

**Biocatalytic synthesis of chiral cyclic  $\gamma$ -oxoesters by sequential C-H  
hydroxylation, alcohol oxidation and alkene reduction**

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## Abstract

A three-step biocatalytic procedure is described for the conversion of methyl and ethyl cyclopentene- and cyclohexenecarboxylates into both the enantiomers of the corresponding chiral 3-oxoesters, which are useful building blocks for the synthesis of active pharmaceutical ingredients. The allylic hydroxylation of the starting cycloalkenecarboxylates is carried out by using *R. oryzae* resting cells entrapped in alginate beads, in acetate buffer solution at 25°C. The oxidation of the intermediate allylic alcohols to unsaturated ketones, performed by the laccase/TEMPO system, and the ene-reductase mediated hydrogenation of the alkene bond were carried out in the same reaction vessel in a sequential mode at 30°C. Being entirely biocatalytic, our multistep procedure provides considerable advantages in terms of selectivity and environmental impact over reported chemical methods.

## Introduction

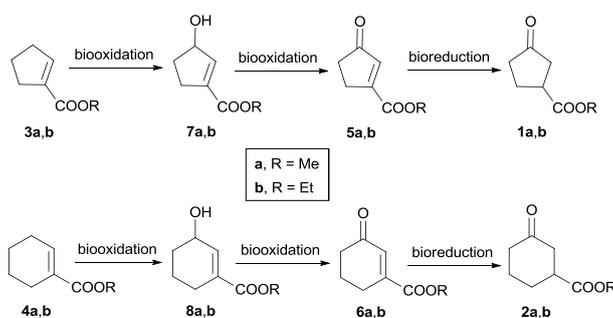
The key role played by biocatalysis in improving the sustainability of fine and bulk chemicals manufacturing is well recognized.<sup>1</sup> The capability of enzymes to catalyze a remarkably wide range of organic reactions with excellent chemo-, regio-, and stereoselectivity under mild conditions (temperature, pH, pressure), limiting the amount of waste and by-products, is greatly appreciated, especially in the pharmaceutical field.<sup>2</sup> Furthermore, the combination of multi-enzymatic reactions in a cascade sequence<sup>3</sup> represents an attractive approach for the production of complex valuable chemicals from simple precursors, taking advantage of the fact that, to date, the biocatalytic counterparts of many classical organic reactions have been established and optimized to work under similar and compatible operating conditions.<sup>4</sup>

For example, the oxyfunctionalization of allylic carbon atoms represents a very useful strategy for converting readily available unsaturated hydrocarbons into high value-added allylic alcohols and  $\alpha,\beta$ -unsaturated ketones.<sup>5</sup> Traditionally, the process has been performed by using stoichiometric oxidants, e.g. chromium(VI)-based reagents, manganese dioxide, potassium permanganate or selenium dioxide. During the last years, the thrust towards more environmentally benign and cost-effective protocols has promoted the search and optimization of catalytic procedures using suitably activated molecular oxygen or peroxides as oxidants.<sup>6</sup> Biocatalysis offers advantageous strategies for this reaction, based on the versatile oxidative capabilities of (mono- and di-) oxygenases, peroxygenases and oxidases.<sup>7</sup> Several examples of selective allylic C-H hydroxylation of steroids, terpenes and terpenoids have been described in the literature using either whole-cell microorganisms or isolated enzymes.<sup>8</sup>

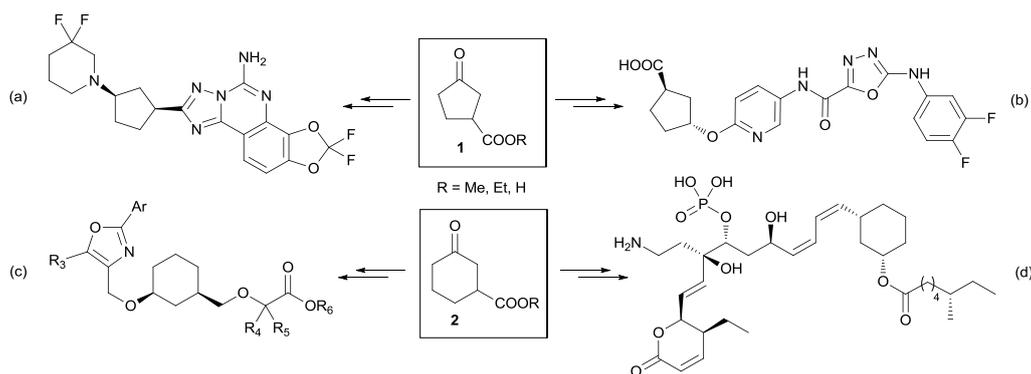
Recently, we envisaged the possibility to employ this reaction as the first step of a multi-enzymatic procedure for the preparation of the enantiomers of cyclic ketoesters **1a,b** and **2a,b** (Scheme 1) from readily available cycloalkenecarboxylates **3a,b** and **4a,b**. Herein, we report on the development and optimization of a completely biocatalytic and environmentally benign system

combining a two-step allylic biooxidation with the biohydrogenation of the oxidized products **5a,b** and **6a,b**, for the preparation of both enantiomers of cyclic  $\gamma$ -oxoesters **1a,b** and **2a,b**.

