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Stereocomplementary Bioreduction of α,β-Unsaturated Dicarboxylic Acids and Dimethyl Esters using Enoate Reductases: Enzyme- and Substrate-Based Stereocontrol

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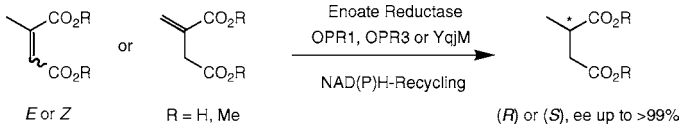
ABSTRACT



Asymmetric bioreduction of α,β-unsaturated dicarboxylic acids, such as 2-methylmaleic/fumaric and 2-methylenesuccinic acid, as well as the corresponding dimethyl esters, using three cloned enoate reductases furnished 2-methylsuccinic acid or dimethyl 2-methylsuccinate, respectively. Opposite stereoisomeric products were obtained in up to >99% ee either by choice of the enzyme or by using *E*/*Z*-configurated substrates. Cofactor-recycling systems (NADH/FDH/formate, NADH/GDH/glucose or NADPH/G6PDH/glucose-6-phosphate) only worked in presence of a divalent metal ion, such as Ca^{2+}, Mg^{2+}, or Zn^{2+}.

The asymmetric conjugate reduction of C=C bonds leads to the creation of up to two stereogenic centers and is thus a key transformation in asymmetric synthesis. Stereoselective bioreduction of activated alkenes is catalyzed by flavin-dependent enoate reductases [EC 1.3.1.X],[1−3] members of the "old yellow enzyme" family[4] at the expense of a nicotinamide cofactor.[1,5] The flavin cofactor transfers a hydride 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 the stereochemistry of this mechanism, the overall addition of [H_2] proceeds in a strict *trans*-fashion. Enoate reductases are able to reduce a wide variety of substrates, such as conjugated enals, enones, α,β-unsaturated carboxylic acids, imides, nitroalkenes, and ynones.[1,3,6,7]

The asymmetric bioreduction of α,β-unsaturated carboxylic acids was predominantly investigated by the group of H. Simon using isolated enzymes or whole cells of strict (or facultative) anaerobes, such as *Clostridium* spp., *Proteus mirabilis*, *Enterobacter agglomerans*, *Acetobacterium woodii*, and *Sporomusa termitida*.[8−11] Although the stereoselectivities reported were impressive, practical application of

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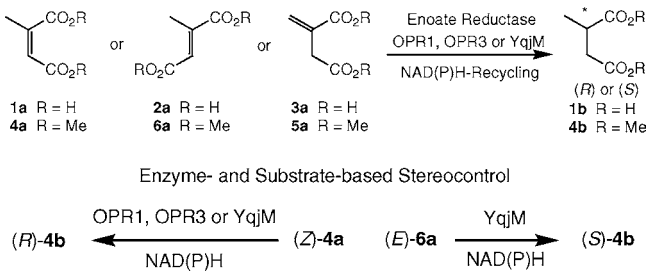
these oxygen-sensitive enzymes/organisms was severely impeded because they required growth and handling of anaerobes and complex electro-microbial or -enzymatic cofactor-recycling systems, based on toxic mediators, such as Paraquat. Overall, these drawbacks rendered industrial applications utopian. During our search for oxygen-stable and durable enoate reductases, we investigated the asymmetric bioreduction of α,β-unsaturated dicarboxylic acids and diesters using 12-oxophytodienoate reductase isoenzymes OPR1 and OPR3 from *Lycopersicum esculentum* (tomato)[6,12] and YqjM from *Bacillus subtilis*.[13]

In order to gain insight into the substrate-selectivity relationship of these enzymes, a structurally related set of diacids, 2-methylmaleic acid ("citraconic acid", **1a**), 2-methylfumaric acid ("mesaconic acid", **2a**), and 2-methylenesuccinic acid ("itaconic acid", **3a**), were chosen, since it was expected that they would yield the same reduction product, 2-methylsuccinic acid (**1b**). The corresponding dimethyl esters (**4a**−**6a**) were tested to compare the activating effects of the carboxyl- versus the alkoxycarbonyl groups (Scheme 1, Table 1).

