DOI: 10.1002/adsc.201100504

Stereochemical Outcome of the Biocatalysed Reduction of Activated Tetrasubstituted Olefins by Old Yellow Enzymes 1–3

Elisabetta Brenna,[a,*] Francesco G. Gatti,[a] Daniela Monti,[b] Fabio Parmeggiani,[a] and Stefano Serra[b]

[a] Politecnico di Milano, Dipartimento di Chimica, Materiali, Ingegneria Chimica, Via Mancinelli 7, I-20131 Milano, Italy
Fax: (+39)-02-2399-3180; phone: (+39)-02-2399-3077; e-mail: elisabetta.brenna@polimi.it
[b] Istituto di Chimica del Riconoscimento Molecolare – CNR, Via Mario Bianco 9, I-20131 Milano, Italy

Received: June 29, 2011; Revised: September 9, 2011; Published online: January 12, 2012

Dedicated to Professor Claudio Fuganti with esteem and affection.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201100504.

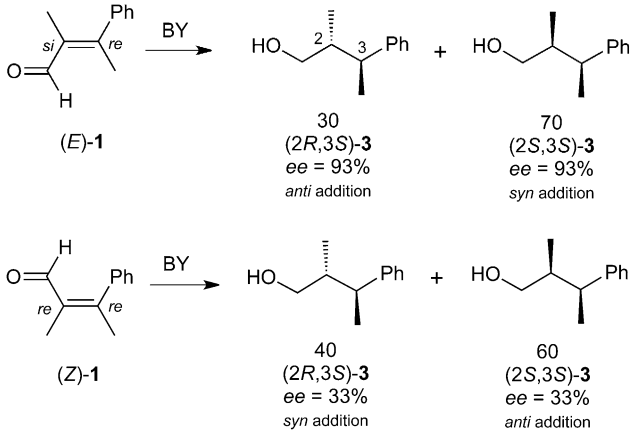
Abstract: The ene-reductase-mediated reactions of (*E*)- and (*Z*)-stereoisomers of tetrasubstituted activated alkenes were performed by means of isolated old yellow enzymes (OYEs) 1–3. The comprehension of the resulting data required a careful analysis of the stereochemical course of this kind of reaction. The investigation of the bioreduction of tetrasubstituted alkenes allowed us to appreciate the contribution of several factors (configuration of the starting alkene, mechanism of hydrogen addition, substrate binding mode) to the overall stereochemistry of the reaction.

Keywords: asymmetric catalysis; baker's yeast; biotransformations; enzyme catalysis; reduction

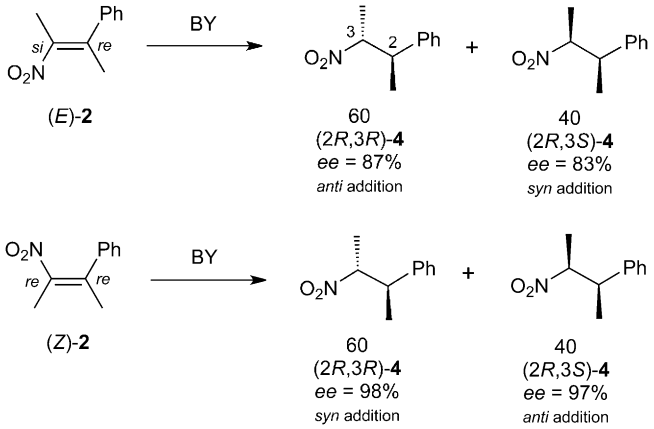
Introduction

The stereoselective reduction of activated C=C double bonds mediated by ene reductases (ER), belonging to the old yellow enzyme (OYE) family,[1] is currently receiving great attention,[2] because the reaction can be exploited in the development of biocatalysed syntheses of enantiopure biologically active molecules.[3] This research activity is supported by the plethora of data collected in the past on the same type of reactions performed by using microbial cell systems, especially by baker's yeast (BY) fermentations.[4] Investigation of the synthetic versatility of this bioreduction[5] and elucidation of mechanicistic aspects (e.g., through deuterium labelling[6]) are fundamental to understand the scope and limitations of OYE-mediated biotransformations.

Recently, we prepared[7] the two unsaturated aldehydes (*E*)- and (*Z*)-**1** (Scheme 1), and submitted them to BY reduction. These substrates have not been deeply investigated, because the synthesis of a tetrasubstituted double bond in a definite configuration is quite a challenging task. The results of the BY biotransformations showed that the reaction occurs with low diastereoselectivity, giving rise to mixtures of the two diastereoisomeric reduction products (2*R*,3*S*)- and (2*S*,3*S*)-**3** in 3/7 and 4/6 ratios, respectively, and that, starting from the two possible diastereoisomers of the alkene, saturated alcohols showing the same absolute configuration were obtained. The concomitant formation of two stereogenic centres allowed us to infer the mechanism of hydrogen addition from the analysis of the relative configurations of the reduction



Scheme 1. BY reduction of unsaturated aldehydes (ref.[7]).



Scheme 2. BY reduction of nitroalkenes (ref.[9]).

products. Compound (2*R*,3*S*)-**3** was the outcome of *anti* hydrogen addition to (*E*)-**1** and of *syn* addition to (*Z*)-**1**. Compound (2*S*,3*S*)-**3** was the diastereoisomer obtained by *syn* hydrogen addition to (*E*)-**1** and *anti* addition to (*Z*)-**1**.

