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# Rationalisation of the stereochemical outcome of ene-reductase-mediated bioreduction of α,β-difunctionalised alkenes

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ABSTRACT

The OYE1-3-mediated reductions of some α,β-difunctionalised alkenes, showing on the double bond a nitrile and ester group, are submitted to a careful stereochemical analysis, in order to identify which of the two electron-withdrawing groups (EWGs) is responsible for the activation of the C=C double bond towards reduction and for establishing hydrogen bond interactions within the binding pocket of the enzymes. The results show that for most of these substrates the activating EWG is the CN moiety linked to the prostereogenic olefinic carbon atom. The final stereochemical outcome can be explained through the empirical model which has been recently developed for difunctionalised alkenes activated by carbonyl/carboxyl containing EWGs.

In a single case the activation is due to the COOR group linked to the less substituted olefinic carbon atom: an alternative empirical model is established for this kind of substrates, taking into consideration the OYE-catalysed reductions of β,β′-disubstituted-α-monofunctionalised alkenes.



## 1. Introduction

The use of ene-reductases (ERs) for the enantioselective reduction of activated alkenes is currently receiving great interest [1], because of the efficacy shown by this kind of transformation in the synthesis of chiral building blocks for organic chemistry applications [2]. The effects due to the stereochemistry of the starting alkene, to the steric and electronic characteristics of the activating electron-withdrawing groups (EWGs), as well as of other substituents are to be carefully investigated, in order to define the limits and potential of this kind of reaction. The information is essential for including this bioreduction into the pool of synthetic tools, into which chemists can delve to select the best strategy for the preparation of their target molecules, just as they are accustomed to do when they search among conventional reagents of organic chemistry. The availability of robust enzymatic procedures can facilitate the introduction of biocatalysed steps in modern production processes, bringing along all the advantages of enzymes for sustainability.

Most of the ERs that have been identified in the last decades belong to the well-known family of Old Yellow Enzymes (OYEs), which are characterised by the presence of a flavin mononucleotide ($FMNH_2$) prosthetic group which imparts a yellow colour to purified protein samples. It has been established that the C=C double bond can only be reduced by these enzymes if it is made susceptible to the nucleophilic attack of a hydride (delivered by the reduced flavin mononucleotide prosthetic group) by the presence of an EWG, which is also able to establish hydrogen bonds within the binding pocket of the enzyme [3]. Investigations are to be devoted to define which EWGs can activate alkenes towards OYE-catalysed reductions by themselves and which ones are to be combined with other groups. Up to now, it has been established that α,β-unsaturated aldehydes and ketones, nitroolefins and maleimides are good substrates for this kind of reactions, whereas the OYE-mediated reduction α,β-unsaturated esters is only possible when another EWG is present on the double bond, *e.g.* a halogen atom linked to the same carbon atom bearing the ester function [4], or an ester [5] or a nitrile [6] group in β position.

We have recently reported [6a] on the reduction of cyano esters (*E*)- and (*Z*)-**1** (Scheme 1), precursors of $\gamma^2$-amino acids for foldamer applications, by means of OYE1-3, and we have carried out a detailed analysis of the stereochemical course of the reaction by means of deuterium labelling. Contemporaneously, a paper has been published [6b] describing the reduction of the carbon–carbon double bond of a class of regioisomeric compounds, *i.e.* (*E*)- and (*Z*)-**2** (Scheme 1), by means of isolated OYEs with the aim of providing a biocatalytic route to precursors of GABA analogues, such as pregabalin.

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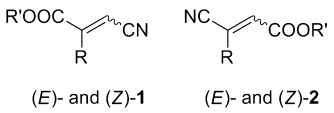



**Scheme 1.** Cyano-substituted unsaturated esters subjected to ER-mediated reduction (Ref. [6a] for (*E*)- and (*Z*)-**1**; Ref. [6b] for (*E*)- and (*Z*)-**2**).

On the basis of our experience on substrates **1**, we decided to investigate in more detail the stereochemistry of the bioreduction of compounds **2** and to establish whether the nitrile or the ester function acted as the activating group of the bioreduction, *i.e.* to determine which of the two EWGs was involved in the formation of hydrogen bonds with the amino acid residues in the active site of OYE1-3.

As a matter of fact, this information is essential to define the binding mode of the substrate, and identify the stereoheterotopic face of the alkene moiety on which the $H^+$ or $H^-$ is delivered for the formation of the new stereogenic centre. Under the practical point of view, it is useful to identify the structural requisites influencing this arrangement, in order to achieve the control on the final stereochemical outcome of the reaction through a guided substrate-engineering strategy.

