori, *Appl. Environ. Microbiol.* **2003**, *69*, 933–937.

[13] The family of flavin-dependent old yellow enzymes represents a quite heterogeneous group of proteins that have been shown to reduce not only conjugated enals, enones, α,β-unsaturated carboxylic acids, and nitroalkenes, but also to reductively cleave nitroesters, and reduce aromatic nitro groups and electron-deficient aromatics; see Ref. [5].

[14] F. Schaller, *J. Exp. Bot.* **2001**, *52*, 11–23.

[15] F. Schaller, C. Biesgen, C. Müssig, T. Altmann, E. W. Weiler, *Planta* **2000**, *210*, 979–984.

[16] General procedures for the asymmetric bioreduction of compounds **1a**–**7a**, for the recycling of cofactors using FDH, GDH, and G6PDH, the sources of enzymes and substrates, as well as

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analytical procedures and proof of absolute configuration of products are given in the Supporting Information.

[17] J. Strassner, A. Fürholz, P. Macheroux, N. Amrhein, A. Schaller, *J. Biol. Chem.* **1999**, *274*, 35067 – 35073.

[18] In contrast to carbonyl reductases that show a rather pronounced preference for either NADH or NADPH, enoate reductases seem to be less specific: their preference for NADH over NADPH may vary significantly, with a ratio of relative activities that range from 0.02:1 to 10:1; see: a) R. E. Williams, D. A. Rathbone, N. S. Scrutton, N. C. Bruce, *Appl. Environ. Microbiol.* **2004**, *70*, 3566 – 3574; b) A. Kurata, T. Kurihara, H. Kamachi, N. Esaki, *Tetrahedron: Asymmetry* **2004**, *15*, 2837 – 2839; c) M. Kataoka, A. Kotaka, A. Hasegawa, M. Wada, A. Yoshizumi, S. Nakamori, S. Shimizu, *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2651 – 2657.

[19] a) H. K. Chenault, G. M. Whitesides, *Appl. Biochem. Biotechnol.* **1987**, *14*, 147; b) U. Kragl, W. Kruse, W. Hummel, C. Wandrey, *Biotechnol. Bioeng.* **1996**, *52*, 309 – 319.

[20] Y. Z. Chen, B. J. Liaw, S. J. Chiang, *Appl. Catal. A* **2005**, *284*, 97 – 104.

[21] E. M. Buque-Taboada, A. J. J. Straathof, J. A. Heijnen, L. A. M. van der Wielen, *Adv. Synth. Catal.* **2005**, *347*, 1147 – 1154.

[22] a) H. Ohta, N. Kobayashi, K. Ozaki, *J. Org. Chem.* **1989**, *54*, 1802 – 1804; b) Y. Meah, V. Massey, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10733 – 10738.

[23] R. R. Bak, A. F. McAnda, A. J. Smallridge, M. A. Trewhella, *Aust. J. Chem.* **1996**, *49*, 1257 – 1260.

[24] 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; C. Breithaupt, J. Strassner, U. Breitinger, R. Huber, P. Macheroux, A. Schaller, T. Clausen, *Structure* **2001**, *9*, 419 – 429.

[25] a) 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.

[26] a) Y. Ijima, K. Matoishi, Y. Terao, N. Doi, H. Yanagawa, H. Ohta, *Chem. Commun.* **2005**, 877 – 879; b) A. O. Magnusson, M. Takwa, A. Hamberg, K. Hult, *Angew. Chem.* **2005**, *117*, 4658 – 4661; *Angew. Chem. Int. Ed.* **2005**, *44*, 4582 – 4585; c) D. Guieysse, G. Sandoval, L. Faure, J. M. Nicaud, P. Monsan, A. Marty, *Tetrahedron: Asymmetry* **2004**, *15*, 3539 – 3543; d) M. T. Reetz, B. Brunner, T. Schneider, F. Schulz, C. M. Clouthier, M. M. Kayser, *Angew. Chem.* **2004**, *116*, 4167 – 4170; *Angew. Chem. Int. Ed.* **2004**, *43*, 4075 – 4078; e) P. F. Mugford, S. M. Lait, B. A. Keay, R. J. Kazlauskas, *ChemBioChem* **2004**, *5*, 980 – 987; f) M. A. Phillips, M. R. Wildung, D. C. Williams, D. C. Hyatt, R. Croteau, *Arch. Biochem. Biophys.* **2003**, *411*, 267 – 276; g) S. Wang, M. M. Kayser, H. Iwaki, P. C. K. Lau, *J. Mol. Catal. B* **2003**, *22*, 211 – 218; h) E. Ferre, M. H. N'Guyen, J. Le Petit, G. Gil, *Biocatal. Biotransform.* **2002**, *20*, 311 – 317; i) W. S. Li, Y. Li, C. H. Hill, K. T. Lum, F. M. Raushel, *J. Am. Chem. Soc.* **2002**, *124*, 3498 – 3499; j) T. Schubert, W. Hummel, M. R. Kula, M. Müller, *Eur. J. Org. Chem.* **2001**, 4181 – 4187; k) D. Zha, S. Wilensek, M. Hermes, K. E. Jaeger, M. T. Reetz, *Chem. Commun.* **2001**, 2664 – 2665; l) T. Hirata, K. Shimoda, T. Gondai, *Chem. Lett.* **2000**, 850 – 851; m) Y. Hirose, K. Kariya, Y. Nakanishi, Y. Kurono, K. Achiwa, *Tetrahedron Lett.* **1995**, *36*, 1063 – 1066.

[27] M. Norin, K. Hult, A. Mattson, T. Norin, *Biocatalysis* **1992**, *7*, 131 – 147.

[28] This value is derived from the difference in energies for the "destruction" of the stereoselectivity for (*R*)-**7b** (99 % *ee*, which amounts to 3.14 kcalM^{-1}) and the "generation" of stereoselectivity in the opposite direction to (*S*)-**7b** (93 % *ee*, which amounts to 1.96 kcalM^{-1}).

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