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Opposite Enantioselectivity in the Bioreduction of (*Z*)-β-Aryl-β-cyanoacrylates Mediated by the Tryptophan 116 Mutants of Old Yellow Enzyme 1: Synthetic Approach to (*R*)- and (*S*)-β-Aryl-γ-lactams

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Abstract: The Trp 116 mutants of Old Yellow Enzyme 1 that catalyse the reduction of (*Z*)-β-aryl-β-cyanoacrylates give the opposite enantioselectivity according to the nature of the amino acid in position 116. Small amino acids (e.g., alanine) make the substrate bind to the enzyme's active site in a "classical" orientation, affording the (*S*)-enantiomer of the reduced product. When the size of the amino acid increases (e.g., leucine), a "flipped" binding mode is adopted by the substrate, which is converted into the corresponding (*R*)-derivative. With bulky amino acids (e.g., tryptophan in the wild-type) the reduction does not occur. The enantiomerically enriched cyanopropanoates thus prepared can be converted into the corresponding (*S*)- and (*R*)-β-aryl-γ-lactams, precursors of inhibitory neurotransmitters belonging to the class of γ-aminobutyric acids, by a simple functional group interconversion in a sequential one-pot procedure.

Keywords: chirality; enantioselectivity; enzyme catalysis; nitriles; reduction

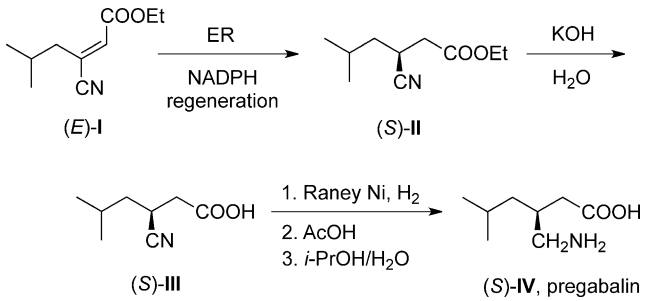
Introduction

A constant need of fine chemicals manufacturers, especially in the pharmaceuticals field, is to develop more sustainable and cost-effective synthetic procedures without impairing the quality and purity of the final products.[1] A possible solution is the replacement of traditional chemical reactions with biocatalytic processes, that generally provide very pure products with improved step and atom economy,[2] under safe conditions, at a reduced cost, by exploiting the typical high chemo- and stereoselectivity of enzymes. Moreover, during the last decade, advancements in protein engineering strategies, in particular directed evolution, have made available robust engineered biocatalysts, that are able to outperform traditional stoichiometric and chemocatalytic methods.[3]

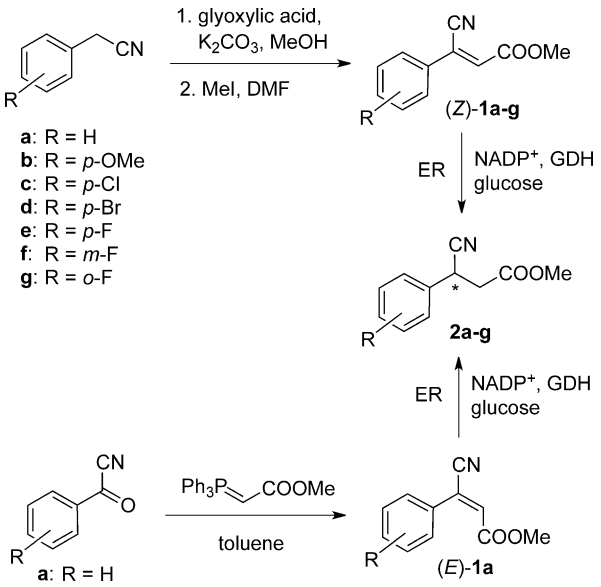
To harness this great potential of enzymes in the development of manufacturing processes, it is essential to enlarge the portfolio of synthetically useful biocatalysed reactions with a well-defined substrate scope, and to understand how they can be combined with classical chemical transformations in the most efficient and productive way.[4]

The enantioselective reduction of suitably substituted C=C double bonds, for example, represents a key chemical transformation for the creation of stereogenic centres in chiral building blocks to be used for fine chemicals production. A very efficient biocatalysed variant of this reaction is based on the use of enzymes called ene-reductases (EC 1.6.99.1, ERs), most of which belong to the family of Old Yellow Enzymes (OYEs).[5] Many efforts have been devoted not only to isolate new ERs,[6] in order to enlarge the collections of the available wild-type catalysts, but also to improve considerably their performance by protein engineering,[7] affording variants with broader substrate scope or opposite enantioselectivity.

Currently, the mechanism[8] of OYE-mediated hydrogenations and the stereoelectronic effects of the



Scheme 1. Chemoenzymatic synthesis of pregabalin.



Scheme 2. Synthesis and bioreduction of substrates (*Z*)-**1a–g** and (*E*)-**1a**.

substituents on the starting alkene,[6,9] have been extensively investigated, in order to define the limits and the potential of the reactions in the field of organic synthesis. It has been established that at least one of the two olefinic carbon atoms must be connected to an electron-withdrawing group (EWG), typically the carbonyl function of aldehydes and ketones, or a nitro group, or an imido functionality.[8b,10] The presence of a CN moiety as the sole EWG of the alkene derivative has been found to promote the bioreduction only in the case of 2-arylacrylonitriles[11] and 2-aryl-2-butenenitriles.[12] Generally, unsaturated esters are not reduced by OYEs, unless a halogen atom is linked in the α position,[13] or another EWG (e.g., an ester,[14] a nitrile[8d,e,15] or a nitro group[16]) is linked to the β olefinic carbon atom.

Indeed, the presence of two EWGs on the C=C double bond makes the bioreduction of these alkenes of particular interest, because it affords chiral building blocks with two functional groups that can be manipulated, broadening the spectrum of the final products that can be prepared. For example, the reduction of cyano ester (*E*)-**I** (Scheme 1) mediated by ERs was carefully investigated by Pfizer in order to prepare the (*S*)-enantiomer of the reduced product **II** on a preparative scale.[17] For this particular cyano ester the best results were achieved by using OPR1 (from *Lycopersicon esculentum*). Derivative **II** (*ee* >99%) was submitted to saponification of the ester group and reduction of the CN moiety, to afford the γ-aminobutyric acid analogue pregabalin (**IV**), developed for the treatment of central nervous system disorders.

The data collected in the literature on the bioreduction of cyano esters of type **I** are limited to compounds bearing an alkyl substituent at the prostereogenic carbon atom.[8d,e,15] Thus, we decided to extend the investigation to those having an aromatic ring in this position, i.e., substrates **1a–g** (Scheme 2), in order to enrich the knowledge of the substrate scope of the reaction. We also envisaged the possibility to convert the resulting reduced compounds into β-aryl-γ-lactams, the synthetic precursors of the inhibitory neurotransmitters belonging to the class of γ-aminobutyric acids (GABA), such as baclofen and phenibut. We report herein on the investigation of the ER-mediated hydrogenation of compounds **1a–g**, and on the exploitation of the corresponding 3-aryl-3-cyanopropanoates **2a–g** by means of simple functional group manipulation.

