Opposite enantioselectivity in the bioreduction of (*Z*)-β-aryl-β-cyanoacrylates mediated by the Trp 116 mutants of Old Yellow Enzyme 1: synthetic approach to (*R*)- and (*S*)-β-aryl-γ-lactams.

Elisabetta Brenna,*[a,b] Michele Crotti,[a] Francesco G. Gatti,[a] Daniela Monti,[b] Fabio Parmeggiani,[a] Robert W. Powell III,[c] Sara Santangelo[a], and Jon. D. Stewart[c]

[a] Prof. E. Brenna, M. Crotti, Prof. F.G. Gatti, Dr. F. Parmeggiani, Miss S. Santangelo. Dipartimento di Chimica, Materiali ed Ingegneria Chimica "Giulio Natta" Politecnico di Milano, P.zza Leonardo Da Vinci 32, 20133, Milano, Italy. Tel: +39-02-2993077, Fax: +39-02-23993180. E-mail: mariaelisabetta.brenna@polimi.it

[b] Dr. D. Monti, Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco, 9, 20131, Milano, Italy; Tel: +39-02-28500038.

[c] Mr R. W. Powell III, Prof. J. D. Stewart, Department of Chemistry, University of Florida, 126 Sisler Hall, Gainesville, Florida 32611, United States

Abstract

The Trp 116 mutants of Old Yellow Enzyme 1 that catalyse the reduction of (*Z*)-β-aryl-β-cyanoacrylates give 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 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, Enzyme catalysis, enantioselectivity, reduction, nitriles, lactams

Introduction

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

Several issues are still to be fully addressed: a) the speed of enzyme optimisation processes, from the screening phase, to the production of kg amounts of enzyme for the proof-of-concept, and finally to the commercial supply; b) the availability of a large portfolio of biocatalysed chemical transformations, for which the substrate scope has been already investigated.

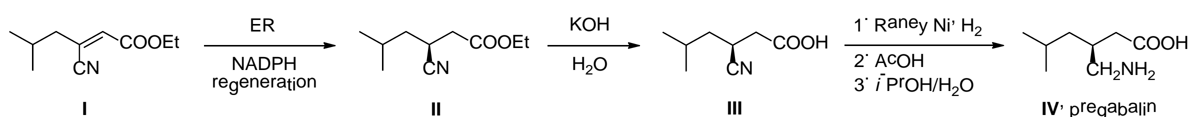
It is also necessary to promote among synthetic chemists a new way of planning the synthesis of target molecules, by introducing the concept of "biocatalytic retrosynthesis".[2] The idea is to take into consideration the possibility to disconnect molecules on the basis that biocatalysts are available for the strategic bond-forming steps, and that the identified building blocks can be prepared by biocatalysed functional groups interconversions. This approach requires the availability of a large array of optimised enzymatic transformations, and the understanding of how bio- and chemocatalysed procedures can be combined in the most efficient and productive way.

The enantioselective reduction of suitably substituted C=C double bonds represents a key chemical transformation for the creation of stereogenic centres in chiral building blocks to be used for fine chemicals manufacturing. The reaction is efficiently catalysed by enzymes called ene-reductases (EC 1.6.99.1, ERs), most of which belong to the family of Old Yellow Enzymes (OYEs).[3] Many efforts have been devoted not only to isolate new ERs,[4] in order to enlarge the collections

of the available wild-type catalysts, but also to improve considerably their performance by protein engineering,[5] affording variants with broader substrate scope or opposite enantioselectivity.

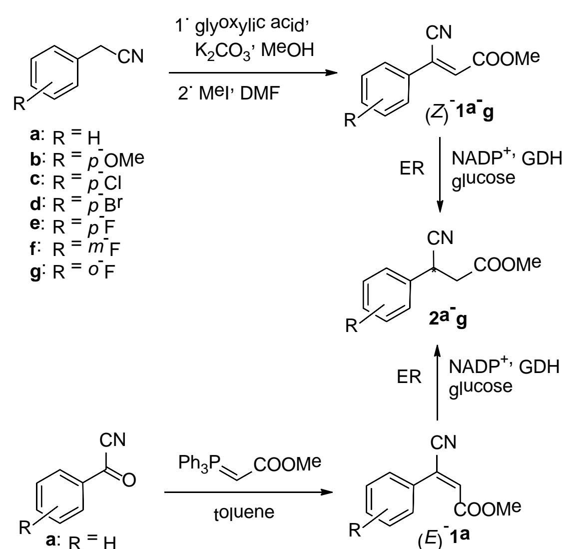
Currently, the mechanism[6] of OYE-mediated hydrogenations and the stereoelectronic effects of the substituents on the starting alkene[4, 7] are extensively investigated, in order to define the limits and the potential of the reactions in the field of organic synthesis. It has been established that at 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.[8] The presence of a single CN group has been found to activate the bioreduction only in the case of α-methylenic derivatives. [9] Generally, unsaturated esters are not reduced by OYEs, unless a halogen atom is linked in α position, [10] or another EWG (*e.g.* an ester, [11] a nitrile[6e, 12] or a nitro group[13]) is linked to the β olefinic carbon atom.