With BY whole cells the concomitant reduction of the aldeyhde group to the corresponding primary alcohol by alcohol dehydrogenases could not be avoided.

These data were in contrast with the commonly accepted ideas that the reaction consists in an *anti* hydrogen addition to the activated carbon double bond,[2b] and that the stereochemistry of the starting alkene plays a key role on the enantioselectivity of the reaction.[2b,8]

Thus, we decided to re-investigate the biocatalysed reduction by using isolated OYEs, in order to verify whether the global stereochemical outcome of the reaction was due to a different stereoselectivity of the ERs active in the whole-cell systems, or if it was a characteristic of the bioreduction of this kind of substrate. We also submitted nitroalkenes (*E*)- and (*Z*)-**2** (Scheme 2) to the same investigation, their BY reductions had been already reported in the literature to occur with low diasteroselectivity,[9] affording the same enantiomers of the two diastereoisomeric nitroalkanes **4**. The same authors investigated the stability of the reduction products under fermentation conditions, in order to exclude a contribution to the diastereoisomeric ratio due to the epimerisation of the C_α stereogenic centre.

We soon realised that the comprehension of the data collected during these latter investigations required a careful analysis of the stereochemical course of OYE-mediated bioreductions. We want to report here on the results of this analysis and on the data concerning the OYE-catalysed reactions of (*E*)- and (*Z*)-isomers of compounds **1** and **2**.

Results and Discussion

General Considerations on the Mechanism of ER-Mediated Reactions

According to the currently postulated mechanism for the OYE-catalysed reduction of α,β-unsaturated aldehydes and ketones,[10] the substrate is located within the active site of the enzyme with the carbonyl group activated by the hydrogen bonds to His191 and Asn194 (Figure 1).

In this way the substrate is stacked above the reduced flavin with its β-carbon atom aligned to N-5

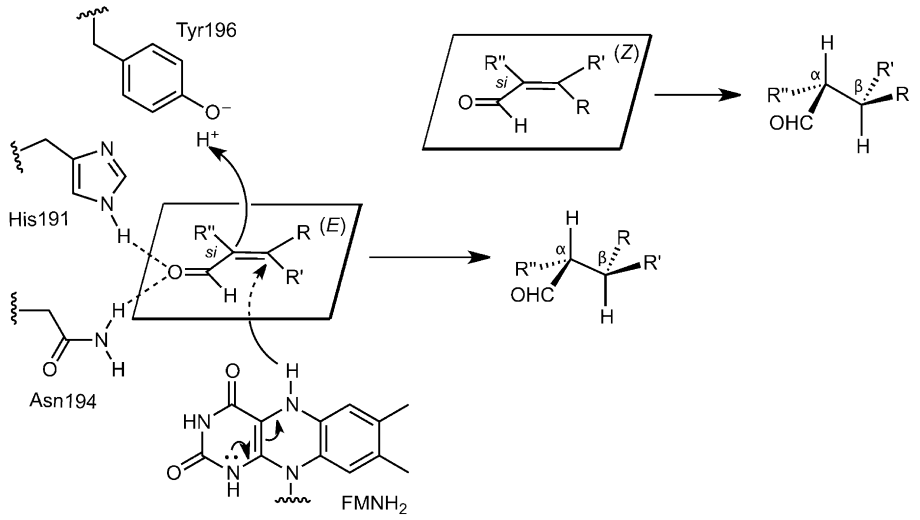


Figure 1. Postulated mechanism of OYE-mediated reduction of C=C double bonds. The priority rules employed to assign stereochemical descriptors are the following ones: for double bonds CHO > R″, R > R′; for stereoheterotopic faces CHO >= CRR′ > R″.

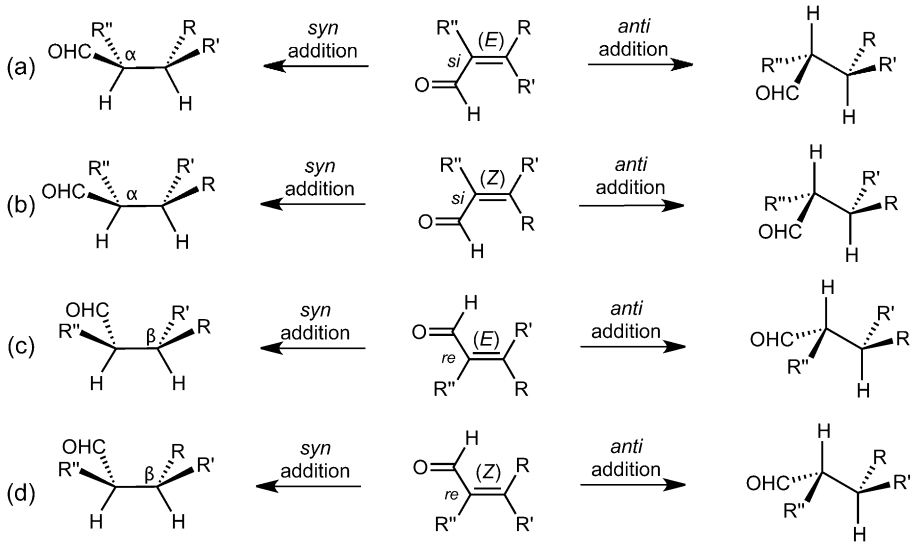


Figure 2. Stereochemical outcome of bioreductions of (*E*)- and (*Z*)-tetrasubstituted olefins. The priority rules employed to assign stereochemical descriptors are the following ones: for double bonds: CHO > R″, R > R′; for stereogenic centres (C_α,C_β): R > CHR″CHO > R′, CHO > CHRR′ > R″; for stereoheterotopic faces: CHO > =CRR′ > R″.