Results and Discussion

Synthesis of Substrates (*Z*)-1a–g and (*E*)-1a

The most convenient synthetic procedure to arylcyanoacrylates **1a–g** was found to be the condensation of the suitable arylacetonitrile derivative with glyoxylic acid and potassium carbonate in methanol solution, first reported by Dean et al. in 1993,[18] to give only the (*Z*)-stereoisomer of the corresponding cyanoacrylic acid potassium salt (Scheme 2). The esterification was then performed by reaction with methyl iodide in DMF. In the case of compound **1a**, the (*E*)-isomer was obtained by Wittig reaction of benzoyl cyanide and methyl (triphenylphosphoranylidene)acetate (Scheme 2): after column chromatography, a sample containing 20% of (*Z*)-stereoisomer was recovered.

The only data concerning the bioreduction of these derivatives were described for the potassium salts of compounds (*Z*)-**1a**, **b**, **c** and **e**,[19] and for the free carboxylic acids of derivatives (*Z*)-**1a**, and **c**.[20] The potassium salts had been reduced by the crude cell extracts of the anaerobic bacteria *Clostridium sporogenes* (DSM 795), *Ruminococcus productus* (DSM 3507), and *Acetobacterium woodii* (DSM 1030), under an H_2 atmosphere to afford the (*S*)-enantiomer of the reduced products in quantitative yields and high

Table 1. Biotransformations of compounds (*Z*)- and (*E*)-**1a** with wild-type OYE1–3.

	(*Z*)-**1a**		(*E*)-**1a**	
ER	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][b]
OYE1	5	–	99	82 (*R*)
OYE2	2	–	99	84 (*R*)
OYE3	8	–	99	70 (*R*)

[a] Conversion, calculated on the basis of GC analysis of the crude mixture after 24 h reaction time.
[b] Calculated on the basis of GC analysis on a chiral stationary phase.

enantiomeric excess values in 3 days reaction time. The carboxylic acids of (*Z*)-**1a**, and **c** had been converted into the corresponding (*R*)-saturated compounds in 80% yields and 98 and 94% *ee* values, respectively, by the whole cells of *Achromobacter* sp. JA81 in 48 h.

OYE1-3-Mediated Reductions of Compounds (*Z*)-1a–g and (*E*)-1a

When substrates (*Z*)-**1a–g** were submitted to OYE1-3-mediated reductions in the presence of glucose and glucose dehydrogenase (GDH) for NADPH regeneration, either very low or no conversion was observed [Table 1 for (*Z*)-**1a**, Table S1 in the Supporting Information for (*Z*)-**1b**–**g**]. On the contrary, when compound (*E*)-**1a** (*de* = 60%) was employed, the (*E*)-alkene was completely reduced to afford (*R*)-**2a** (*ee* = 70–84%) (Table 1) and the (*Z*)-stereoisomer was left unreacted. The absolute configuration of compound **2a** was assigned by comparison with the specific optical rotation value reported for (*S*)-**2a** in the literature.[19]

In order to understand the marked preference of OYE1-3 for the reduction of (*E*)-**1a** with respect to the (*Z*)-stereoisomer, we investigated the stereochemical course of the reaction. First of all, deuteration experiments were performed in order to establish whether the CN or the ester moiety was the activating EWG, i.e., the one establishing hydrogen bonds with the amino acids residues within the enzyme binding pocket. OYE1-mediated reduction of (*E*)-**1a** was carried out in D_2O in the presence of a stoichiometric quantity of NADH to afford (*R*)-**2a**-d_1 (Figure 1). These reaction conditions are known[21] to promote the delivery of a H^- to the olefinic carbon atom in the β position to the activating EWG, and a D^+ to the one in the α position. In Figure 1 the proton NMR spectrum of (*R*)-**2a**-d_1 (Figure 1b) is compared to that of the unlabelled racemic compound **2a** prepared by $NaBH_4$ reduction (Figure 1a): the nearly complete disappearance of the multiplet at 4.28 ppm due to the

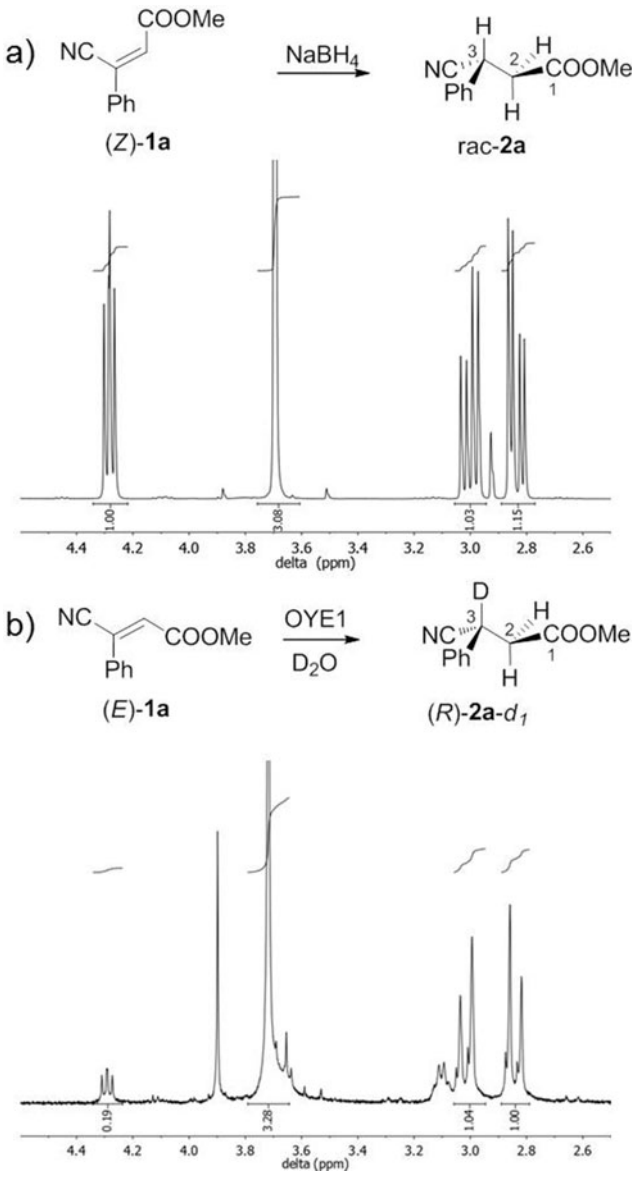
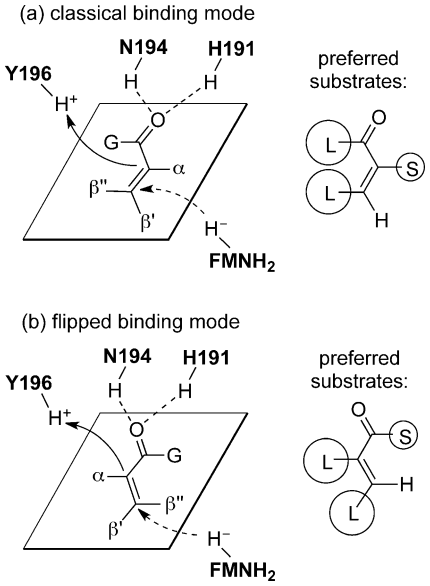


Figure 1. a) ^{1}H NMR spectrum of *rac*-**2a**; **b)** ^{1}H NMR spectrum of (*R*)-**2a**-d_1 obtained from (*E*)-**1a** (*de* = 60%) by OYE1-mediated reduction in D_2O in the presence of stoichiometric NADH (c = 80%, *ee* = 80%); the singlet at 3.90 ppm is due to the COOMe of unreacted (*Z*)-**1a**. All spectra are recorded in $CDCl_3$.