The bioreduction of these difunctionalised alkenes is 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 compound **I** (Scheme 1) mediated by OPR1 (from *Lycopersicon esculentum*) was carefully optimized by Pfizer to afford the (*S*)-enantiomer of the reduced product **II** with ee >99% on a preparative scale. [14] The latter 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.



Scheme 1. Chemoenzymatic synthesis of pregabalin.

The data collected in the literature on the bioreduction of derivatives of type **I** are limited to compounds bearing an alkyl substituent at the prostereogenic carbon atom.[6e, 12] We decided to extend the investigation to those having an aromatic ring in this position, *i.e.* substrates **1a-g** (Scheme 2), because we 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.



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

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, 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 aryl cyanoesters were those described for the potassium salts of compounds (*Z*)-**1a**, **b**, **c** and **e**. [15] These derivatives 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 H_2 atmosphere to afford the (*S*)-enantiomer of the reduced products in quantitative yields and high enantiomeric excess values in 3 days reaction time.

*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 conversions were observed (Table S1, Supporting Information). On the contrary, when compound (*E*)-**1a** (de = 60 %) was employed, the (*E*)-alkene was completely reduced to afford (*R*)-**2a** (ee = 70-84 %) and the (*Z*)-stereoisomer was left unreacted (Table 1).

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

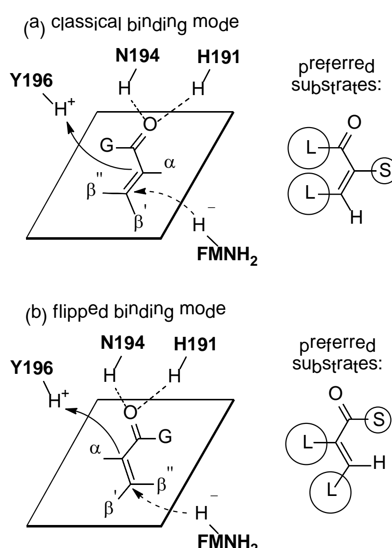
	(*Z*)-**1a**		(*E*)-**1a**	
ER	**c (%)[a]**	**ee (%)[b]**	**c (%)[a]**	**ee (%)[b]**
OYE1	5	–	99	82 (*R*)
OYE2	2	–	99	84 (*R*)
OYE3	8	–	99	70 (*R*)

[a] conversion, calculated by GC analysis of the crude mixture after 24 h reaction time; [b] calculated by GC analysis on a chiral stationary phase.

In the case of (*E*)-**1a**, we proved that the CN moiety was the activating EWG, *i.e.* the one establishing hydrogen bonds with the amino acids residues of the enzyme binding pocket. As a

matter of fact, the ^{1}H NMR spectrum of the reduced product (*R*)-**2a**-d_1, obtained by OYE1-mediated reduction of (*E*)-**1a** in D_2O in the presence of a stoichiometric quantity of NADH, [16] showed the incorporation of a deuterium atom at the carbon atom in α position to the nitrile (Figure S1a-b, Supporting Information).

This marked preference for the OYE-mediated reduction of (*E*)-**1a** with respect to the (*Z*)-stereoisomer is in agreement with the conclusions we have recently drawn on the stereochemistry of OYE1-3 mediated reductions, [12c, 17] by considering the two possible substrate orientations already described in the literature: the "classical" (or "normal") binding mode[6a] and the "flipped" binding mode[7b] (Scheme 3). The first was inferred from the structure of oxidised OYE1, bearing *p*-hydroxybenzaldehyde in the active site; the latter was obtained 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 β position to the activating moiety.



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).