which transfers the hydride. Tyr196 lies above the substrate and donates a proton to the α-carbon atom. The delivery of a hydride to C_β from below the plane of the double bond, and of a proton to C_α from above accounts for the *anti* mode of hydrogen addition, which is the stereochemical course usually observed in this kind of reductions. In the model proposed by Massey et al.[10c] the unsaturated carbonyl compound is bound in such a way that the proton is delivered to the C_α-*si* face (Figure 1).

If this binding mode is satisfied, and the addition occurs with *anti* stereochemistry, the switch from (*E*)- to (*Z*)-alkenes will be characterised by inversion of configuration of C_β (Figure 1).

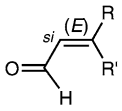
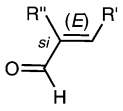
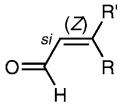
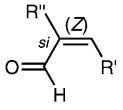
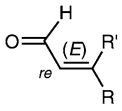
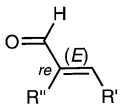
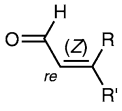
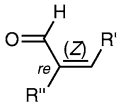
When these expectations are disappointed, the following justifications may be given: (i) (*E*)/(*Z*) isomerisation occurs in the reaction medium; (ii) the location of the electron-withdrawing group in the binding site is different from the one described for the carbonyl moiety of unsaturated aldehydes; (iii) the hydrogen addition step is characterised by a different stereospecific mechanism; (iv) the alkene is arranged in the binding site exposing the other stereoheterotopic face to the reduced flavin.

As for the hydrogen addition mode, the protonation step of the α-carbon atom can be provided also by the solvent water, with delivery of the proton on the C_α-*re* face from the same side of the hydride, thus allowing a hydrogen *syn* addition to the double bond. The final product of *syn* addition [Figure 2 (a) for (*E*)-olefins, Figure 2 (b) for (*Z*)-olefins] is characterised by opposite configuration at C_α, if compared with the *anti* reduction product obtained from the same olefin arranged in the same binding mode. A few examples of *syn* reduction of activated carbon double bonds have been reported by using BY as a biocatalyst.[6e,f,9] Through deuterium labelling experiments, it was demonstrated[6b] that the chiral α,β-unsaturated aldehyde perillaldehyde was reduced by baker's yeast in either a *syn* or *anti* mode according to its absolute configuration.

As for the substrate binding mode, a 180° flipping of the olefin around the carbon double bond [Figure 2, (c) and (d)] has been assumed in some cases[2d,11] to explain the experimental data. According to this flipped arrangement, hydrogen addition takes place on the other stereoheterotopic face of the olefin, with delivery of the proton on the C_α-*re* and C_α-*si* faces in the *anti* and *syn* addition modes, respectively. The enantioselectivity of this reaction is opposite to that occurring with the same substrate in the classical binding mode [Figure 2 (a) *vs.* (c); Figure 2 (b) *vs.* (d)], with inversion of configuration at C_α as well as at C_β, given the same stereospecificity of hydrogen addition.

The effects on stereochemistry due to the three highlighted factors, i.e., configuration of the starting alkene, mechanism of hydrogen addition, and substrate binding mode, can be summarised as follows: the other two factors being fixed, a switch from (*E*)- to (*Z*)-alkenes causes inversion of configuration of C_β, a switch from *anti* to *syn* addition causes inversion of configuration at C_α, while a switch from classical to flipped binding mode causes inversion of configuration both at C_α and C_β. Only the concomitant variation of all the three factors affords the same enantiomer.

Table 1. Stereochemical outcome of bioreductions of (*E*)- and (*Z*)-trisubstituted olefins. The priority rules employed to assign stereochemical descriptors are the following ones: for double bonds: CHO > R″, R > R′; for stereogenic centres: R > CH_2CHO > R′, CHO > CH_2R' > R″; for stereoheterotopic faces: CHO > = CRR′, = CHR′ > R″.

Entry	Binding mode	Substrate	Product CHO−CH_2−CHRR′		Substrate	Product CHO−CHR″−CH_2R′	
			A) *anti* addition	B) *syn* addition		C) *anti* addition	D) *syn* addition
1	*Classical*		C_β-(*S*)	C_β-(*S*)		C_α-(*R*)	C_α-(*S*)
2	*Classical*		C_β-(*R*)	C_β-(*R*)		C_α-(*R*)	C_α-(*S*)
3	*Flipped*		C_β-(*R*)	C_β-(*R*)		C_α-(*S*)	C_α-(*R*)
4	*Flipped*		C_β-(*S*)	C_β-(*S*)		C_α-(*S*)	C_α-(*R*)

General Considerations on the Stereochemistry of ER-Mediated Reduction of Trisubstituted Activated Alkenes

The most common substrates for ER reductions are trisubstituted olefins, in particular unsaturated aldehydes (Table 1). According to these considerations, in the hypothesis of a classical binding mode of the substrate, and of a hydrogen *anti* addition mechanism, it is expected that (*E*)- and (*Z*)-stereoisomers of β,β-disubstituted unsaturated aldehydes will afford compounds with opposite configuration at C_β (Table 1, entries 1A and 2A), whereas (*E*)- and (*Z*)-stereoisomers of α,β-disubstituted unsaturated aldehydes will be converted into derivatives showing the same configuration at C_α (Table 1, entries 1C and 2C). As a matter of fact, only the configuration of C_β should be affected by the change in the alkene stereochemistry.