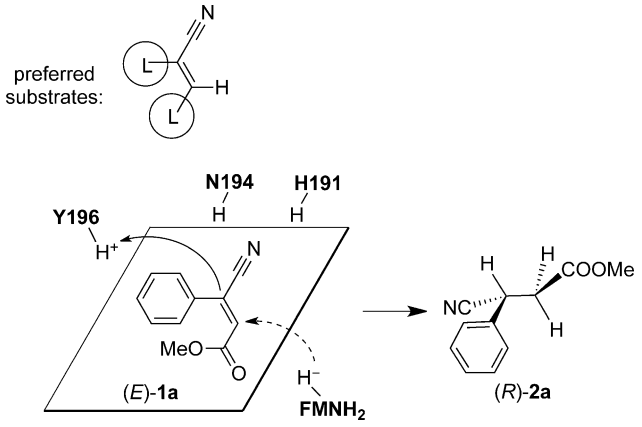
hydrogen atom at C-3 highlights the incorporation of a deuterium atom in this position.

We drew the conclusion that the CN moiety was the activating EWG in the OYE-mediated reduction of (*E*)-**1a**. The following step was to define the productive binding mode adopted by the substrate within the enzyme active site during the reaction. There are two possible substrate orientations described in the literature: the "classical" (or "normal") binding mode and the "flipped" binding mode (Scheme 3).

The former was inferred by Massey et al.[8a] for 2-cyclohexenone from the crystal structure of oxidised



Scheme 3. Substrate binding modes in OYE active site. G is the substituent linked to the C=O moiety of the EWG; α and β are the substituents in alpha and beta position with respect to the EWG; S and L stand for small and large, respectively.



Scheme 4. OYE-meditated reduction of (*E*)-**1a** in a flipped binding mode with the CN as the activating EWG, affording (*R*)-**2a**.

OYE1 with the inhibitor *para*-hydroxybenzaldehyde bound to the active site, by assuming that the carbonyl group would be positioned in an analogous way to the phenolate oxygen of the aromatic aldehyde. The latter[9b] was deduced by a 180° rotation of the substrate about the axis passing through the oxygen atom of the carbonyl group and the carbon atom of the double bond in the β position to the activating moiety. The experimental data collected in the literature for acyclic trisubstituted olefins activated by an EWG linked to the prostereogenic carbon atom and efficiently converted by OYE1-3 enzymes, have already shown a connection[8c,d] between the preferred reactive binding mode and the relative size of the groups shown as G and α in Scheme 3. If G is bulky (large, L) and α has a modest steric hindrance (small, S), a classical binding mode is adopted and the alkene stereoisomer best reduced is the one with the large substituent (L) at C_β on the same side of the EWG. If G is small, and α is large, then a flipped binding mode is preferred, and the favoured alkene stereoisomer is the one with the large substituent (L) at C_β on the opposite side of the EWG. These considerations could be also extended to alkenes for which the activating EWG is a CN moiety without a G group.[8e]

As for compound (*E*)-**1a**, the information on the activating EWG was combined with the absolute stereochemistry of the reduced compound, which defines the stereoheterotopic face of the alkene on which the addition of the proton has occurred. A flipped binding mode could be inferred for (*E*)-**1a** (Scheme 4) based on the hypothesis of an *anti* hydrogen addition (a hydride from below the plane of the alkene to C_β and a proton from above the plane to C_α) and on the evidence that the stereogenic centre was created in (*R*) configuration with the CN involved in the hydrogen bonds within the active site.

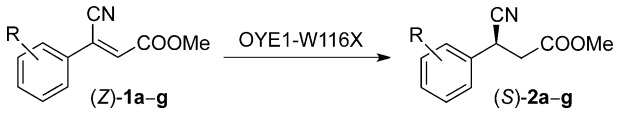
Then, the marked preference for the OYE-mediated reduction of (*E*)-**1a** with respect to the (*Z*)-stereoisomer could be explained by the fact that the hindrance of the phenyl ring favoured a flipped orientation, which was optimal for the (*E*)-stereoisomer, affording the (*R*) enantiomer of the reduced product.

OYE1-W116X-Mediated Reductions of Compounds (*Z*)-1a–g

From a synthetic route planning perspective, the aryl cyano esters **1a–g** were much more easily obtained as (*Z*)- rather than (*E*)-stereoisomers, so it was more advantageous to find a way to get the former accepted by the enzyme, e.g., by protein engineering, rather than struggling to synthesise the latter. The use of Trp 116 mutants of *S. pastorianus* OYE1 represented an attractive option,[7c] because literature data, based also on crystallographic studies,[22] had shown that the Trp 116 residue (conserved in OYE2–3) played a critical role in the stereochemistry of OYE-mediated alkene reductions by influencing the substrate orientation within the active site.

Therefore, we decided to investigate the transformation of cyano esters (*Z*)-**1a–g** by using the complete set of Trp 116 mutants of OYE1. The results of the biocatalysed reductions highlighted the possibility to organise the twenty variants in three different groups: (A) Ala, Cys, Gly, Ile, Asn, Gln, Ser, Thr and Val mutants, affording (*S*)-**2a–g** (Table 2); (B) His, Leu and Met mutants giving (*R*)-**2a–g** derivatives

Table 2. Biotransformations of compounds (*Z*)-**1a–g** affording (*S*)-**2a–g**.