These compounds, along with their corresponding ketoacids, are exceptionally useful building blocks in the synthesis of active pharmaceutical ingredients (APIs), because they make available rigid cyclic cores bearing two functional handles for further manipulation. Such structures are often inserted into the skeleton of drug candidates, in order to investigate the effects due to limited conformational freedom and reduced rotation around single bonds. Representative examples of APIs incorporating such cores are reported in Figure 1.



**Scheme 1.** Synthetic plan to chiral cyclic ketoesters **1** and **2**.



**Figure 1.** Structures of active pharmaceutical ingredients incorporating a 1,3-difunctionalised  $C_5$  or  $C_6$  ring: (a) antagonists of the  $A_{2A}$  receptor, employed for the treatment of neurological disorders;<sup>9</sup> (b) inhibitors of acetyl-CoA:diacylglycerol acyltransferase activity, employed for the treatment of type II diabetes;<sup>10</sup> (c) PPAR (peroxisome proliferator-activated receptor) ligands, employed for the treatment of hyperlipidemia and diabetes;<sup>11</sup> (d) leustroducsin B, a potent colony-stimulating factor inducer isolated from the culture broth of *Streptomyces platensis* SANK 60191.<sup>12</sup>

## Results and discussion

### Allylic hydroxylation of methyl and ethyl cycloalkenecarboxylates

A survey of chemical literature on the allylic oxidations of esters **3** and **4** highlighted that chemical reagents afford directly the corresponding unsaturated oxo-derivatives **5** and **6**. The following methods have been described: (i) stoichiometric chromium(VI) oxide, with acetic anhydride and acetic acid in CH<sub>2</sub>Cl<sub>2</sub> at 0-5°C;<sup>13,14</sup> (ii) *t*-butyl hydroperoxide as stoichiometric oxidant, in the presence of either 20% Pd(OH)<sub>2</sub>/C (5 mol%)<sup>15</sup> or dirhodium complexes<sup>16,17</sup> as catalysts in CH<sub>2</sub>Cl<sub>2</sub> solution. The only example of biocatalysed oxyfunctionalisation is the hydroxylation of derivatives **3a** and **4a** mediated by suitable mutants of monooxygenase P450-BM3, which was studied by Reetz *et al.* with the aim of preparing both enantiomers of allylic alcohols **7a** and **8a**.<sup>18</sup> In a following work,<sup>19</sup> the same authors employed designer cells to achieve a multi-enzymatic cascade transformation to convert substrate **4a** into (*S*)- and (*R*)-**2a**, by using two different types of engineered *Escherichia coli* cells: one producing P450-BM3 mutants, for the regioselective oxidation of **4a** to **6a**, and the other producing variants of the ene-reductase YqjM, suitable for the (*S*)- or (*R*)-selective hydrogenation of the alkene bond.

The importance of chiral building blocks **1a,b** and **2a,b** in the field of medicinal chemistry, and the lack of cost-effective, sustainable synthetic routes for these compounds at a preparative scale, prompted us to investigate the combination of redox enzymes in a sequential procedure to achieve the conversion of derivatives **3** and **4** into chiral  $\gamma$ -oxoesters **1** and **2**.

Methyl cyclopentenecarboxylate **3a** was chosen as a model compound to investigate the oxidative capability of a set of four filamentous fungi, *Colletotrichum lini*, *Rhizopus oryzae*, *Aspergillus niger* and *Alternaria tenuis*, which had been described to perform the allylic C-H hydroxylation of steroids and terpenes.<sup>20-23</sup> Screening experiments were carried out by incubating substrate **3a** with the fungal cell cultures, and monitoring the biotransformations by GC/MS analysis. After 5 days,