In the case of (*E*)- and (*Z*)-β,β-disubstituted enals the switch from *anti* to *syn* addition either in the classical (Table 1, entries 1/2A *vs.* 1/2B) or flipped binding mode (Table 1, entries 3/4A *vs.* 3/4B) will have no effect on the absolute configuration of the products, because C_α is not a stereogenic centre. Given the same addition mode, opposite enantioselectivity will be obtained by a flipping of the substrate in the active site (Table 1, entries 1A/B *vs.* 3A/B, and 2A/B *vs.* 4A/B).

As for α,β-disubstituted enals, *syn* addition with the substrate located in a classical binding mode (Table 1, entries 1D and 2D), and *anti* addition on a flipped substrate (Table 1, entries 3C and 4C) will afford products showing the same absolute configuration, opposite to those obtained with *anti* addition in the classical binding mode (Table 1, entries 1C and 2C) or *syn* addition in the flipped accommodation (Table 1, entries 3D and 4D).

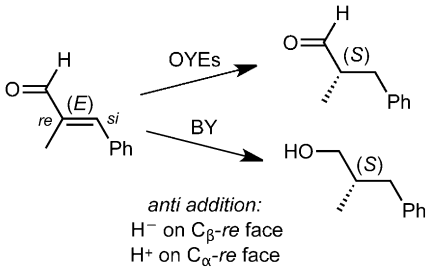


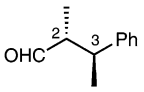
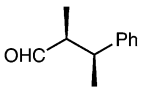
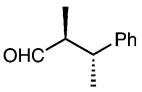
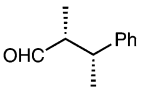
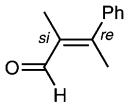
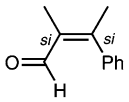
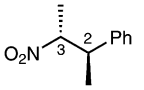
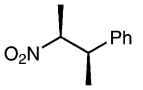
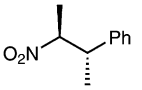
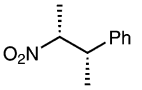
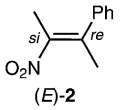
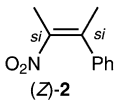
Figure 3. Bioreduction of α-methylcinnamaldehyde.

For example, the ER-catalysed reduction of (*E*)-α-methylcinnamaldehyde (Figure 3) either by isolated OYEs or by baker's yeast, afforded, respectively, (*S*)-2-methyl-3-phenylpropanal[12] (*ee* = 94, 96, 90% with OYE1–3, respectively) and (*S*)-2-methyl-3-phenylpropanol (*ee* = 70%),[13] in contrast with the expectation of Table 1 entry 1C. Recently, deuterium labelling experiments[7] showed a preferential (85:15) *anti* hydrogen addition to this substrate in BY reaction, thus a flipped binding mode can be assumed to explain the enantioselectivity of the reduction (Table 1, entry 3C).

Analysis of the Stereochemical Outcome of the ER-Mediated Reduction of Tetrasubstituted Activated Alkenes

In the analysis of the overall stereochemistry of bioreductions, the contribution due to the arrangement of the substrate within the binding site can be discriminated experimentally from that due to the stereospe-

Table 2. Experimental data of BY and OYE-mediated reductions of tetrasubstituted alkenes (*E*)- and (*Z*)-**1**, and (*E*)- and (*Z*)-**2**.

Substrate	Biocatalyst	Hydride on C_β-*si* face		Hydride on C_β-*re* face		*anti/syn* addition mode,[a] conversion[a] [%]
						
		(2*R*,3*S*)-**5**	(2*S*,3*S*)-**5**	(2*S*,3*R*)-**5**	(2*R*,3*R*)-**5**	
		***anti* addition**	***syn* addition**	***anti* addition**	***syn* addition**	
		***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	
 (*E*)-**1**	BY[c]	93	93			30/70,[d] 18[e]
	OYE1	72	85			45/55, 100
	OYE2	60	90			50/50, 100
	OYE3			90	62	75/25, 100
		***syn* addition**	***anti* addition**	***syn* addition**	***anti* addition**	
		***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	
 (*Z*)-**1**	BY[c]	33	33			60/40,[d] 19[e]
	OYE1	0	0			50/50, 62
	OYE2			40	46	50/50, 65
	OYE3			90	95	70/30, 100
						
		(2*R*,3*R*)-**4**	(2*R*,3*S*)-**4**	(2*S*,3*S*)-**4**	(2*S*,3*R*)-**4**	
		***anti* addition**	***syn* addition**	***anti* addition**	***syn* addition**	
		***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	
 (*E*)-**2**	BY	87	83			60/40, 54
	OYE1			88	76	40/60, 87
	OYE2		16	96		13/87, 37
	OYE3			87	50	30/70, 42
		***syn* addition**	***anti* addition**	***syn* addition**	***anti* addition**	
		***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	***ee* [%]**[b]	
 (*Z*)-**2**	BY	98	97			40/60, 72
	OYE1	99	99			40/60, 100
	OYE2	82	99			25/75, 100
	OYE3	78	99			30/70, 88