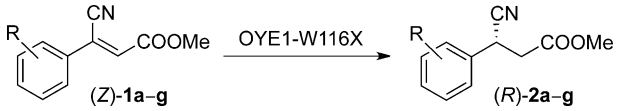


(*Z*)-**1a–g** (*S*)-**2a–g**

Variant	**1a**, R = H		**1b**, R = *p*-OMe		**1c**, R = *p*-Cl		**1d**, R = *p*-Br		**1e**, R = *p*-F		**1f**, R = *m*-F		**1g**, R = *o*-F	
	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][c]	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][b])	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][c]
W116A	100	48	77	56	92	60	95	68	94	90	100	56	48	68
W116C	60	70	32	*rac*	40	50	95	68	41	72	100	48	74	66
W116G	31	84	45	38	44	6	81	70	33	94	50	60	29	66
W116I	23	rac	68	60 (*R*)	86	42	90	30	88	30	100	rac	65	52
W116N	80	50	76	28	79	42	82	60	25	26	65	40	66	60
W116Q	70	36	38	64 (*R*)	68	26	68	*rac*	64	*rac*	94	16	81	6
W116S	42	6	40	28	25	64	58	*rac*	51	73	100	56	47	64
W116T	58	58	39	34 (*R*)	23	54	95	60	82	60	88	60	86	74
W116V	85	70	95	24	28	48	88	50	90	70	100	56	100	72

[a] Conversion calculated on the basis of GC analysis of the crude mixture after 24 h reaction time (isolation yields are reported in the Supporting Information).
[b] Calculated on the basis of GC analysis on a chiral stationary phase.
[c] Calculated on the basis of HPLC analysis on a chiral stationary phase. Reactions were performed in pH 7 buffer solution.

Table 3. Biotransformations of compounds (*Z*)-**1a–g** affording (*R*)-**2a–g**.



(*Z*)-**1a–g** (*R*)-**2a–g**

Variant	**1a**, R = H		**1b**, R = *p*-OMe		**1c**, R = *p*-Cl		**1d**, R = *p*-Br		**1e**, R = *p*-F		**1f**, R = *m*-F		**1g**, R = *o*-F	
	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][c]	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][c]	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][b]	c [%][a]	*ee* [%][c]
W116H	42	74	44	99	13	67	40	54	18	99	5	–	7	–
W116L	91	88	95	99	84	66	100	66	93	80	100	60	100	64
W116M	83	29	79	84	17	14	95	40	25	2	100	*rac*	86	46 (*S*)

[a] Conversion calculated on the basis of GC analysis of the crude mixture after 24 h reaction time (isolation yields are reported in the Supporting Information).
[b] Calculated on the basis of GC analysis on a chiral stationary phase.
[c] Calculated on the basis of HPLC analysis on a chiral stationary phase. Reactions were performed in pH 7 buffer solution.

(Table 3); (C) Trp (wild-type), Asp, Glu, Phe, Lys, Pro, Arg and Tyr mutants characterised by very low or no conversions (Table S2, Supporting Information).

As for the enantioselectivity of the bioreductions with enzymes of groups A and B, the only exceptions were observed in the reactions of the *para*-methoxy derivative (*Z*)-**1b**, catalysed by the W116I, W116Q and W116T variants which afforded compound (*R*)-**2b**, although with poor selectivity, and in the reduction of *ortho*-fluoro cyano ester (*Z*)-**1g** with the W116M mutant giving derivative (*S*)-**2g**.

As for the efficiency of the reactions with the mutants of group C, exceptions were observed with the *para*-bromo derivative, which was reduced by these enzymes, even if with modest yields and very low enantiomeric excess values.

The absolute configuration of compounds **2a**, **b**, **c**, and **e** was established by comparison with the specific optical rotation values reported in the literature; it was assigned by chemical correlation to compound **2d**, and by analogy to derivatives **2f** and **2g** (see the Supporting Information).

We determined the activating group for substrate (*Z*)-**1a** in the reactions with W116L and W116A, characterised by opposite enantioselectivity, by performing the bioreductions in deuterated water in the presence of stoichiometric NADH. In both cases the presence of a deuterium atom at the carbon atom bearing the CN group was highlighted by the analysis of the corresponding ^{1}H NMR spectrum (Figure 2).

Thus, a classical binding mode (Scheme 5a) could be deduced for the W116A mutant (or any of the var-

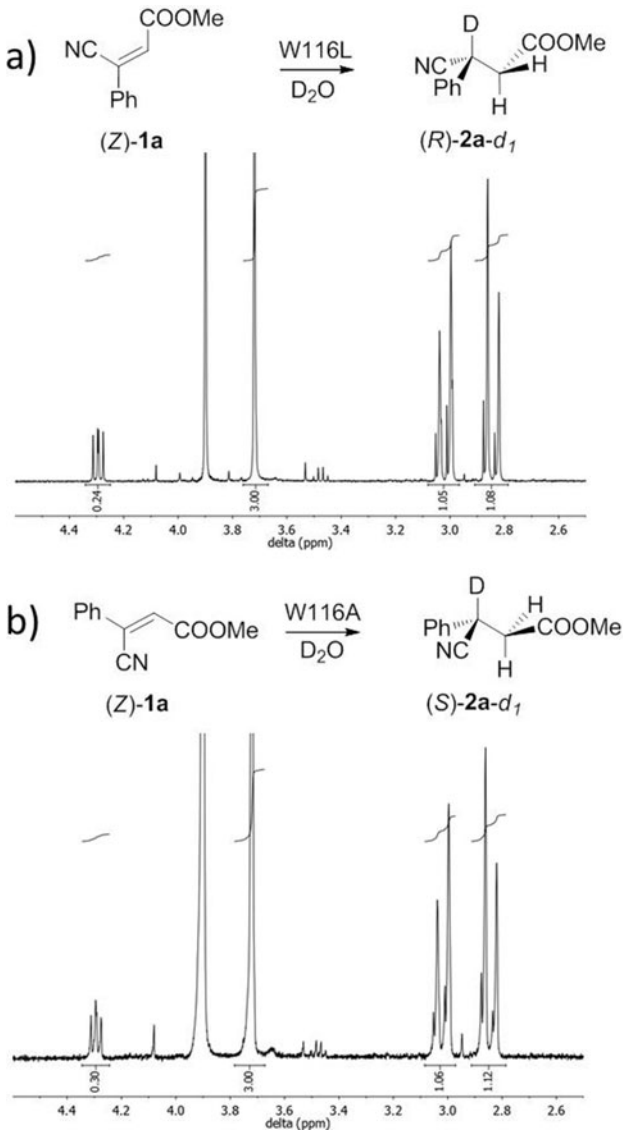
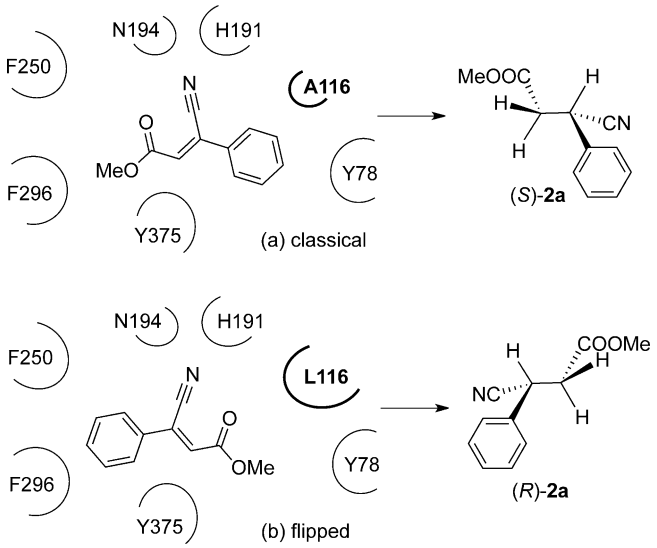


Figure 2. a) ^{1}H NMR spectrum of (*R*)-**2a**-d_1 obtained from (*Z*)-**1a** by OYE1-W116L-mediated reduction in D_2O in the presence of stoichiometric NADH (c = 65%, *ee* = 90%); **b)** ^{1}H NMR spectrum of (*S*)-**2a**-d_1 obtained from (*Z*)-**1a** by OYE1-W116A-mediated reduction in D_2O in the presence of stoichiometric NADH (c = 32%, *ee* = 65%). The singlet at 3.72 ppm is due to the COOMe of the reduced product, and that at 3.90 ppm is due to the COOMe of the unreacted starting compound. All spectra are recorded in $CDCl_3$.



