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Enantioselective C=C bond reduction of unsaturated α-chloro esters by old yellow enzymes

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ABSTRACT

The reduction of the C=C double bond of some unsaturated α-chloro esters was investigated by means of cloned and overexpressed enoate reductases. The results were compared with those obtained by employing baker's yeast whole cells. The *anti* stereochemistry of hydrogen additions was confirmed by deuterium labeling experiments.

1. Introduction

The efficiency of baker's yeast (BY) in the stereoselective C=C reduction of unsaturated aldehydes, ketones and nitroalkenes has been known for years and it has been conveniently exploited for the preparation of useful chiral intermediates [1]. The synthetic value of this biocatalytic reduction is mainly due to its high enantioselectivity and to the *anti* mode of hydrogen addition [2], which makes it complementary to the enantioselective hydrogenation catalysed by chiral metal complexes. Most of the work has been carried out using whole cell systems, whose employment is easy and cheap, as no cofactor is needed.

Among the plethora of papers published on this BY mediated reduction, only a successful example of application of the method to unsaturated esters was reported. In 1987 Utaka et al. [3] described the enantioselective BY reduction of (*Z*)- and (*E*)-methyl trichlorobutenoates (*Z*)- and (*E*)-**1** to (*S*)- and (*R*)-trichloropropanoic acids **2** (Scheme 1).

They showed the synthetic usefulness of this procedure by converting these saturated α-chloro acids into the enantiomers of a naturally occurring antibiotic agent. Then they extended the investigation [4] to other 2-chloro-2-alkenoates, *i.e.* compounds **3**–**7** (Fig. 1).

The authors established that the chlorine atom in α position to the carboxylic group was essential for the C=C reduction, because neither the corresponding 2-alkenoates nor 3-chloro-2-alkenoates could be converted by BY. All (*Z*)-unsaturated α-chloro esters gave saturated (*S*)-chloro acids with high enantiomeric excess (ee > 98%), while the (*E*)-stereoisomers afforded (*R*)-chloro acids with lower ee values.

Recently, cloned and overexpressed enoate reductases belonging to the family of old yellow enzymes (OYEs) have become available in sufficient amounts. This has prompted the investigation of the enoate reductase capability of some of these isolated enzymes [2,5], *e.g.* OYE1 from *Saccharomyces pastorianus* (formerly *carlsbergensis*) and OYE2-3 from *S. cerevisiae* [6], in order to evaluate the synthetic significance of the reduction process, when this is carried out without the concomitant action of other enzymes, which are inevitably present in whole cell systems. In particular, we report herein an update on the bioreduction of substrates (*Z*)-**1**, (*Z*)-**3**, (*E*)-**3**, and (*Z*)-**7** by means of cloned and overexpressed OYEs 1-3.

2. Experimental

2.1. General methods

GC–MS analyses were performed using a HP-5MS column (30 m × 0.25 mm × 0.25 μm). The following temperature program was employed: 60 °C (1 min)/6 °C min^{-1}/150 °C (1 min)/12 °C min^{-1}/280 °C (5 min). ^{1}H and ^{13}C NMR spectra

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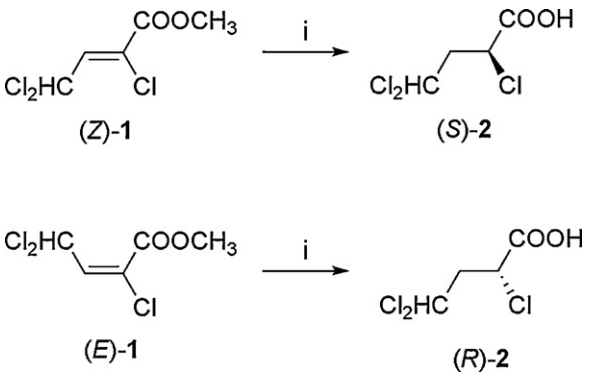


Scheme 1. BY-mediated biotransformations of (*E*)- and (*Z*)-**1**. i, dry baker's yeast, water, glucose.

were recorded on a 400 MHz spectrometer. The chemical shift scale was based on internal tetramethylsilane.