The results obtained from this study have been also used to better define the empirical model we have recently developed [6a] to rationalise the experimental data on OYE1-3 mediated reductions of activated alkenes, and to describe the structural prerequisites for the optimal arrangement of the substrates within the binding site of the enzymes. A distinction could be made between trisubstituted alkenes for which the activating EWG was linked to the prostereogenic carbon atom, and those activated by the other functional group.

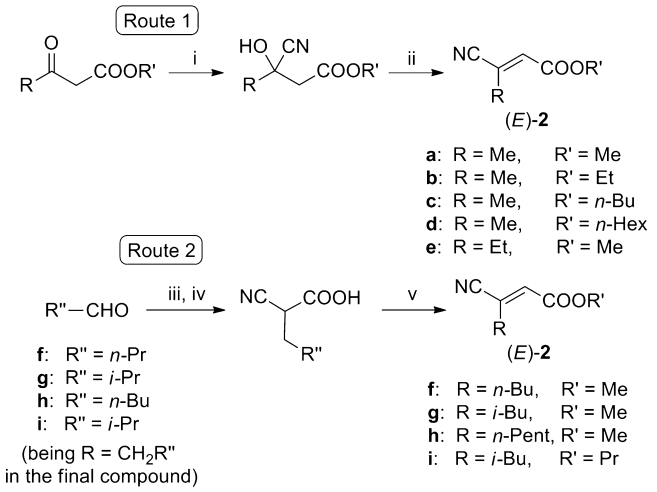
## 2. Experimental

### 2.1. General procedure for OYE1–3 mediated biotransformations of substrates (E)-*2a–i* (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 OYE (0.89 μM). 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 (15,000 × *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.

### 2.2. General procedure for OYE1–3 mediated biotransformations (50 mg scale)

For substrates (*E*)-**2a–h** a similar protocol was followed on a larger scale, employing the OYE which 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 7.0) containing the required OYE (0.89–2.66 μM), 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) and submitted to column chromatography (*n*-hexane with increasing amount of EtOAc), when conversion was not complete in order to purify the reduced product from the starting material.




**Scheme 2.** Synthesis of cyanoesters (*E*)-**2**. Reagents and conditions: (i) $Me_3SiCN$, DMSO-water 5:1; (ii) $SOCl_2$, Py, 100 °C; (iii) cyanoacetic acid, cat. $NH_4OAc$ in toluene; (iv) $NaBH_4$ in water and $NaHCO_3$; and (v) CHOCOOR′, $Ac_2O$, Py.

### 2.3. 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 OYE (1.11 μM). 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 $Na_2SO_4$.

## 3. Results and discussion

The known data [6b] of the bioreductions of compounds **2** can be summarised as follows. The best values of conversion and enantioselectivity were achieved by using OYE1-3. (*E*)-stereoisomers were generally converted into (*S*)-reduction products with high enantioselectivity, whereas the conversion of (*Z*)-cyano esters into the corresponding (*R*)-enantiomers decreased with the increasing steric demand of the substrates. Reducing the size of the ester moiety from ethyl to methyl had a strong positive impact on conversions, thus suggesting activation due to the ester group. An interesting case of exceptional behaviour of OYE3 was observed as for the enantioselectivity of the reduction of the cyano ester precursor of pregabalin.

We decided to start a systematic investigation of the stereochemical course of these reactions, by preparing other substrates of type **2**, showing (*E*) stereochemistry at the double bond and increasing steric hindrance either at the ester moiety (**2a–d**) or at the prostereogenic olefinic carbon atom (**2e–h**). Compound **2i** was also prepared to investigate the peculiar enantioselectivity of OYE-mediated reductions of isobutyl substituted cyanoesters.

A different synthetic approach was chosen with respect to that described in ref. 6b, in order to optimise the preparation of (*E*)-stereoisomers of cyano esters **2**. Derivatives (*E*)-**2a–e** were synthesised starting from the corresponding β-keto ester according to route 1 (Scheme 2) which afforded only the desired configuration of the C=C double bond. Route 2 was employed to obtain compounds (*E*)-**2f–i**, which were recovered from the reaction mixture and separated from the (*Z*)-stereoisomer by column chromatography.

The absolute configuration of the reduced products was determined by conversion either in the dimethyl ester or in the diacid derivatives (by treatment with refluxing methanol and a catalytic