Scheme 5. Binding modes of (*Z*)-**1a** in the active site of OYE1-W116A **(a)** and OYE1-W116L **(b)** deduced by analysis of the bioreduction results. The flavin prosthetic group lies below the plane of the substrate, while the catalytic residue Y196 lies above.

iants belonging to group A) in the reaction to (*S*)-**2a**, and a flipped one (Scheme 5b) for the W116L mutant (or any of the variants belonging to group B) in the reduction affording (*R*)-**2a**.

We attempted to support these conclusions on the reactive binding mode of β-aryl-β-cyano esters and to obtain information on the coordination geometry of the nitrile group by crystallographic studies. Soaking experiments of (*Z*)-**1a** and (*Z*)-**1e** were successful only with crystals of OYE1-W116A. The analysis of the collected X-ray data of the crystals showed the presence in the active site of an electron density best fit, respectively, by substrates (*Z*)-**1a** (PDB code: 4YNC) and (*Z*)-**1e** (PDB code: 4YIL). The resulting orientations of these two substrates in the active site were found to be very similar, and in both cases they showed features suggesting that they did not represent catalytically productive arrangements (Figure 3).

First of all, the activating nitrile group was not directed towards Nε2 of H191 and Nδ2 of N194 (Figure 3), but it was positioned between Y82 and Y375. The hydrogen bonds with H191 and N194 are known to be essential for the catalytic turnover by OYE1.[23] Then, the orientation of the alkene with respect to the FMN cofactor was not optimal: the angle formed between C_β of the cyano ester and the plane of the FMN is 121.5° for **1a** and 112.9° for **1e**; the distance between C_β of the cyano ester and N-5 of FMN is 4.70 Å for **1a** and 4.78 Å for **1e**. These values are outside the range observed by Fraaije et al. in their survey of flavoproteins bound to their respective substrates and/or products.[24]

No information could be obtained on the geometry of the hydrogen bonds accepted by the nitrogen atom of the nitrile within the active site.

Understanding the Stereoselectivity of OYE1-W116X Variants in the Reactions with Cyano Esters (*Z*)-1a–g

In a previous work,[22] the stereochemistry of the bioreductions of (*R*)- and (*S*)-carvone with wild-type

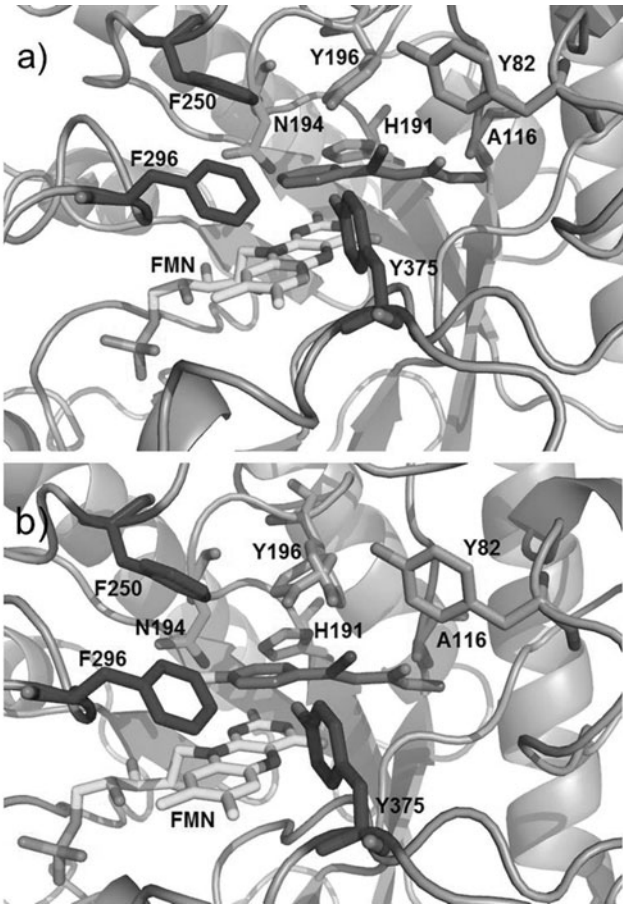


Figure 3. Crystal structures of the complexes between OYE1-W116A mutant and **(a)** compound (*Z*)-**1a** (PDB code: 4YNC), or **(b)** compound (*Z*)-**1e** (PDB code: 4YIL).

OYE1 and OYE1-W116X variants was successfully investigated by solving the X-ray crystal structures of *pseudo*-Michaelis complexes formed *in cristallo* by soaking each protein individually with the substrate. This study concluded that in the active site of these enzymes there is a pocket lined entirely by hydrophobic residues (i.e., T37, M39, F74, Y82, A85, and L118) where large substituents of the substrate can be favourably accommodated (Figure 4). The access to this pocket can be physically blocked by the presence of a bulky amino acid in position 116. In this case the substrate adopts a different orientation in which the large substituent is projected into a portion of the active site partially defined by the side-chains of F250, F296, and Y375 (Figure 4). This accommodation is generally less favourable because it induces a rearrangement of the side-chains of both F296 and Y375 from their preferred positions in the apoprotein.[22]

These observations are crucial to understand the stereochemical course of OYE-mediated reactions of cyano esters (*Z*)-**1a–g**. For these substrates the bulkiest substituent is the aryl ring linked to the carbon atom in the α position to the nitrile, whose best accommodation is within the hydrophobic pocket in the right side of the active site. The achievement of this orientation, made accessible by small amino acids, implies a classical binding mode for the substrate (Scheme 5a). When larger amino acids, such as Leu, His and Met, hinder the occupancy of this pocket, a flipped orientation is adopted by the substrate with the aromatic ring pointing towards the left side (Scheme 5b).