The enantiomeric excess values were determined by GC analyses, which were performed on a DANI HT 86.10 gas chromatograph, according to the following conditions:

a) methyl 2-chloro-4-methylpentanoate: Chirasil Dex CB, 25 m × 0.25 mm (Chrompack) column, 60 °C (2 min)/1 °C min^{-1}/70 °C/30 °C min^{-1}/180 °C (2 min); (*R*)-enantiomer t_R = 10.07 min, (*S*)-enantiomer t_R = 10.33 min.
b) methyl 2,4,4,4-tetrachlorobutanoate: Megadex 5, 25 m × 0.25 mm (Mega) column, 60 °C (3 min)/2 °C min^{-1}/190 °C/20 °C min^{-1}/215 °C; (*R*)-enantiomer t_R = 19.38 min, (*S*)-enantiomer t_R = 20.17 min.

2.2. Synthesis of substrates (*Z*)-**1**, (*Z*)-**3**, (*E*)-**3**, and (*Z*)-**7**

These substrates were prepared according to the literature [7]. Condensation of ethyl 2-chloroacetoacetate (3.28 g, 0.02 mol) with the suitable aldehyde (0.024 mol) was performed in THF solution (50 ml) in the presence of potassium carbonate (4.14 g, 0.030 mol). The reaction mixture was stirred at room temperature for 2 days, diluted with water and extracted with diethyl ether. After removal of the solvent under reduced pressure, the residue was purified by column chromatography, to give the corresponding ethyl ester which was converted into the methyl ester by reaction in refluxing methanol, in the presence of a catalytic amount of sulfuric acid. The assignment of the (*E*) or (*Z*) configuration of the unsaturated esters was done according to Ref. [4].

2.2.1. (*Z*)-methyl 2,4,4-trichlorobut-2-enoate ((*Z*)-**1**)

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.23 (1H, d, *J* = 9.3 Hz, CH=), 6.56 (1H, d, *J* = 9.3 Hz, $CHCl_2$), 3.89 (s, 3H, $COOCH_3$). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 161.5, 137.0, 125.2, 64.8, 53.7. GC/MS: t_R = 10.63 min, *m/z* 202 (M^+, 3), 167 (100), 139 (51).

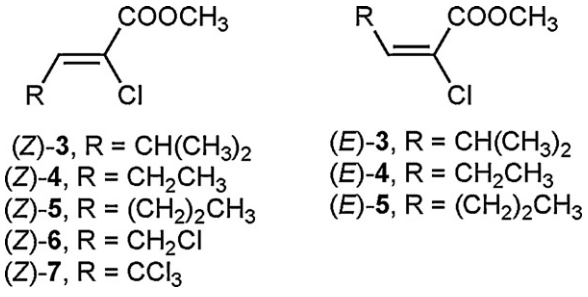


Fig. 1. Substrates for BY-mediated bioreduction.

2.2.2. (*Z*)-methyl 2-chloro-4-methylpent-2-enoate ((*Z*)-**3**)

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 6.89 (1H, d, *J* = 9.4 Hz, CH=), 3.82 (s, 3H, $COOCH_3$), 2.90 (m, 1H, $CH(CH_3)_2$), 1.08 (6H, d, *J* = 6.7 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 163.2, 148.6, 122.5, 52.8, 29.1, 21.0. GC/MS: t_R = 7.82 min, *m/z* 162 (M^+, 100), 147 (75), 131 (50), 115 (65).

2.2.3. (*E*)-methyl 2-chloro-4-methylpent-2-enoate ((*E*)-**3**)

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 6.24 (1H, d, *J* = 10.3 Hz, CH =), 3.82 (s, 3H, $COOCH_3$), 3.31 (m, 1H, $CH(CH_3)_2$), 1.05 (6H, d, *J* = 6.7 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 163.1, 151.3, 120.2, 52.5, 29.3, 22.1. GC/MS: t_R = 6.92 min, *m/z* 162 (M^+, 100), 147 (64), 131 (40), 115 (56).

2.2.4. (*Z*)-methyl 2,4,4,4-tetrachlorobut-2-enoate ((*Z*)-**7**)

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.75 (1H, s, CH=), 3.82 (s, 3H, $COOCH_3$). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 161.6, 141.8, 129.3, 89.1, 53.9. GC/MS: t_R = 14.01 min, *m/z* 201 (M^+-Cl, 100), 173 (93), 123(88), 107 (56).

2.2.5. Methyl 2,4,4-trichlorobutanoate

Reduction of (*Z*)-**1** afforded the corresponding saturated compound which was identified by GC/MS spectroscopy: t_R = 10.63 min, *m/z* 173 (M^+–OCH_3, 6), 145 (18), 108 (82), 59 (100).