The analysis of the literature data collected for those acyclic trisubstituted olefins, which are activated by an EWG linked to the prostereogenic carbon atom and are efficiently converted by OYE1-3 enzymes, allowed the following observations to be made.[12c] The preferred reactive binding mode seems to be determined by the relative size of the groups shown as G and α (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 at C_β (L) 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 at C_β (L) on the opposite side of the EWG.

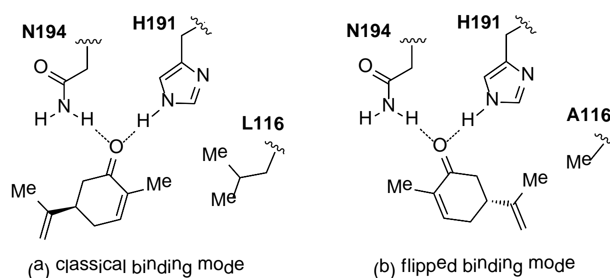
These considerations can be extended also to alkenes for which the activating EWG is a CN moiety without G group.[6e] Thus, for the stereoisomers of cyanoester **1a**, the hindrance of the phenyl ring favours a flipped orientation, which is optimal for the (*E*)-stereoisomer, and it is in agreement with the (*R*) configuration of the reduced product.

*OYE1-W116X-mediated reductions of compounds (Z)-**1a-g***

From a synthetic route planning perspective, the aryl cyanoesters **1a-g** are much more easily obtained as (*Z*)- rather than (*E*)-stereoisomers, so it would be highly advantageous to find a way to get the former accepted by the enzyme, *e.g.* by protein engineering, rather than struggling to synthesize the latter.

It has been recently established that the Trp 116 residue in *S. pastorianus* OYE1 (conserved in OYE2-3) plays a critical role in the stereochemistry of OYE-mediated alkene reductions by influencing the substrate orientation within the active site.[18] The stereochemistry of the bioreductions of (*R*)- and (*S*)-carvone with wild-type OYE1 and OYE1-W116X variants has been investigated in details by considering the X-ray crystal structures of *pseudo*-Michaelis complexes formed *in cristallo* by soaking each protein individually with the substrate. [19]

For example, the crystallographic studies of the W116L mutant of OYE1 with (*R*)-carvone bound in the active site (PDB code 4GWE; Figure S2a, Supporting Information) showed that the presence of Leu in position 116 favors the classical orientation of the substrate (Scheme 4a) with the bulky isopropenyl substituent pointing in the opposite direction. When the indole ring of Trp is replaced by the methyl group of Ala, a hydrophobic pocket is made available in the active site for the location of the isopropenyl group (PDB code 4K7V; Figure S2b, Supporting Information) and (*R*)-carvone adopts a flipped binding mode (Scheme 4b).



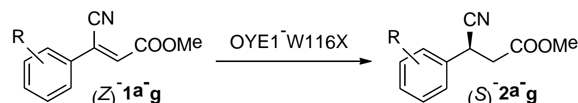
Scheme 4: Binding modes of (*R*)-carvone in the active site of OYE1-W116L (a) and OYE1-W116A (b), from ref. [19].

Therefore, we decided to investigate the transformation of cyanoesters (*Z*)-**1a**-**g** by using the complete set of Trp 116 mutants of OYE1.[17] The results of the biocatalysed reductions highlighted the possibility to organize 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 3); C) Trp (wild-type), Asp, Glu, Phe, Lys, Pro, Arg and Tyr mutants characterized 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 *p*-methoxy derivative (*Z*)-**1b**, catalyzed by the W116I, W116Q and W116T variants which afforded compound (*R*)-**2b**, although with poor

selectivity, and in the reduction of *o*-fluoro cyanoester (*Z*)-**1g** with the W116M mutant giving derivative (*S*)-**2g**.

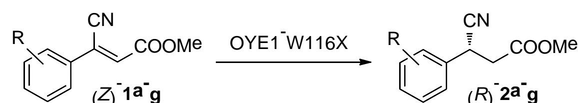
Table 2. Biotransformations of compounds (*Z*)-**1a-g** affording (*S*)-**2a-g** (pH = 7.0).



	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	
Variant	**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] (%)**
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 by GC analysis of the crude mixture after 24 h reaction time (isolation yields are reported in the Supporting Information); [b] calculated by GC analysis on a chiral stationary phase; [c] calculated by HPLC analysis on a chiral stationary phase.