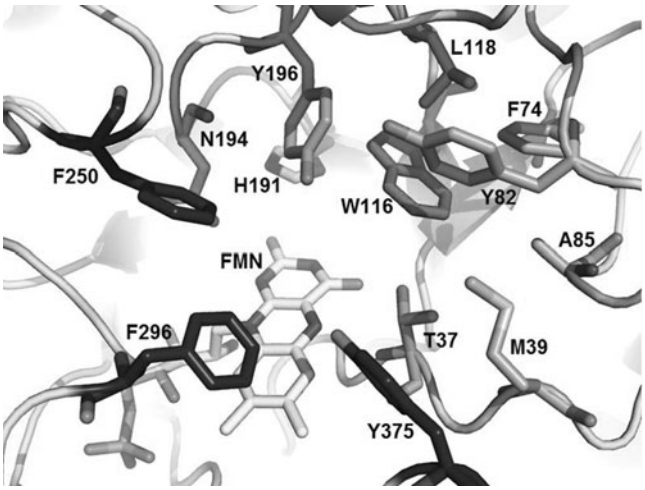


Figure 4. Structure of active site of oxidized OYE1 from the crystal data of Fox and Karplus[25] (PDB code: 1OYA).

We could make some considerations on these results by taking into account the different size of the amino acid residues represented by the numerical values of their Van der Waals volumes (Figure 5).[26]

The presence in position 116 of an amino acid characterised by a side-chain volume higher than 130 Å^3 (W116F, K, Y and R variants and wild-type) inhibited the reduction of cyano esters (*Z*)-**1a–g**: the substrate was allowed to bind the active site neither in a classical nor in a flipped orientation. The first was forbidden by the size of the residue in position 116, the latter by the necessity to re-orient the side chains of some residues on the opposite side of the binding pocket. The reactions did not occur also with W116D and E, in which the tryptophan was replaced by an acidic amino acid, and with W116P, characterised by the presence of the sterically constrained ring of proline.

With amino acids showing a volume lower than 100 Å^3, the reactive substrate binding mode was the classical one, affording the (*S*)-enantiomer of the reduced products. 120 Å^3 represents a kind of threshold for the transition from (*S*)-selectivity (with W116Q and I) to (*R*)-selectivity (with W116H, L and M). Modest *ee* values were obtained with all the substrates and W116Q, H, I, and M variants, whereas the presence of the leucine residue created the optimal situation for a flipped binding mode.

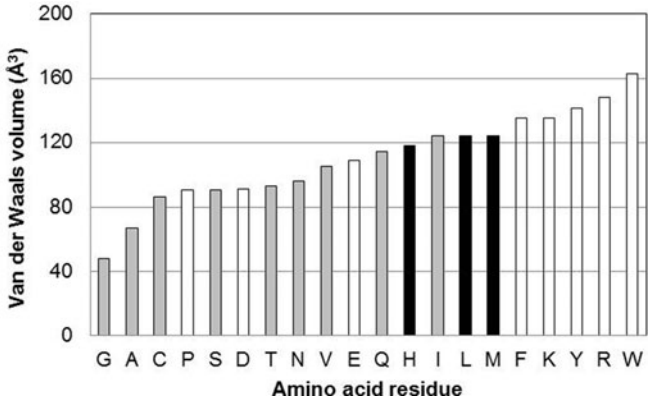


Figure 5. Van der Waals volumes of amino acid residues *vs.* outcome of the bioreduction catalysed by the corresponding OYE1-W116X variant [grey bars: (*S*)-selective bioreductions; black bars: (*R*)-selective bioreductions; white bars: bioreductions with low or no conversions].

Table 4. Effect of pH on the bioreduction of (*Z*)-**1a** and **c** mediated by OYE1-W116A and OYE1-W116L.

pH[a]	(*Z*)-**1a**, R = H		(*Z*)-**1c**, R = *p*-Cl	
	c [%][b]	*ee* [%][c]	c [%][b]	*ee* [%][c]
		OYE1-W116A		
4.0	0	–	0	–
5.0	43	86 (*S*)	33	86 (*S*)
6.0	100	90 (*S*)	85	82 (*S*)
7.0	100	48 (*S*)	92	60 (*S*)
8.0	96	40 (*S*)	97	22 (*S*)
9.0	93	*rac*	90	*rac*
		OYE1-W116L		
4.0	0	–	0	–
5.0	18	90 (*R*)	15	70 (*R*)
6.0	100	94 (*R*)	92	88 (*R*)
7.0	91	88 (*R*)	84	66 (*R*)
8.0	100	60 (*R*)	100	40 (*R*)
9.0	100	40 (*R*)	97	*rac*

[a] Buffers: acetate for pH 4.0–5.0, phosphate for pH 6.0–8.0, tris for pH 9.0.
[b] Conversion, calculated on the basis of GC analysis of the crude mixture after 24 h reaction.
[c] Calculated on the basis of GC analysis on a chiral stationary phase.

Synthesis of (*R*)- and (*S*)-β-Aryl-γ-lactams

One of the aims of this work is to optimise efficient synthetic procedures to enantiopure chiral building blocks for fine chemicals production. A two-step sequence, consisting in the biocatalysed hydrogenation of the alkene followed by the chemical reduction of the nitrile, can be employed to convert substrates (*Z*)-**1a**–**g** into chiral β-aryl-γ-lactams,[27] whose structural pattern is inserted in many active pharmaceutical ingredients such as baclofen,[28] phenibut[29] and rolipram.[30] For these drugs the (*R*)-enantiomer has been identified to be the most active one.

The intermediate reduced compounds **2a**–**g** are characterised by the presence of a benzylic stereogenic centre which could be configurationally unstable because of the acidity of the hydrogen atom in the α position to the CN group. In a first screening evaluation the bioreductions of compounds (*Z*)-**1a–g** were performed in pH 7.0 buffer solutions, as it is usually reported in the literature. The dependence of the enantiomeric purity of the reduced compounds on the reaction pH was investigated in the range 4–9 for substrates (*Z*)-**1a** and **c**, precursors of phenibut and baclofen, with two mutants of opposite enantioselectivity, OYE1-W116A and OYE1-W116L (Table 4). The acidic medium inhibited the reduction, whereas basic conditions caused a significant loss of the enantiomeric purity of compounds **2a** and **c**, as a consequence of the configurational lability of the stereocentre.

The optimal value was established to be pH 6.0, thus the screening of OYE1-W116X variants in the reduction of cyano esters (*Z*)-**1a**–**g** was repeated at this pH value (see Tables S3 and S4 in the Supporting Information for the complete set of data). Figure 6 gives at a glance the general increase of the enantioselectivity of the reaction of all the substrates with a selection of mutants. Conversions were in general lower, but the decrease was not substantial.

OYE1-W116A afforded the best results for the synthesis of (*S*)-**2a**, **c**–**g** in nearly quantitative yields and *ee* values in the range 82–95% in 24 h reaction time. Derivative (*S*)-**2b** could be obtained only with poor enantiomeric enrichment. OYE1-W116L was selected for the preparation of the (*R*)-enantiomers of derivatives **2**, in particular (*R*)-**2a** and (*R*)-**2c**, precursors of the active forms of phenibut and baclofen.