[a] Evaluated by GC analysis of the crude reaction mixture.
[b] Evaluated by GC analysis on a chiral stationary phase.
[c] Data for BY reduction are referred to saturated alcohols *syn*- and *anti*-**3**, obtained as a consequence of the presence of alcohol dehydrogenases within cells.[7]
[d] From ref.[7]
[e] Isolated yields from ref.[7]

cificity of hydrogen addition by investigating the ER-mediated reactions of tetrasubstituted olefins, in which two stereogenic centres are created upon reduction. As a matter of fact, in this case tetrasubstitution allows the distinction between the products of *syn* and *anti* hydrogen addition to be made by the analysis of their relative configuration, avoiding labelling experiments.

The results of BY reductions of tetrasubstituted olefins (*E*)- and (*Z*)-**1** and **2** shown in Scheme 1 and Scheme 2 can be now reconsidered under the light of the previously reported observations, on the assumption that the ene-reduction activity of BY is due to OYEs 2–3,[14] even if the presence of other enzymes capable of the same reaction cannot be excluded.

BY reduction of (*E*)-**1** (Scheme 1) showed a slight preference for *syn* addition with the alkene located in order to offer the C_β-*si* face to H^- attack.

An *anti* addition seemed to be slightly favoured in the BY reduction of (*Z*)-**1**. The absolute configuration of both diastereoisomers can be explained by the addition of the hydride on the C_β-*si* face, which is here possible in a flipped binding mode. However, very poor enantioselectivity was observed.

As for nitroalkenes (Scheme 2), BY reduction of both diastereoisomers was characterised by very

scarce diastereoselectivity. However, rather high enantiomeric excess values were obtained especially for the two products of (*Z*)-**2** biotransformation. Even in this case the absolute configuration of the nitroalkanes could be explained by assuming the exposure of the C_β-*si* face to hydride addition for both (*E*)- and (*Z*)-**2**, in a classical and flipped binding mode, respectively.

The data of OYE-mediated biotransformations of olefins (*E*)- and (*Z*)-**1**, and (*E*)- and (*Z*)-**2** are reported in Table 2. The regeneration of the catalytic NADPH cofactor was performed by using glucose dehydrogenase (GDH) with glucose as a sacrificial substrate.

All the starting tetrasubstituted alkenes were diastereoiosomerically pure. The occurrence of double bond isomerisation was monitored by GC/MS during the biotransformations. No isomerisation was observed for (*E*)- and (*Z*)-**1** and for (*Z*)-**2**. A low percentage of (*Z*)-**2** was detected in the course of OYE1–3 reductions of substrate (*E*)-**2**: in these reactions final conversion values were less than 100%, and the unreacted (*E*)-olefin showed the following final values of diastereoisomeric excess: *de* = 82% (OYE1), 76% (OYE2), and 77% (OYE3).

The configurational stability of the saturated aldehydes **5** was verified by incubating a sample of (2*R*,3*S*)-**5** under the bioconversion conditions. No appreciable formation of the other diastereoisomer due to epimerisation at C_α was detected after 24 h.

OYE1- and OYE2-mediated reductions of (*E*)-**1** proceeded in agreement with the BY biotransformation as regards the enantioselectivity, with addition of the hydride on the C_β-*si* face in a classical substrate arrangement, but without any diastereoselectivity. The results obtained with OYE3 can be explained by admitting a flipped binding mode of (*E*)-**1** in the active site of the enzyme, promoting addition of H^- on the C_β-*re* face. A preference for *anti* addition (75:25) was also shown in this case.

BY and OYE1 catalysed the reduction of (*Z*)-**1** with poor and no enantioselectivity, respectively, affording 1:1 mixtures of the two diastereoisomers: in the case of BY, addition of H^- on the C_β-*si* face in a flipped binding mode is slightly prevailing. The results obtained by using OYE2 and OYE3 revealed addition of H^- on the C_β-*re* face, in a classical binding mode, with *anti* addition being a little more favoured than the *syn* mode in the case of OYE3.

As for (*Z*)-**2**, BY- and OYE1-3-mediated reactions gave reduced products with fairly high enantioselectivity, always adding the hydride to the C_β-*si* face in a flipped binding mode, and with *syn* addition prevailing over the *anti* mode.

The results achieved in the OYE-mediated biotransformations of (*E*)-**2** could be partially altered by the occurrence of double bond isomerisation to a small extent: in particular, the effect could be that of lowering the enantiomeric excess values of the reduction products. However, in spite of this alteration, they were decidedly discordant with those obtained with BY. OYE1-3 afforded the reduction products expected for H^- attack on the C_β-*re* face in a flipped binding mode, except for *syn*-**4** recovered from OYE2 reaction, showing indeed a very low enantiomeric excess. The *anti* addition was disfavoured with respect to the *syn* mode, even though nitroalkanes obtained by an *anti* reduction mode were characterised by enantiomeric excess values higher than those of *syn* reduction products.

As a general remark, the enantioselectivity of these reductions is not usually high, with some interesting exceptions. The formation of the other enantiomer can be due to a lack of selectivity in the binding mode of the diastereoisomeric olefin in the enzyme site, with the hydrogen addition occurring with the same mechanism. It is plausible that an increase in the steric hindrance of the substituents at C_α and/or C_β could improve binding specificities and lead to higher enantioselectivities.