Table 3. Biotransformations of compounds (*Z*)-**1a-g** affording (*R*)-**2a-g** (pH = 7.0)

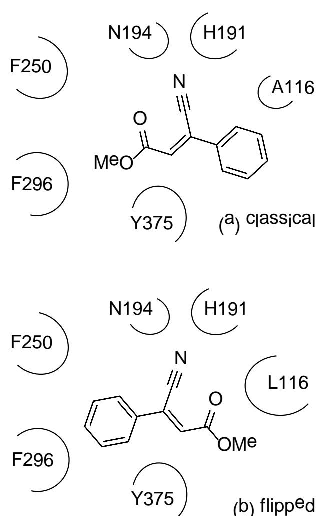


	1a, R = H		**1b**, R = *p*-OMe		**1c**, R = *p*-Cl		**1d**, R = *p*-Br		**1e**, R = *p*-F		**1f**, R = *m*-F		**1g**, R = *o*-F	
Variant	**c[a] (%)**	**ee[b] (%)**	**c[a] (%)**	**ee[c] (%)**	**c[a] (%)**	**ee[b] (%)**	**c[a] (%)**	**ee[c] (%)**	**c[a] (%)**	**ee[b] (%)**	**c[a] (%)**	**ee[b] (%)**	**c[a] (%)**	**ee[c] (%)**
W116H	42	74	44	99	13	67	40	54	18	99	5	–	7	–
W116L	91	88	95	99	34	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 by GC analysis of the crude mixture after 24 h reaction time (isolation yields are reported in the Supporting Information); [b] calculated by GC analysis on a chiral stationary phase; [c] calculated by HPLC analysis on a chiral stationary phase.

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

We determined the activating group for substrate (*Z*)-**1a** in the reactions with W116L and W116A, characterized 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 S1c-d, Supporting Information). If an *anti* hydrogen addition is hypothesized, the absolute configuration of the reduced products allows the substrate binding mode in the enzyme active site to be inferred. A classical binding mode (Scheme 5a) can be deduced for the W116A mutant (or any of the variants belonging to group A) and a flipped one (Scheme 5b) for the W116L mutant (or any of the variants belonging to group B).



Scheme 5: Binding modes of (*Z*)-**1a-g** in the active site of OYE1-W116A (a) and OYE1-W116L (b) deduced by analysis of the bioreduction results.

Thus, also for cyanoesters **1a-g** the amino acid mutation in position 116 controls the orientation of the substrate in the active site, in a manner that appears to be reversed with respect to (*R*)-carvone. If only the mutants which are able to reduce to a certain extent (>20%) both (*R*)-carvone and (*Z*)-**1a** are considered, it is possible to notice that: i) the presence of Ala or Val in position 116 promotes a productive binding mode which is flipped for (*R*)-carvone and normal for **1a**; ii) with Leu, His and Met, (*R*)-carvone adopts a classical orientation and compound **1a** a flipped one.

The conclusion drawn on the bioreduction of carvone enantiomers was that the flipped orientation was the preferred binding mode for the substrate. The X-ray structures showed that the presence of a small amino acid in position 116, such as Ala or Val, enabled the accommodation of the bulky isopropenyl group of carvone into a pocket lined by hydrophobic protein residues on the eastern side of the active site (Figure S3). When this arrangement became sterically forbidden by a certain mutation of the amino acid in position 116, the enzyme accepted the alternative classical binding mode, which required the re-orientation of the side chains of some residues (*i.e.* Phe 250, Phe 296, and Tyr 375, see Figure S2) in the western side of the active site.

Cyanoesters (*Z*)-**1a-g** are characterized by different molecular geometry and alkene substitution pattern. The activating EWG is a linear nitrile moiety instead of a trigonal carbonyl group, and the bulkiest substituent, whose best accommodation is within the hydrophobic pocket in the eastern side of the active site, is the aryl ring linked to the carbon atom in α position to the nitrile. For these substrates, 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 western side (Scheme 5b).