Scheme 1. Asymmetric Bioreduction of Dicarboxylic Acids **1a**−**3a** and Dimethyl Esters **4a**−**6a**



Substrate **1a** was not converted by OPR3 but was quantitatively reduced to (*R*)-**1b** in >99% ee by OPR1 and YqjM using NADH or NADPH in molar amounts (entries 1 and 2). Much to our surprise, cofactor recycling using well-established systems (NADH/FDH/formate, NADH/GDH/glucose, NADPH/G6PDH/glucose-6-phosphate) completely failed (data not shown). Since FDH, GDH, and G6PDH depend on essential metal ions,[14] we suspected that this deactivation might be caused by removal of the essential metal through complexation by diacid **1a** acting as strong chelating agent. In order to neutralize this effect, the medium was supplemented with Ca^{2+}, Mg^{2+}, or Zn^{2+} equimolar to the substrate, which completely restored the activity of the recycling enzymes (entries 3−5). Since the nature of the bivalent metal did not have any influence on the outcome of the reaction regarding conversion and/or stereoselectivity, this is a strong hint that the deactivation of the recycling enzyme occurs via the aforementioned mechanism. The addition of Fe^{3+} as supplementing metal failed; no conversion was observed (data not shown). The simplicity of this system is in strong contrast with the complex conditions required for *Clostridia*.[9]

A striking influence of the substrate configuration was found for the *E*-configurated substrate analog **2a**, which proved to be unreactive with all three enzymes (entry 6). A related (however, opposite) trend was reported with *Clostridium formicoaceticum*, which reduced (*E*)-**2a** to (*S*)-**1b** while the (*Z*)-counterpart **1a** turned out to be unreactive.[10]

The *exo*-methylene analog **3a** proved to be a difficult substrate, which was only converted to (*R*)-**1b** by YqjM in low conversion, albeit with excellent stereoselectivity (entries 8 and 9).

Although α,β-unsaturated esters were suspected to be good substrates for enoate reductases,[3] it turned out that ester hydrolysis occurred first when whole cells were used, which rendered the corresponding α,β-unsaturated carboxylic acids as the actual substrates.[10,15] This drawback was successfully circumvented by using isolated enzymes (entries 10−21). Overall, diesters **4a**−**6a** proved to be superior in comparison to the corresponding diacids **1a**−**3a**. In contrast to the dicarboxylic acids **1a**−**3a**, diesters **4a**−**6a** had no adverse effects on the cofactor-recycling systems.

The *cis*-configurated diester **4a** was reduced by all enzymes with perfect stereoselectivity furnishing (*R*)-**4b** in >99% ee (entries 10−13). Among them, OPR3 displayed the highest activity ($c = 99\%$). In contrast, the *exo*-methylene analogue **5a** turned out to be more cumbersome (entries 14−17): it was not accepted by OPR3 and also OPR1 showed moderate activity (up to 66% conversion); only YqjM gave sufficient conversion (up to 91%).

Depending on the enzyme, the (*E*/*Z*)-configuration of the diester substrate had a strong impact on the stereochemical outcome of the reaction. Whereas OPR1 converted both (*Z*)-**4a** and (*E*)-**6a** with similar activities yielding (*R*)-**4b**, OPR3 accepted only the (*Z*)-substrate **4a** and was completely inactive on (*E*)-**6a**. A related behavior was observed for dimethyl maleate reductase isolated *from Clostridium formicoaceticum*, which was inactive on the (*E*)-configurated substrate dimethyl fumarate.[10]

Although YqjM showed comparable activities on both stereoisomers, it produced *opposite enantiomers* with perfect stereoselectivity (ee >99%). This substrate-based stereocontrol is remarkable in magnitude since the stereochemical outcome of the reaction could be completely controlled by

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(11) Rohdich, F.; Wiese, A.; Feicht, R.; Simon, H.; Bacher, A. *J. Biol. Chem.* **2001**, *276*, 5779−5787.
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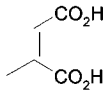
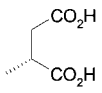
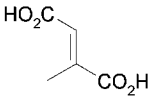
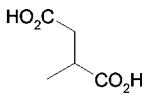
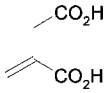
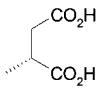
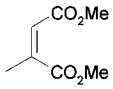
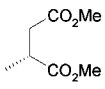
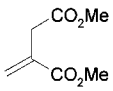
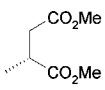
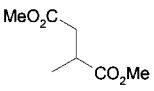
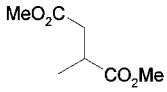
(14) The following essential metals were reported. (i) NADH-dependent formate dehydrogenase: Fe^{2+}, Mo^{6+} (http://www.brenda.uni-koeln.de/php/result_flat.php4?ecno=1.2.1.2). (ii) NADP-dependent glucose 6-phophate dehydrogenase: Ca^{2+}, Mg^{2+}, Mn^{2+} (http://www.brenda.uni-koeln.de/php/result_flat.php4?ecno=1.1.1.49). (iii) NAD(P)H-dependent D-glucose dehydrogenase: Ca^{2+}, Mg^{2+}, Mn^{2+}, K^{+}, Na^{+}, Ni^{2+}, Zn^{2+} (http://www.brenda.uni-koeln.de/php/result_flat.php4?ecno=1.1.1.47).
(15) Ferraboschi, P.; Grisenti, P.; Casati, R.; Fiecchi, A.; Santaniello, E. *J. Chem. Soc., Perkin Trans. 1* **1987**, 1743−1748.