The reduction of the nitrile by treatment with $NiCl_2 \cdot 6H_2O$ and $NaBH_4$ (Scheme 6), already employed by Fryszkowska et al.,[19] was performed in this case directly in the aqueous medium of the biocatalysed reduction, without isolation of the intermediate cyano esters **2**, to afford, after extraction and isolation, the corresponding lactams (*R*)-**3a** and (*R*)-**3c**.

This procedure represents a very effective route to the pharmacologically active enantiomers of phenibut and baclofen. The bioreductions, performed in pH 6.0 buffer solution in the presence of OYE1-W116L, afford (*R*)-**2a** (*ee* = 94%) and (*R*)-**2c** (*ee* = 90%), respectively, in 2.7 and 2.0 $g\,L^{-1} \cdot d^{-1}$ space-time yields, showing potential scalability. The use of the system $NiCl_2/NaBH_4$ has the advantage to afford the reduction of the nitrile quantitatively and almost instantaneously, and to be compatible with the water medium of the enzymatic reactions. Thus, the two subsequent reduction steps, the first catalysed by the enzyme and the second promoted by the chemical reducing agent, can be easily telescoped, without impairing the enan-

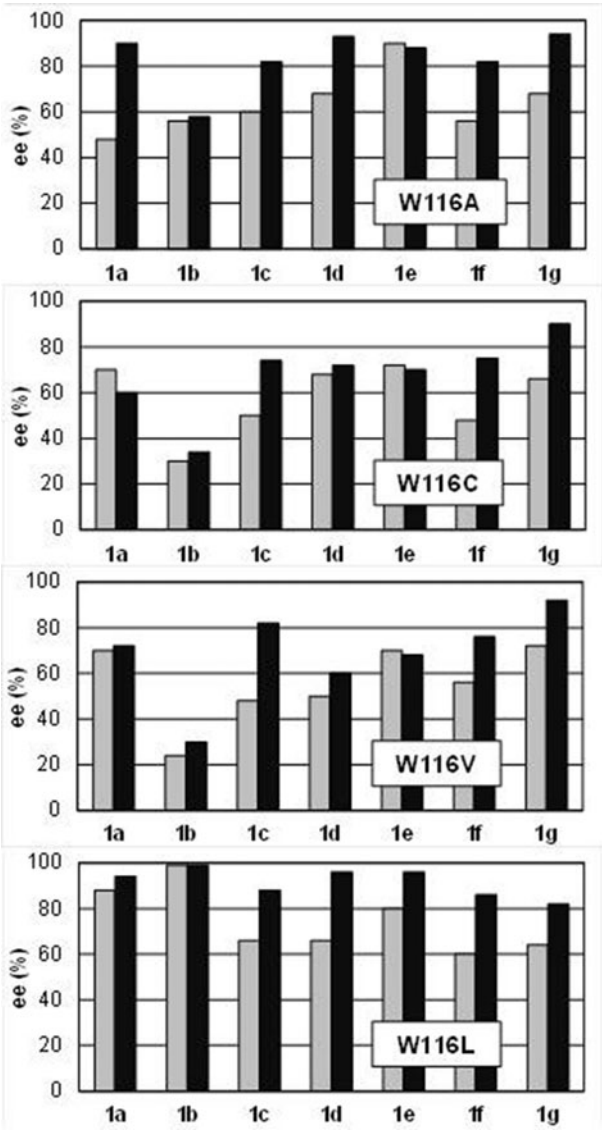
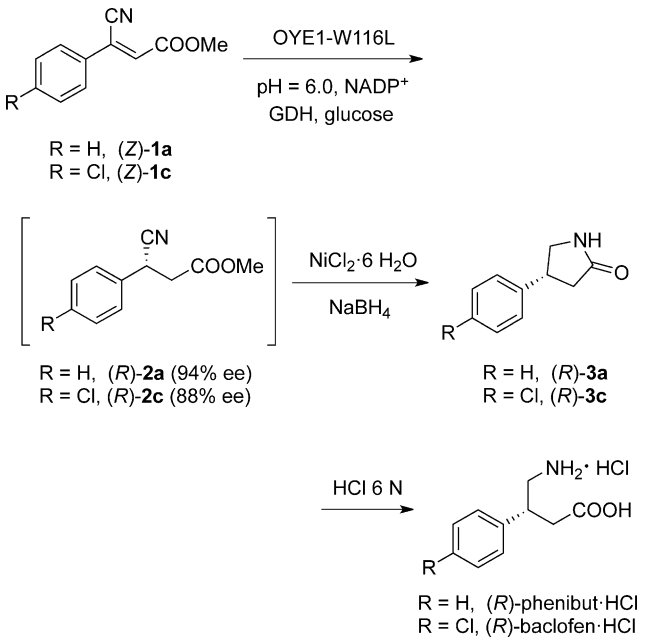


Figure 6. Effect of pH on the enantioselectivity of the bioreduction of (*Z*)-**1a–g** with selected variants (grey bars: *ee* values obtained at pH 7.0; black bars: *ee* values obtained at pH 6.0).

tiomeric excess of the final compounds, with the advantage of diminishing the number of purification operations, and contributing significantly to decrease cycle time, solvent usage and waste.

Conclusions

The reductions of cyano esters (*Z*)-**1a–g** catalysed by OYE1-W116X mutants confirm the strategic role played by the amino acid in position 116 on the stereochemical course of these reactions by controlling the access to a hydrophobic pocket that favourably hosts bulky substituents.



Scheme 6. Synthetic procedure to the lactams (*R*)-**3a** and (*R*)-**3c**, precursors of (*R*)-phenibut and (*R*)-baclofen.

These results are in agreement with the considerations made on the importance of the relative size of the G and α groups in establishing the reactive binding mode for acyclic alkenes with the EWG linked to the prostereogenic carbon atom (Scheme 3). When wild-type OYE1-3 are employed, a Trp occupies position 116, and the favoured substrate orientation is the one in which a small group, either G or α, is located within the hydrophobic pocket in the right side of the active site.

OYE1-W116A and OYE1-W116L were selected for the preparation of the (*S*)- and (*R*)-enantiomer, respectively, of derivatives **2a–g**. We showed the possibility to convert these reduced enantiomerically enriched intermediates into the corresponding (*S*)- and (*R*)-β-aryl-γ-lactams by a simple functional group interconversion to be performed according to a sequential one-pot procedure by addition of the chemical reagent for the reduction of the CN group directly to the biotransformation medium.