Conclusions

The stereochemical outcome of ER-mediated reductions of activated alkenes is influenced by the configuration of the C=C double bond, the arrangement of the substrate in the active site, and the stereospecificity of the hydrogen addition step. If suitable tetrasubstituted olefins are employed as substrates, it is possible to evaluate the contribution of each factor to the overall stereochemical course of the reaction.

In the BY-mediated reactions of compounds **1** and **2** the hydride attack occurs exclusively on the C_β-*si* face with both (*E*)- and (*Z*)-stereoisomers: a flipped substrate binding mode has to be assumed for (*Z*)-**1** and (*Z*)-**2**.

When isolated OYEs 1–3 were employed, some cases of hydride addition to the C_β-*re* face were observed. This reduction stereochemistry requires a flipped arrangement for (*E*)-olefins, and a classical accomodation for (*Z*)-isomers.

The reaction performed either with whole-cell system or with isolated enzymes is characterised by low values of diastereoselectivity: no definite preference for *syn* or *anti* addition was observed.

It is rather difficult to rationalise the data so far collected: probably the presence of steric hindrance on both C_α and C_β prevents the substrate from finding an optimal binding mode within the enzyme active site, which would be necessary to achieve higher stereoselectivity in the reduction process.

The detailed stereochemical analysis performed in this work highlights all the parameters to be consid-

ered, in order to explain the stereochemical outcome of ER-mediated reductions. The configuration of the starting double bond is not always the only factor controlling stereochemistry: the possibility of a *syn* addition mechanism, and of a flipped binding mode in the active site may play a crucial role.

Experimental Section

General Methods

TLC analyses were performed on Merck Kieselgel 60 F254 plates. All the chromatographic separations were carried out on silica gel columns. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer (Bruker ARX 400). The chemical shift scale was based on internal tetramethylsilane. GC/MS analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 μm). The following temperature programme was employed: 60 °C (1 min)/6 °C/min/150 °C (1 min)/12 °C/min/280 °C (5 min).

Compounds *anti*- and *syn*-**4** and **5** were detected in the reaction mixtures by GC/MS. The identity and absolute configuration of *anti*- and *syn*-**5** were established by GC/MS and GC analysis on a chiral stationary phase, respectively, by comparison with samples of (2*R*,3*S*)-**5** and (2*S*,3*S*)-**5**, prepared by Swern oxidation of the two mixtures of (2*R*,3*S*)-**3** and (2*S*,3*S*)-**3** obtained by BY reduction of (*E*)-**1** and (*Z*)-**1**, according to ref.[7] The identity and absolute configuration of *anti*- and *syn*-**4** were established by GC/MS and GC analysis on a chiral stationary phase, respectively, by comparison with a 60/40 mixture of (2*R*,3*R*)-**4** and (2*R*,3*S*)-**4**, obtained by BY reduction of (*E*)-**2**, according to ref.[9] The GC/MS data of the reduced products are as follows: *anti*-**4** t_R = 15.21 min, *m*/*z* (%) = 179 (M^+, 1), 132 (46), 117 (23), 105 (17), 91 (100); *syn*-**4** t_R = 16.08 min, *m*/*z* (%) = 179 (M^+, 1), 132 (62), 117 (33), 105 (42), 91 (100); *anti*-**5** t_R = 13.05 min, *m*/*z* (%) = 162 (M^+, 17), 147 (4), 105 (100); *syn*-**5** t_R = 13.56 min, *m*/*z* (%) = 162 (M^+, 25), 147 (8), 105 (100).

The enantiomeric excess values were determined by GC analysis, performed using a DAcTBSil.BetaCDX 0.25 μm × 0.25 mm × 25 m column (Mega, Italy), according to the following conditions: (i) 60 °C (1 min)/5 °C min^{-1}/93 °C/0.05 °C min^{-1}/97 °C/30 °Cmin^{-1}/220 °C: (2*R*,3*S*)-*syn*-**4** t_R = 47.0 min; (2*S*,3*R*)-*syn*-**4** t_R = 53.9 min; (2*R*,3*R*)-*anti*-**4** t_R = 65.4 min; (2*S*,3*S*)-*anti*-**4** t_R = 66.6 min; (ii) 70 °C (1 min)/0.30 °C min^{-1}/90 °C/30 °Cmin^{-1}/220 °C: (2*R*,3*S*)-*anti*-**5** t_R = 33.4 min; (2*S*,3*R*)-*anti*-**5** t_R = 35.4 min; (2*S*,3*S*)-*syn*-**5** t_R = 40.3 min; (2*R*,3*R*)-*syn*-**5** t_R = 41.2 min.

Synthesis of Tetrasubstituted Alkenes

Aldehydes (*E*)- and (*Z*)-**1** were prepared according to ref.[7] Nitro derivatives (*E*)- and (*Z*)-**2** were prepared according to ref.[9]

(*E*)-2-Methyl-3-phenylbut-2-enal [(*E*)-1]: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.66 (q, *J* = 1.4 Hz, 3H), 2.46 (q, *J* = 1.4 Hz, 3H), 7.15–7.20 (m, 2H), 7.29–7.43 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 12.9, 19.3, 126.7, 127.8, 128.4, 133.2, 142.8, 155.3, 191.7; GC/MS (EI): t_R = 15.19 min: *m*/*z* (%) = 160 (M^+, 50), 159 (100), 145 (22), 131 (16), 115 (32), 103 (4), 91 (24), 77 (10).