2.2.6. Methyl 2-chloro-4-methylpentanoate

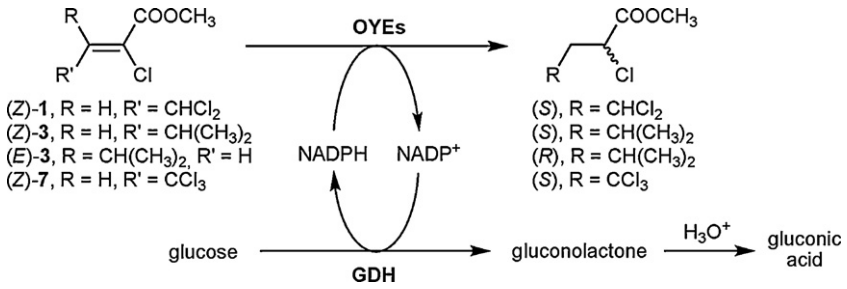
Reduction of (*Z*)-**3** and (*E*)-**3** afforded, respectively, the (*S*)- and (*R*)-enantiomers of the title compound. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.32 (1H, t, *J* = 6.9 Hz, CHCl), 3.78 (s, 3H, $COOCH_3$), 1.90–1.70 (m, 3H, CH_2 + $CH(CH_3)_2$), 0.96 (3H, d, *J* = 6.9 Hz, CH_3), 0.93 (3H, d, *J* = 6.9 Hz, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 170.3, 55.5, 52.6, 43.5, 25.1, 22.4, 21.3. GC/MS: t_R = 7.07 min, *m/z* 129 (M^+–Cl, 33), 108 (100), 87 (30).

2.2.7. Methyl 2,4,4,4-tetrachlorobutanoate

Reduction of (*Z*)-**5** afforded the (*S*)-enantiomer of the title compound. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.62 (1H, dd, *J* = 7.9, 3.8 Hz, CHCl), 3.84 (s, 3H, $COOCH_3$), 3.78 (1H, dd, *J* = 15.3, 7.9 Hz, CHH), 3.24 (1H, dd, *J* = 15.3, 3.8 Hz, CHH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 168.6, 95.3, 58.2, 53.4, 51.4. GC/MS: t_R = 12.24 min, *m/z* 203 (M^+–Cl, 4), 167 (25), 109 (84), 59 (100).

2.3. Preparation of enantiomerically enriched products for the determination of the absolute configuration

Reference standards of (*S*)-saturated methyl esters for GC analysis on a chiral phase column were prepared by BY fermentation of substrates (*Z*)-**3** and (*Z*)-**7**, followed by treatment with a solution of CH_2N_2 in diethyl ether. To a fermenting BY mixture (100 g yeast in 1 L tap water) the suitable substrate (2.5 g) was added. After 24 h stirring, acetone (1 L) was added to the mixture. After stirring overnight, ethyl acetate (1 L) was added and the mixture was stirred 1 h. The two phases were separated in a separatory funnel and the aqueous phase was extracted twice with a 9:1 mixture of ethyl acetate-hexane. The combined organic phases were concentrated under vacuum to a small volume. After addition of 10 vol of ethyl acetate–hexane 9:1, the organic phase was washed repeatedly with water and brine. The oily residue obtained upon evaporation of the dried (Na_2SO_4) organic phase was purified by SiO_2 chromatography. Deuterium incorporation experiments were performed by submitting substrate (*Z*)-**7** to BY fermentation, with the addition of *ca.* 5% of 99% D_2O to the fermenting mixture at the beginning of the reaction.



Scheme 2. OYE 1-3-mediated biotransformations of unsaturated α-chloro esters.

2.4. Source of enzymes

All the enzymes employed were overexpressed in *E. coli* BL21 (DE3) strains harboring a specific plasmid prepared according to standard molecular biology techniques: pET30a-OYE1 from the original plasmid kindly provided by Bruce [8], pET30a-OYE2 and pET30a-OYE3 from *S. cerevisiae* BY4741 and pKTS-GDH from *B. megaterium* DSM509 (detailed steps reported in Ref. [9]).

Overexpression of the enzymes in E. coli BL21 (DE3). A 5 mL culture in LB medium 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 overnight at 37 °C and 220 rpm. This starter culture was used to inoculate a 200 mL culture, which was in turn grown overnight at the same conditions and used to inoculate a 1.5 L culture. The latter 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 homogenized (Haskel high-pressure homogenizer). The cell-free extract, after centrifugation (20,000 × *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 dialyzed 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.