Experimental Section

Sources of Enzymes

Wild-type Old Yellow Enzymes fused with a His_6-tag (OYE1 from *Saccharomyces pastorianus,* OYE2 and OYE3 from *Saccharomyces cerevisiae*), OYE1-W116X site-saturation mutagenesis library fused with a GST tag (OYE1-W116X from *Saccharomyces pastorianus)* and glucose dehydrogenase fused with a His_6-tag (GDH from *Bacillus megaterium*) were overproduced in *Escherichia coli* BL21(DE3)

strains, harbouring the following plasmids: pET-30a-OYE1 containing the *oye1* gene provided by Neil C. Bruce;[31] pET-30a-OYE2 and pET-30a-OYE3 containing the *oye2* and *oye3* genes cloned from *S. cerevisiae* BY4741;[32] pDJB5-OYE1-W116X provided by J. D. Stewart;[7c] pKTS-GDH containing the *gdh* gene cloned from *B. megaterium* DSM509.[32]

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

LB medium (15 mL) supplemented with the appropriate antibiotic (50 μg mL^{-1} kanamycin for pET-30a and pDJB5, 100 μg mL^{-1} ampicillin for pKTS) was inoculated with a single colony from a fresh plate and grown overnight at 37 °C and 220 rpm. This starter culture was used to inoculate 1.5 L of LB medium, which was incubated at 37 °C and 220 rpm until OD_{600} reached 0.4–0.5, then enzyme expression was induced by the addition of IPTG (0.1 mM final concentration). For the pKTS-GDH plasmid anhydrotetracycline was also added (50 ng mL^{-1} final concentration). After 5 h, the cells were harvested by centrifugation (5000 × *g*, 20 min, 4 °C), resuspended in 50 mL of lysis buffer (20 mM potassium phosphate buffer pH 7.0, 300 mM NaCl, 10 mM imidazole), disrupted by sonication (Omni Ruptor 250 ultrasonic homogeniser, five sonication cycles, 15 s each, 50% duty) and centrifuged (20000 × *g*, 20 min, 4 °C). The cell-free extracts of GST-tagged OYE1-W116X mutants were aliquoted and stored frozen at −80 °C. The cell-free extracts of His-tagged OYE1–3 and GDH, were purified by affinity chromatography on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM potassium 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 50 mM potassium 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 Enzyme-Mediated Biotransformations of Substrates 1a–g (Screening)

A solution of the substrate in DMSO (10 μL, 500 mM) was added to a potassium phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μmol), $NADP^+$ (0.1 μmol), GDH (4 U) and the required purified or cell-free extract OYE (80–120 μg). 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. Two replicates were performed for each biotransformation: no significant differences (less than 5%) were observed for conversion and enantiomeric excess values.

General Procedure for OYE-W116X Cell-Free Extracts-Mediated Biotransformations

For substrates (*Z*)-**1a–g** a similar protocol was followed on a larger scale (50 mg), employing the appropriate OYE1-W116X mutant that provided the best conversion and/or *ee*, in order to isolate and characterise the corresponding reduced product. A solution of the suitable cyano ester in *i*-PrOH (1 mL, 250 mM) was added to a potassium phosphate buffer solution (25 mL, 50 mM, pH 6.0 or pH 7.0) containing the required OYE (4–5 mg), GDH (100 U), glucose (1 mmol, 180 mg) and $NADP^+$ (5 μmol, 3.7 mg). The reaction was monitored by GC until complete conversion (generally 24 h). The mixture was then extracted with EtOAc (3 × 10 mL), dried over anhydrous Na_2SO_4 and purified by column chromatography (*n*-hexane with increasing amount of EtOAc).

Bioreduction Procedure for the Preparation of Monodeuterated Samples

A solution of the substrate in *i*-PrOH (100 μL, 500 mM) was added to potassium phosphate buffer solution (5.0 mL, 50 mM in D_2O, pH 7.0) containing glucose (20 μmol), NADH (75 μmol) and the required OYE1 mutant (1–3 mg). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30 °C). The solution was extracted with EtOAc (3 × 5.0 mL), centrifuging after each extraction (3000 × *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

In situ Preparation of (*R*)- and (*S*)-β-Aryl-γ-lactams[19]

General procedure: The enantioselective reduction of the suitable (*Z*)-methyl 3-cyano-3-arylacrylate was performed with the required OYE1-GST fusion protein mutant as it has been already described for biotransformations on the 50-mg scale. The reaction was monitored by GC/MS. When the reduction was complete, $NiCl_2 \cdot 6H_2O$ (1 equiv.) and $NaBH_4$ (3 equiv.) were added cautiously to the reaction mixture under vigorous stirring. After 1 h, the mixture was extracted with EtOAc (3 × 10 mL) and purified by column chromatography (*n*-hexane with increasing amount of EtOAc).

(*R*)-4-Phenylpyrrolidin-2-one [(*R*)-3a]: From the corresponding unsaturated compound (*Z*)-**1a** (50.0 mg, 0.27 mmol) by reaction with OYE1-W116L in buffer solution (pH 6.0, followed by *in situ* nitrile reduction, derivative (*R*)-**3a** was obtained; yield: 26.5 mg (61%); $[\alpha]_D$: −34.1 (*c* 0.55, MeOH) {lit.[33] $[\alpha]_D$: −36 (*c* 0.5, MeOH) for (*R*)-**3a** with *ee* = 99.9%}.^{1}H NMR (400 MHz, $CDCl_3$):[33] δ = 7.40–7.30 (m, 5H), 3.72 (m, 1H), 3.65 (m, 1H), 3.36 (dd, *J* = 9.0, 7.1 Hz, 1H), 2.68 (dd, *J* = 17.0, 8.9 Hz, 1H), 2.45 (dd, *J* = 17.0, 9.0 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$):[33] δ = 177.3, 142.1, 128.9, 127.2, 126.7, 49.3, 40.4, 37.7; GC-MS (EI): t_r = 21.56 min, *m/z* (EI) = 161 (M^+, 50), 133 (7), 104 (100).

(*R*)-4-(4-Chlorophenyl)pyrrolidin-2-one [(*R*)-3c]: From the corresponding unsaturated compound (*Z*)-**1c** (50.0 mg, 0.23 mmol) by reaction with OYE1-W116L in buffer solution (pH 6.0), followed by *in situ* nitrile reduction, derivative (*R*)-**3c** was obtained; yield: 30.4 mg (68%); $[\alpha]_D$: −34.7 (*c* 0.78, EtOH) {lit.[33] $[\alpha]_D$: −38.0 (*c* 1. EtOH) for (*R*)-**3c** with *ee* = 99.9%}; ^{1}H NMR (400 MHz, $CDCl_3$):[33] δ = 7.31 (m, 2H), 7.18 (m, 2H), 3.77 (m, 1H), 3.66 (m, 1H), 3.37 (dd, *J* = 8.5, 7.5 Hz, 1H), 2.72 (dd, *J* = 17.0, 8.9 Hz, 1H), 2.45 (dd, *J* = 17.0, 8.0 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$):[33] δ = 177.1, 140.7, 133.0, 129.0, 128.1, 127.1, 49.3, 37.7; GC-MS (EI): t_r = 24.34 min, *m/z* (EI) = 195 (M^+, 46), 138 (100), 103 (23).

Supporting Information

Tables S1–S4, characterization data of cyano esters **1a–g** and reduced products **2a–g**, analytical methods for the determination of conversion and enantiomeric excess, crystallographic data, and copies of the NMR spectra are given in the Supporting Information.

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