(*Z*)-2-Methyl-3-phenylbut-2-enal [(*Z*)-1]: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.92 (q, *J* = 1.0 Hz, 3H), 2.27 (q, *J* = 1.0 Hz, 3H), 7.18–7.25 (m, 2H), 7.31–7.41 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 11.1, 23.4, 128.2, 128.7, 134.4, 140.2, 157.4, 193.5; GC/MS (EI): t_R = 15.36 min: *m*/*z* (%) = 160 (M^+, 57), 159 (100), 145 (22), 131 (17), 115 (32), 103 (4), 91 (25), 78 (10).

(*E*)-(3-Nitrobut-2-en-2-yl)benzene [(*E*)-2]: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.09 (q, *J* = 1.5 Hz, 3H), 2.29 (q, *J* = 1.5 Hz, 3H), 7.10–7.40 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 16.1, 21.0, 126.2, 127.9, 128.5, 136.9, 139.5, 144.5; GC/MS (EI): t_R = 16.77 min; *m*/*z* (%) = 176 (M^+−1, 6), 160 (25), 115 (56), 91 (100).

(*Z*)-(3-Nitrobut-2-en-2-yl)benzene [(*Z*)-2]: ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.99 (q, *J* = 1.5 Hz, 3H), 2.12 (q, *J* = 1.5 Hz, 3H), 7.20–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 16.9, 21.7, 127.0, 128.1, 128.5, 137.9, 139.7, 145.3; GC/MS (EI): t_R = 15.92 min; *m*/*z* (%) = 176 (M^+−1, 7), 160 (21), 115 (57), 91 (100).

Strains

Baker's yeast from Lesaffre Italia (code number 30509) was employed for the preparation of reference compounds. All the enzymes employed were overexpressed in *Escherichia coli* BL21 (DE3) strains harbouring a specific plasmid prepared according to standard molecular biology techniques: pET30a-OYE1 from the original plasmid kindly provided by Neil C. Bruce,[15] pET30a-OYE2 and pET30a-OYE3 from *Saccharomyces cerevisiae* BY4741 and pKTS-GDH from *Bacillus megaterium* DSM509 (detailed steps reported in ref.[16]).

Overexpression of the Enzymes in *E. coli* BL21 (DE3)

LB medium (5 mL) containing the appropriate antibiotic (50 μg mL^{-1} kanamycin for pET-30a, 100 μg mL^{-1} ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown 8 h at 37 °C and 220 rpm. This starter culture was used to inoculate 200 mL medium, which was incubated for 8 h at the same conditions and used to inoculate 1.5 L medium. The latter culture was shaken at 37 °C and 220 rpm until OD_{600} reached 0.4–0.5, then enzyme expression was induced by the addition of 0.1 mM IPTG (50 ng mL^{-1} anhydrotetracycline was also added in the case of the pKTS-GDH plasmid). After 5–6 h the cells were harvested by centrifugation (5000 *g*, 20 min, 4 °C), resuspended in 50 mL of lysis buffer (20 mM phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole) and homogenised (Haskel high-pressure homogenizer). The cell-free extract, after centrifugation (20000 *g*, 20 min, 4 °C), was chromatographed on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM phosphate buffer, pH 7.0, 300 mM NaCl and a 10–300 mM imidazole gradient. Protein elution was monitored at 280 nm, the fractions were collected according to the chromatogram and dialysed twice against 1.0 L of 20 mM phosphate buffer pH 7.0 (12 h each, 4 °C) to remove imidazole and salts. Purified protein aliquots were stored frozen at −80 °C.

General Procedure for the OYE-Mediated Bioreduction

The substrate (*E*)- and (*Z*)-**1**, and (*E*)- and (*Z*)-**2**, (5 μmol) dissolved in DMSO (10 μL) was added to a phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μmol), $NADP^+$ (0.1 mM), GDH (4 U) and the required OYE (75 $\mu g\,mL^{-1}$). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (2×250 μL), centrifuging after each extraction (15000 *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

Acknowledgements

Prof. Claudio Fuganti is warmly acknowledged for fruitful discussions. Neil C. Bruce (Department of Biology, University of York) is kindly acknowledged for the gift of plasmid pT7-OYE1. Sven Panke and Christian Femmer (ETH Zürich Department of Biosystems Science and Engineering, Basel) are kindly acknowledged for the help provided in the preparation of the plasmids and the overexpressing strains.