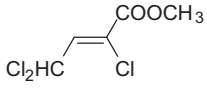
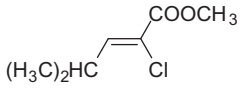
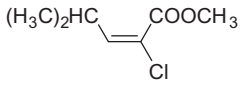
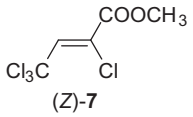
2.5. General procedure for the OYE mediated bioreduction

The substrate (*Z*)-**1**, (*Z*)-**3**, (*E*)-**3** or (*Z*)-**7** (5 μmol) dissolved in DMF (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 (40 μg 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 (15,000 × *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4.

2.6. Acquisition of the ^2H spectra

The ^2H spectra were measured on a Bruker Avance 500 spectrometer equipped with a 10-mm probe head and ^{19}F lock channel under CPD (Waltz 16 sequence) proton decoupling conditions at the temperature of 305 K. The solutions were prepared dissolving 20–100 mg of material in *ca.* 3.0 mL of C_6H_6 adding about 40 μL

Table 1
Conversion and enantiomeric excess values obtained by biotransformation of unsaturated α-chloro esters (*Z*)-**1**, (*Z*)-**3**, (*E*)-**3**, and (*Z*)-**7**.

	OYE1		OYE2		OYE3		Baker's yeast[a]	
	c (%)	ee (%)	c (%)	ee (%)	c (%)	ee (%)	c (%)	ee (%)
 (*Z*)-**1**	100	n.d.[b]	100	n.d.[b]	100	n.d[b]	60	>98 (*S*)
 (*Z*)-**3**	90	91 (*S*)	91	82 (*S*)	89	83 (*S*)	20	>98 (*S*)
 (*E*)-**3**	13	n.d.[c]	6	n.d.[c]	92	79 (*R*)	10	68 (*R*)
 (*Z*)-**7**	78	95 (*S*)	95	94 (*S*)	95	94 (*S*)	50	>98 (*S*)

c, conversion; n.d., not determined.
[a] Data from Ref. [4] (conversion calculated by the authors on the methyl ester prepared by diazomethane treatment of the recovered carboxylic acid).
[b] We could not prepare a sample of racemic reduced compound to be used as a reference.
[c] Conversions were too low to allow the determination of the enantiomeric excess of the reduced product.

of C_6F_6 for the lock. The spectra were run collecting 1024–4096 scans depending on the solution concentration using the following acquisition parameters: 5.4 s acquisition time, 1530 Hz spectral width, 16 K time domain and 1 s delay. The spectra were Fourier transformed with a line broadening of 0.3 Hz, manually phased and integrated after an accurate correction of the base line. For partially overlapped signals the peak areas were determined through the deconvolution routine of the Bruker TopSpin NMR software using a Lorentzian line shape.

3. Results and discussion

BY reduction of methyl 2-chloroalkenoates was described to afford the corresponding saturated 2-chloroalkanoic acids. When the ethyl esters were employed, no conversion was observed [4]. The authors concluded that the real substrates for BY reduction were the unsaturated carboxylic acids, generated in the reaction medium by the hydrolytic enzymes of the yeast cells, and that the hydrolysis of methyl esters was faster than that of ethyl esters.

We prepared substrates (*Z*)-**1**, (*Z*)-**3**, (*E*)-**3**, and (*Z*)-**7** according to the literature method [7], by reaction of the suitable aldehyde with ethyl α-chloroacetoacetate and in potassium carbonate in THF. The ethyl esters were then transesterified in refluxing methanol with a catalytic amount of sulfuric acid. The substrates were submitted to biotransformations (Scheme 2) in the presence of OYEs 1-3 and the results are reported in Table 1.

Cloned and overexpressed enoate reductases afforded reaction conversions which were higher than those achieved by means of baker's yeast whole cells. In the conditions of OYE-mediated biotransformation no hydrolysis could occur: in spite of this fact, the unsaturated α-chloro methyl esters could be effectively reduced, thus showing they are the real substrates of this enzyme-mediated reduction. We also verified that the ethyl ester derivatives could not be reduced by isolated enzymes. Unexpectedly, in the case of substrate (*E*)-**3** only OYE3 resulted to be effectively working in the reduction process, affording the corresponding saturated product with an enantiomeric excess value higher than that achieved by using BY.