We attempted to support these conclusions on the reactive binding mode of β-aryl-β-cyanoesters 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 to be obtained) and (*Z*)-**1e** (PDB code to be obtained). 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 S3, Supporting Information). First of all, the activating nitrile group was not directed towards Nε2 of His 191 and Nδ2 of Asn 194 (Figure S3), but it was positioned between Tyr 82 and Tyr 375. The hydrogen

bonds with His 191 and Asn 194 are known to be essential for the catalytic turnover by OYE1.[20] Then, the orientation of the alkene with respect to the FMN cofactor was not optimal: the angle formed between C_β of the cyanoester and the plane of the FMN is 121.5° for **1a** and 112.9° for **1e**; the distance between C_β of the cyanoester and N5 of FMN is 4.70 Å for **1a** and 4.78 Å for **1e**. These values are outside the range observed by Fraaije in his survey of flavoproteins bound to their respective substrates and/or products. [21]

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

Synthesis of (R)- and (S)-β-aryl-γ-lactams

One of the aims of this work is to optimize efficient synthetic procedures to enantiopure chiral building blocks for fine chemicals production. Compounds **2a-g**, obtained by bioreduction of substrates (*Z*)-**1a-g**, can be converted by reduction of the nitrile moiety into chiral β-aryl-γ-lactams, [22] whose structural pattern is inserted in many active pharmaceutical ingredients such baclofen, [23] phenibut[24] and rolipram. [25] For these drugs the (*R*)-enantiomer has been identified to be the most active one.

The bioreductions of compounds (*Z*)-**1a-g** were routinely performed in pH = 7.0 buffer solutions. To exclude effects due to the possible configurational instability of the reduced compounds **2a-g** on the enantioselectivity values of the reactions, some experiments were also performed in pH = 6.0 buffer solution. The slightly acidic reaction medium was generally not detrimental for the conversions, and with some variants, *i.e.* W116A, C, L, and V, produced an increase of the enantiomeric purity of all the final compounds **2a**-**g** (Figure 1 and Table 4). The complete data set of the reactions run in pH = 6.0 buffer solution is reported in the Supporting Information (Tables S3-S4).

Table 4. Selected biotransformations of compounds (*Z*)-**1a-g** in pH = 6.0 buffer solution.

	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	
Variant	**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] (%)**
(*S*)-selective														
W116A	100	90	85	58	85	82	100	93	100	88	100	82	100	94
W116C	31	60	78	34	84	74	85	90	72	70	100	75	53	90
W116V	100	72	100	30	100	82	80	60	100	68	100	76	100	92
(*R*)-selective														
W116L	100	94	100	99	92	88	100	96	100	96	100	86	94	82

[a] conversion, calculated by GC analysis of the crude mixture after 24 h reaction time (isolation yields are reported in the Supporting Information); [b] calculated by GC analysis on a chiral stationary phase; [c] calculated by HPLC analysis on a chiral stationary phase.

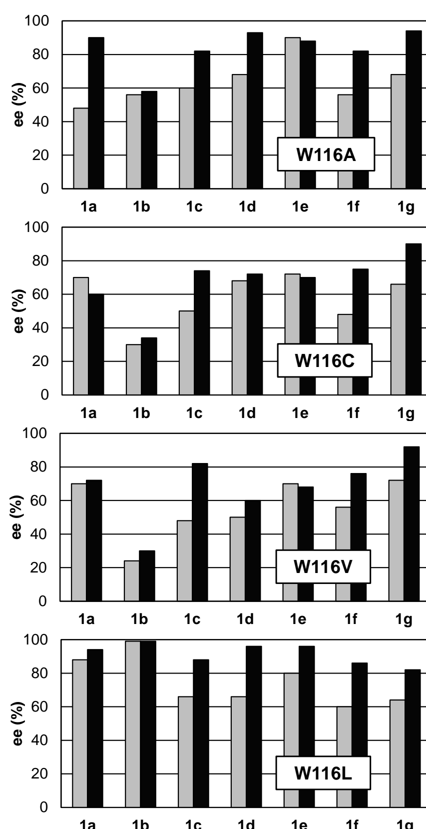
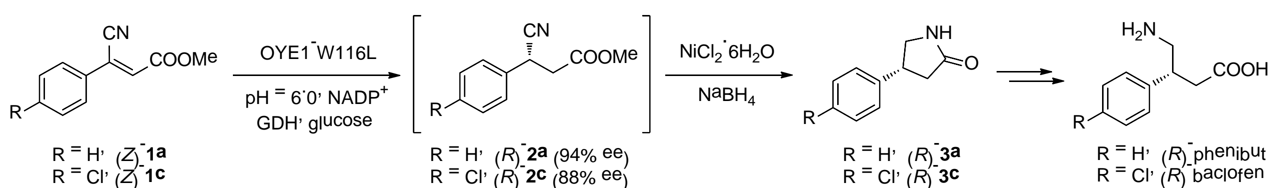


Figure 1: Effect of pH on the enantioselectivity of the bioreduction of (*Z*)-**1a-g** with selected variants (grey bars: ee values obtained at pH = 7.0; black bars: ee values obtained at pH = 6.0).