References

[1] O. Warburg, W. Christian, *Biochem. Z.* **1933**, *266*, 377–411.
[2] a) R. E. Williams, N. C. Bruce, *Microbiology* **2002**, *148*, 1607–1614; b) R. Stuermer, B. Hauer, B. Hall, K. Faber, *Curr. Opin. Chem. Biol.* **2007**, *11*, 203–213; c) M. Hall, C. Stueckler, B. Hauer, R. Stuermer, T. Friedrich, M. Breuer, W. Kroutil, K. Faber, *Eur. J. Org. Chem.* **2008**, 1511–1516; d) H. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892–914.
[3] H.-P. Meyer, *Org. Process Res. Dev.* **2011**, *15*, 180–188.
[4] a) C. Fuganti, *Pure Appl. Chem.* **1990**, *62*, 1449–1452; b) C. Fuganti, P. Grasselli, *Baker's Yeast-Mediated Synthesis of Natural Products*, in: *Biocatalysis in Agricultural Biotechnology*, Chapter 25, ACS Symposium Series, Vol. 389, pp 359–370, **1989** American Chemical Society, Washington; c) S. Servi, *Synthesis* **1990**, 1–25; d) R. Csuk, B. I. Glänzer, *Chem. Rev.* **1991**, *91*, 49–97; e) E. Brenna, C. Fuganti, F. G. Gatti, F. Parmeggiani, *Tetrahedron: Asymmetry* **2009**, *20*, 2694–2698.
[5] a) E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Eur. J. Org. Chem.* **2011**, 4015–4022; b) E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Org. Proc. Res. Dev.* DOI: 10.1021/op200086t; c) C. Stueckler, C. K. Winkler, M. Hall, B. Hauer, M. Bonnekessel, K. Zangger, K. Faber, *Adv. Synth. Catal.* **2011**, *353*, 1169–1173; d) C. Stueckler, C. K. Winkler, M. Bonnekessel, K. Faber, *Adv. Synth. Catal.* **2010**, *352*, 2663–2666.
[6] a) E. Brenna, G. Fronza, C. Fuganti, F. G. Gatti, *Eur. J. Org. Chem.* **2010**, 5077–5084; b) G. Fronza, C. Fuganti, M. Pinciroli, S. Serra, *Tetrahedron: Asymmetry* **2004**, *15*, 9383–9388; c) G. Fronza, C. Fuganti, P. Grasselli, A. Mele, *J. Org. Chem.* **1991**, *56*, 6019–6023; d) G. Fronza, C. Fuganti, P. Grasselli, S. Lanati, R. Rallo, S. Tchilibon, *J. Chem. Soc. Perkin Trans. 1* **1994**, 2927–2930; e) G. Fogliato, G. Fronza, C. Fuganti, S. Lanati, R. Rallo, R. Rigoni, S. Servi, *Tetrahedron* **1995**, *51*, 10231–10240; f) G. Fronza, C. Fuganti, M. Mendozza, R. Rallo, G. Ottolina, D. Joulain, *Tetrahedron* **1996**, *52*, 4041–4052; g) G. Fronza, C. Fuganti, P. Grasselli, M. Barbeni, *Tetrahedron Lett.* **1992**, *33*, 6375–6378; h) G. Fronza, C. Fuganti, P. Grasselli, A. Mele, A. Sarra, G. Allegrone, M. Barbeni, *Tetrahedron Lett.* **1993**, *34*, 6467–6470.
[7] G. Fronza, C. Fuganti, S. Serra, *Eur. J. Org. Chem.* **2009**, 6160–6171.
[8] C. Stueckler, M. Hall, H. Ehammer, E. Pointner, W. Kroutil, P. Macheroux, K. Faber, *Org. Lett.* **2007**, *9*, 5409–5411.
[9] Y. Kawai, Y. Inaba, N. Tokitoh, *Tetrahedron: Asymmetry* **2001**, *12*, 309–318.
[10] a) K. M. Fox, P. A. Karplus, *Structure* **1994**, *2*, 1089–1105; b) B. J. Brown, Z. Deng, P. A. Karplus, V. Massey, *J. Biol. Chem.* **1998**, *273*, 32753–32762; c) R. M. Kohli, V. Massey, *J. Biol. Chem.* **1998**, *273*, 32763–32770.
[11] a) S. K. Padhi, D. J. Bougioukou, J. D. Stewart, *J. Am. Chem. Soc.* **2009**, *131*, 3271–3280; b) A. Müller, B. Hauer, B. Rosche, *Biotechnol. Bioeng.* **2007**, *98*, 22–29; c) H. S. Toogood, A. Fryszkowska, V. Hare, K. Fisher, A. Roujeinikova, D. Leys, J. M. Gardiner, G. M. Stephens, N. S. Scrutton, *Adv. Synth. Catal.* **2008**, *350*, 2789–2803.
[12] C. Stueckler, N. Mueller, C. K. Winkler, S. M. Glueck, K. Gruber, G. Steinkellner, K. Faber, *Dalton Trans.* **2010**, *39*, 8472–8476.
[13] P. D′Arrigo, C. Fuganti, G. Pedrocchi Fantoni, S. Servi, *Tetrahedron* **1998**, *54*, 15017–15026.
[14] a) K. Saito, D. J. Thiele, M. Davio, O. Lockridge, V. Massey, *J. Biol. Chem.* **1991**, *266*, 20720–20724; b) K. Stott, K. Saito, D. J. Thiele, V. Massey, *J. Biol. Chem.* **1993**, *268*, 6097–6106; c) Y. S. Niino, S. Chakraborty, B. J. Brown, V. Massey, *J. Biol. Chem.* **1995**, *270*, 1983–1991.
[15] R. E. Williams, D. A. Rathborne, N. S. Scrutton, N. C. Bruce, *Appl. Environ. Microbiol.* **2004**, *70*, 3566–3574.
[16] M. Bechtold, E. Brenna, C. Femmer, F. G. Gatti, S. Panke, F. Parmeggiani, A. Sacchetti, *Org. Process Res. Dev.* DOI:10.1021/op200085k.