We also confirmed the *anti* stereochemistry of hydrogen addition in the BY mediated reduction by submitting substrate (*Z*)-**7** to BY fermentation in the presence of deuterated water (5%), to give compound **8** (Fig. 2). The deuterium spectrum of **8** showed deuterium incorporation in the positions 2 and 3 of the substrate. The incorporation resulted to be highly stereoselective affording the [2*S*,3*S*] [2,3-$^2H_2$] derivative. In fact, of the two C-3 methylene protons H-3a (3.58 ppm, benzene solution) and H-3b (2.92 ppm) only H-3b appeared to be enriched in deuterium nuclei (Fig. 2). The assignment of H-3a *vs*. H-3b was the prerequisite for the stereochemical determination of the deuterium nucleus at position 3. The vicinal coupling constants $J(H_{3a},H_2)$ and $J(H_{3b},H_2)$ showed values of 7.9 and 3.8 Hz, respectively, indicating that the molecule had a preferential conformation where the nuclei H-3a and H-2 were mainly pseudo-*anti* oriented. Such a preferred conformation had to be independently determined to remove any ambiguity in the assignment of the methylene protons.

To this aim we performed some *ab initio* energy calculations of the molecule using the Gaussian 03 program [10]. As input geometries of Gaussian we used that of the two most probable conformers having the CCl_3 substituent gauche to the ester or chlorine groups. These structures were minimized without imposing any symmetry constraint. The iterations converged in the same minimum energy structure for both input geometries thus proving that the molecule had an absolute minimum. In this structure the CCl_3 and $COOCH_3$ substituents were approximately gauche oriented showing dihedral angles $CCl_3–C_3–C_2–COOCH_3 = -92.2°$ and

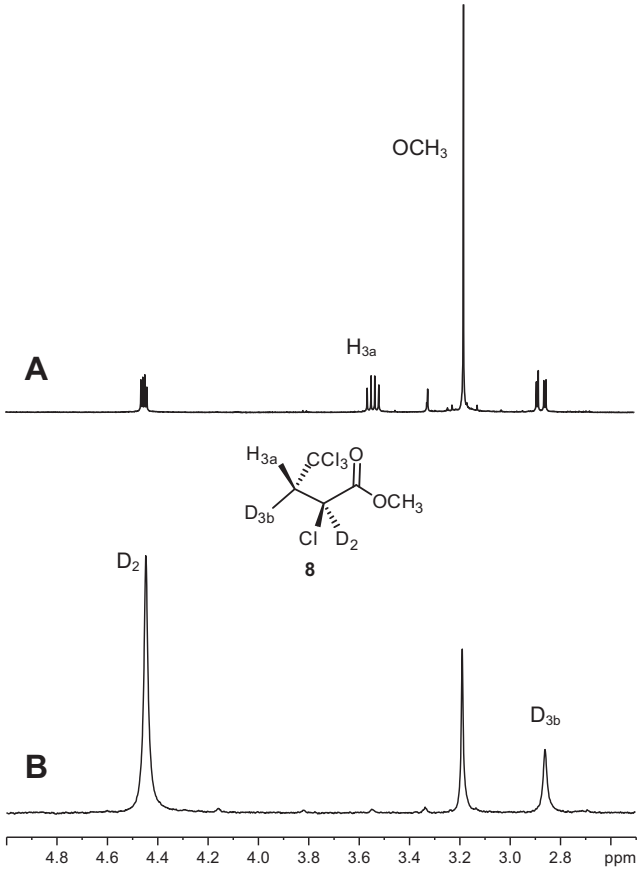


Fig. 2. (A) Proton and (B) deuterium NMR spectra in C_6H_6 solution of compound **8** obtained by bioreduction of (*Z*)-**7** in $H_2O + D_2O$ (5%). The signal at 3.22 ppm in spectrum B is due to the natural abundance deuterium nuclei of the methoxy group.

$CCl_3–C_3–C_2–Cl = 151.2°$. We concluded that the carbon C-3 in the deuterated molecules had *S* configuration. *Anti* addition to the conjugated double bond was thus confirmed.

4. Conclusions

The use of isolated OYEs in the reduction of C=C double bonds shows great advantages under the synthetic point of view, because side reactions, such as ester hydrolysis, can be avoided. In these conditions the real substrate of the biocatalysed transformation can be identified. Furthermore, the recovery of the ester from the biotransformation crude mixture can be less troublesome than the isolation of the acid from BY biomass. The reaction is particularly useful for the optimisation of synthetic procedures to valuable enantiomerically enriched chiral synthons [11].

Another advantage is that the enzyme which is the best catalyst for the reduction of a particular substrate can be highlighted, and optimisation of the reaction conditions can be performed to increase product loading. The amount of biocatalyst added can be controlled, and conversions can be improved.

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