Table 1. Asymmetric Bioreduction of α,β-Unsaturated Dicarboxylic Acids **1a**−**3a** and Corresponding Diesters **4a**−**6a** Using Enoate Reductases OPR1, OPR3, and YqjM

entry	substrate	product	cofactor	OPR1		OPR3		YqjM	
				convn %	ee %	convn %	ee %	convn %	ee %
1			NADH	>99	(*R*) >99	nc[b]	-	>99	(*R*) >99
2			NADPH	>99	(*R*) >99	nc	-	>99	(*R*) >99
3			NAD$^+$-FDH-M^{2+}[a]	>99	(*R*) >99	nd	-	>99	(*R*) >99
4	**1a**	(*R*)-**1b**	NAD$^+$-GDH-M^{2+}[a]	>99	(*R*) >99	nd	-	>99	(*R*) >99
5			NADP$^+$-G6PDH-M^{2+}[a]	>99	(*R*) >99	nd	-	>99	(*R*) >99
6	 **2a**	 **1b**	NADH or NADPH	nc	-	nc	-	nc	-
7			NADH	nc	-	nc	-	nc	-
8			NADPH	nc	-	nc	-	3	(*R*) >99
9	**3a**	(*R*)-**1b**	NADP$^+$-G6PDH	nd	-	nd	-	14	(*R*) >99
10			NADH	59	(*R*) >99	96	(*R*) >99	83	(*R*) >99
11			NADPH	48	(*R*) >99	87	(*R*) >99	88	(*R*) >99
12			NAD$^+$-FDH	91	(*R*) >99	99	(*R*) >99	93	(*R*) >99
13	**4a**	(*R*)-**4b**	NADP$^+$-G6PDH	28	(*R*) >99	75	(*R*) >99	78	(*R*) >99
14			NADH	28	(*R*) >99	3	(*R*) >99	91	(*R*) >99
15			NADPH	29	(*R*) >99	nc	-	77	(*R*) >99
16			NAD$^+$-FDH	66	(*R*) >99	nd	-	88	(*R*) >99
17	**5a**	(*R*)-**4b**	NADP$^+$-G6PDH	14	(*R*) >99	nd	-	64	(*R*) >99
18			NADH	65	(*R*) 79	nc	-	57	(*S*) >99
19			NADPH	58	(*R*) 80	nc	-	70	(*S*) >99
20			NAD$^+$-FDH	99	(*R*) 77	nd	-	69	(*S*) >99
21	**6a**	(*R*)- or (*S*)-**4b**	NADP$^+$-G6PDH	33	(*R*) 80	nd	-	36	(*S*) >99

[a] Medium supplemented with Ca^{2+}, Mg^{2+}, or Zn^{2+} equimolar to the substrate; for details see the electronic Supporting Information. [b] nc = no conversion; nd = not determined.

choosing an (*E*)- or (*Z*)-configurated substrate. Whereas (*Z*)-**4a** furnished (*R*)-**4b**, (*E*)-**6a** gave the mirror image (*S*)-**4b** counterpart in >99% ee. A related phenomenon was observed during the reduction of (*E*/*Z*)-2-chloroacrylic acids using baker's yeast[16] and (*E*/*Z*)-enals using enoate reductases from yeast and *Zymomonas mobilis*.[17] In addition, an enzyme-based stereocontrol was also observed during the reduction of **6a**, where the absolute configuration of the product could be controlled by the choice of biocatalyst: whereas OPR1 delivered (*R*)-**4b** (ee_{max} 80%), YqjM furnished (*S*)-**4b** (ee >99%).

In conclusion, we have shown that α,β-unsaturated dicarboxylic acids and their corresponding diesters were stereoselectively reduced by 12-oxophytodienoate reductase isoenzymes OPR1 and OPR3 from *Lycopersicon esculentum* (tomato) and the "old yellow enzyme" homolog YqjM from *Bacillus subtilis* with virtually absolute stereoselectivites (ee up to >99%). In contrast to enoate reductases from anaerobes, standard protocols for cofactor recycling could be used with these oxygen-stable enzymes and undesired ester hydrolysis was entirely eliminated. Most remarkably, the position of the C=C double bond within the substrate and its (*E*/*Z*)-configuration played a crucial role in the substrate recognition. Whereas *exo*-methylene substrates were rather difficult to reduce, stereochemical control was achieved by the choice of enzyme or the (*E*/*Z*)-configuration of the substrate.

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Supporting Information Available: General synthetic and analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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