well-defined oxygenated metabolites could be identified only in the biotransformation of **3a** with *R. oryzae*, while the other strains afforded complex mixtures of products. Preliminary experiments with *R. oryzae* were performed also on substrates **3b** and **4a,b** and the results are reported in Figure 2, Procedure A. The identification of the components of each reaction mixture was performed by comparison with authentic samples prepared by chemical synthesis (see Supporting Information). Interestingly, the biotransformation of compound **3a** showed also the formation of the ethyl ester **3b** during the first day of fermentation, as a consequence of a transesterification reaction. After five days (Figure 2a, Procedure A) the reaction mixture contained cyclopentanemethanol **9** (14%), obtained by reduction of both the C=C double bond and the ester moiety, and the two allylic alcohols **7a** (41%) and **7b** (30%). Neither the corresponding ketones **5a** and **5b**, nor the other possible regioisomeric allylic alcohols could be detected by GC/MS among the other components of the reaction mixture (unknown products 15%), not even in trace amounts. When ethyl ester **3b** was employed as a substrate (Figure 2b, Procedure A), after 5 days reaction time, *R. oryzae* gave cyclopentanemethanol **9** (10%), allylic alcohol **7b** (22%), and the corresponding saturated derivative ethyl 3-hydroxycyclopentane-1-carboxylate (45%), besides other unidentified compounds (10%). Derivative **4a** was hydroxylated very neatly by *R. oryzae* (Figure 2c, Procedure A), to give only allylic alcohol **8a** (95 %), and 5% of cyclohexanemethanol (**10**). Finally, the biotransformation of the ethyl ester **4b** (Figure 2d, Procedure A) was found to be rather slow, albeit quite selective: after 9 days a mixture containing 55% of the starting substrate and 45% of the hydroxylated derivative **8b** was recovered.

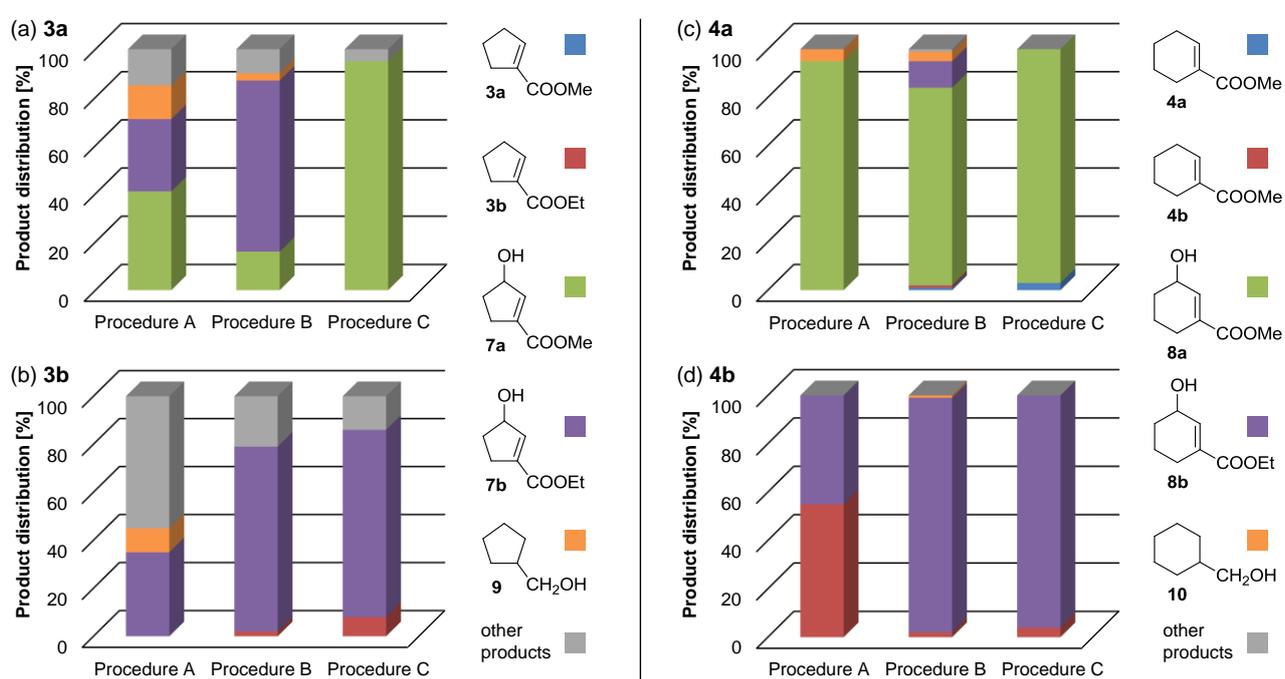
After these positive preliminary results on the oxidative capability of *R. oryzae* towards cycloalkenecarboxylates, we tried to improve the reaction conditions by entrapping the microorganism in calcium alginate beads. The long mycelia of *R. oryzae* are known to form large pellets or clumps in free cell cultivation, causing a severe mass transfer resistance. Immobilization has been described to be a useful method to control the length of mycelia, facilitating oxygen mass