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) was performed directly in the aqueous medium of the biocatalysed reduction, without isolation of the intermediate cyanoesters **2**, to afford, after extraction and isolation, the corresponding lactams (*R*)-**3a** and (*R*)-**3c**.



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

This procedure represents a very effective route to the pharmacologically active enantiomers of phenibut and baclofen. The two subsequent reduction steps can be telescoped, without impairing the enantiomeric 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 cyanoesters (*Z*)-**1a**-**g** catalyzed 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 favorably hosts bulky substituents. We could make some considerations on our 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 2).[26]

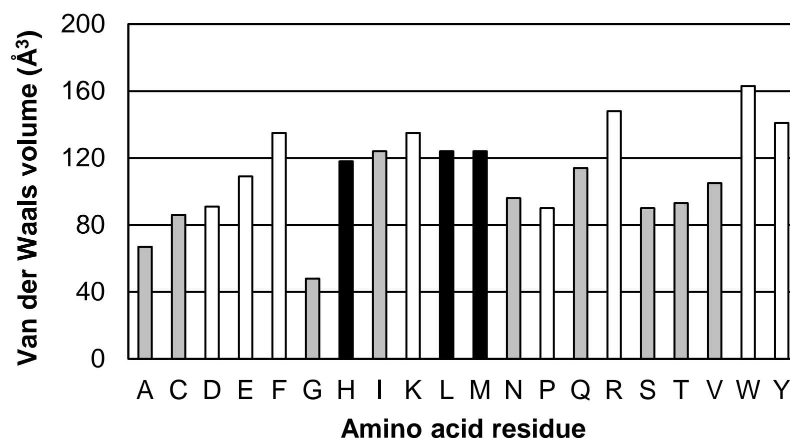


Figure 2. Van der Waals volumes of amino acid residues *vs.* outcome of the bioreduction catalyzed by the corresponding OYE1-W116 variant (grey bars: (*S*)-selective bioreductions; black bars: (*R*)-selective bioreductions; white bars: bioreductions with low or no conversions).

The presence in position 116 of an amino acid characterised by a side-chain volume higher than 130 Å^3 (wild-type and W116F, R K and Y variants) inhibited the reduction of cyanoesters (*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 Trp unit 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. 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 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 eastern 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

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 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-GST fusion protein 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. 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.

Supporting Information

Tables S1-4, Figures S1-3, descriptions of strains and enzymes, characterization data of cyanoesters **1a**-**g**, reduced products **2a-g**, and lactam derivatives (*R*)-**3a** and (*R*)-**3c**, analytical procedures for the determination of conversion, and enantiomeric excess, crystallographic data, copies of NMR spectra are given in the Supporting Information.

References

[1] J. Lalonde, *Speciality Chemicals Magazine,* on line issue October 2013.

[2] N. J. Turner, E. O'Reilly, *Nature Chem. Biol.* **2013**, *9*, 285 – 288.

[3] K. Saito, D. J. Thiele, M. Davio, O. Lockridge, V. Massey, *J. Biol. Chem.* **1991**, *266*, 20720-20724; R. Stuermer, B. Hauer, M. Hall, K. Faber *Curr. Opin. Chem. Biol.* **2007**, *11*, 203-213; R. E. Williams, N. C. Bruce *Microbiology*, **2002**, *148*, 1607-1614.

[4] F. G. Gatti, F. Parmeggiani, A. Sacchetti in *Synthetic methods for biologically active molecules – Exploiting the potential of bioreductions* (Ed.: E. Brenna) Wiley-VCH, Weinheim, **2014**, pp. 49-84.

[5] S. Kille, M.T. Reetz, in *Synthetic methods for biologically active molecules – Exploiting the potential of bioreductions* (Ed.: E. Brenna) Wiley-VCH, Weinheim, **2014**, pp. 113-138; S. K. Padhi, D. J. Bougioukou, J. D. Stewart, J. Am. Chem. Soc. **2009**, *131*, 3271–3280; Y. A. Pompeu, B. Sullivan, A. Z. Walton, J.D. Stewart, *Adv. Synth. Catal.* **2012**, *354*, 1949 – 1960.

[6] a) R. M. Kohli, V. Massey, J. Biol. Chem. **1998**, *273,* 32763 – 32770; b) H. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892–914; c) S. K. Padhi, D. J. Bougioukou, J. D. Stewart, *J. Am. Chem. Soc.* **2009**, *131,* 3271 – 3280; d) E. Brenna, F. G. Gatti, D. Monti, F. Parmeggiani, S. Serra *Adv. Synth Catal.* **2012**, *354*, 105–112; e) E. Brenna, M. Crotti, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, A. Pugliese, D. Zampieri, *J. Mol. Catal. B: Enzym.* **2014**, *101*, 67-72.

[7] a) H. Toogood, N. S. Scrutton, *Catal. Sci. Technol*. **2013**, *3*, 2182-2194; b) H. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892–914; c) D. J. Bougioukou, A. Z. Walton, J. D. Stewart, *Chem. Commun.,* **2010**, *46*, 8558–8560.

[8] M. Hall, C. Stueckler, B. Hauer, R. Stuermer, T. Friedrich, M. Breuer, W. Kroutil, K. Faber, *Eur. J. Org. Chem*. **2008**, 1511–1516.

[9] E. Brenna, M. Crotti, F.G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, S. Santangelo, D. Zampieri, *ChemCatChem* **2014**, 2425-2431

[10] E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Eur. J. Org. Chem.* **2011**, 4015 – 4022; E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Org. Process Res. Dev.* **2012**, *16*, 262 – 268.

[11] E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Adv. Synth Catal.* **2012**, *354*, 2859 – 2864.

[12] a) C. K. Winkler, D. Clay, S. Davies, P. O'Neill, P. McDaid, S. Debarge, J. Steflik, M. Karmilowicz, J. W. Wong, K. Faber, *J. Org. Chem.* **2013**, *78*, 1525–1533; b) E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, Catal. Sci. Technol. **2013**, *3*, 1136-1146.

[13] M. A. Swiderska, J. D. Stewart, *Org. Lett.* **2006**, *8*, 6131-6133.

[14] S. Debarge, P. McDaid, P. O'Neill, J. Frahill, J. W. Wong, D. Carr, A. Burrell, S. Davies, M. Karmilowicz, J. Steflik, *Org. Process Res. Dev*. **2014**, *18*, 109−121.

[15] A. Fryszkowska, K. Fisher, J. M. Gardiner, G. M. Stephens, *Org. Biomol. Chem.* **2010**, *8*, 533-535.

[16] C. Stueckler, C. K. Winkler, M. Hall, B. Hauer, M. Bonnekessel, K. Zangger, K. Faber, *Adv. Synth Catal.* **2011**, *353*, 1169-1173.

[17] E. Brenna, M. Crotti, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, A. Pugliese, D. Zampieri, *J. Mol. Catal. B: Enzym.* **2014**, *101*, 67-72.

[18] S. K. Padhi, D. J. Bougioukou, J. D. Stewart *J. Am. Chem. Soc.* **2009**, *131*, 3271-3280.

[19] Y. A. Pompeu, B. Sullivan, J. D. Stewart, *ACS Catal.* **2013**, *3*, 2376-2390.

[20] B. J. Brown, Z. Deng, P. A. Karplus, V. Massey, *J. Biol. Chem.* **1998**, *273*, 32753−32762.

[21] M. W. Fraaije, A. Mattevi, *Trends Biochem. Sci.* **2000**, *25*, 126−132.

[22] M. Ordóñez, C. Cativiela, *Tetrahedron: Asymmetry* **2007**, *18*, 3‑99

[23] E. Falch, A. Hedegaard, L. Nielsen, B. R. Jensen, H. Hjeds, P. Krogsgaard-Larsen, *J. Neurochem.* **1986,** *47*, 898-903.

[24] M. Dambrova, L. Zvejniece, E. Liepinsh, H. Cirule, O. Zharkova, Grigory Veinberg, I. Kalvinsh, *Eur. J. Pharmacol.* **2008**, *583*, 128–134.

[25] H.H. Schneider, R. Schmiechen, M. Brezinski, J. Seidler, *Eur. J. Pharmacol.* **1986**, *127*, 105-115.

[26] N. J. Darby, T.E. Creighton, *Protein Structure*, Oxford University Press, Oxford, **1993**.

Graphical abstract