transfer.<sup>24</sup> Thus, we studied the biocatalysed oxydation of **3a,b** and **4a,b** by using Ca-alginate entrapped *R. oryzae*. The spore suspension was mixed with Na-alginate and then added to a sterilized solution of CaCl<sub>2</sub>, to give cell beads which were filtered, washed and pre-cultured for 2 days at 25°C. A dense layer of mycelia was formed on the outer surface of the beads.<sup>24</sup> In separate experiments **3a,b** and **4a,b**, dissolved in DMSO, were added to the flasks containing the *R. oryzae* beads suspended in the culture medium. The biotransformations were monitored by GC/MS and the results are reported in Figure 2, Procedure B. The oxidation of **3a** (Figure 2a, Procedure B) occurred with a limited quantity of by-products (3% of **9**, and 10% of unknown compounds), affording a 1:4.4 mixture of allylic alcohols **7a** and **7b** in 4 days reaction time. The reaction of ethyl ester **3b** (Figure 2b, Procedure B) was nearly complete in 2 days, affording compound **7b** as the main component (77%) of the reaction together with a small quantity of ethyl 3-hydroxycyclopentane-1-carboxylate (10%) and of unidentified products (11%). When derivative **4a** was employed as the starting compound (Figure 2c, Procedure B), allylic alcohol **8a** was the main product (82%, 4 days), but the formation of ethyl ester **8b** (11%) was also observed. The hydroxylation of **4b** to allyl alcohol **8b** was nearly complete (97%) in 3 days without formation of by-products (Figure 2d, Procedure B).

Further experiments were performed by resuspending the beads in acetate buffer solution (pH 6.0), in order to minimize the effects of extracellular enzymes and residual activities present in the culture medium. The biotransformations were monitored by GC/MS and the results improved considerably, as reported in Figure 2, Procedure C. For the biotransformation of **3a**, methanol was used as a cosolvent instead of DMSO, in order to prevent the formation of ethyl ester **7b** (Figure 2a, procedure C): in 5 days the reaction afforded 95% of methyl ester **7a**, along with 5% of unknown products. The biotransformation of **3b** (Figure 2b, Procedure C) occurred smoothly, and afforded after 2 days the allylic alcohol **7b** (78%) with a small quantity of the corresponding saturated alcohol (4%, lower than that observed in culture medium). Under the same conditions, substrates **4a**

and **4b** were converted nearly quantitatively into allyl alcohols **8a** (97%) and **8b** (96%) in 3 days (Figure 2c and 2d, Procedure C).

The allylic alcohols **7a,b** and **8a,b** were found to be enantiomerically enriched (Supporting Information, Table S1): the enantiomeric excess values of the cyclohexene derivatives (60-86%) were generally higher than those of the cyclopentene compounds (35-75%).



**Figure 2.** Results of *R. oryzae* mediated biotransformations of compounds **3a** (a), **3b** (b), **4a** (c), **4b** (d), according to the following experimental procedures. Procedure A: 5 mM substrate (DMSO as cosolvent), *R. oryzae* whole cells, 25°C; Procedure B: 5 mM substrate (DMSO as cosolvent), *R.oryzae* beads suspended in culture medium, 25°C; Procedure C: 5 mM substrate (MeOH or DMSO as cosolvent), *R.oryzae* beads suspended in buffer pH 6.0, 25°C.

In contrast with most of the reported chemical methods, the *R. oryzae* oxidation was demonstrated to stop cleanly at the allylic alcohol stage without formation of unsaturated ketoesters **5** and **6**. While this may be a desirable chemoselectivity trait in different synthetic plans, a further oxidation step had to be implemented to obtain **5** and **6** prior to the final bioreduction.

### **Biocatalysed oxidation of allylic alcohols 7a,b-8a,b**

Allylic alcohols **7a,b-8a,b** were converted into ketones **5a,b-6a,b** by using molecular oxygen as the oxidant, in the presence of a laccase and a suitable chemical mediator. Laccases are blue multicopper oxidases able to catalyze the four-electron reduction of molecular oxygen to water, coupled with the concomitant oxidation of organic aromatic substrates.<sup>25</sup> They are not effective towards non-phenolic derivatives, so to carry out the oxidation of primary and secondary alcohols they require electron transfer mediators, such as 2,2',6,6'-tetramethylpiperidine-*N*-oxyl (TEMPO).<sup>26</sup> The treatment of allylic alcohols **7a,b-8a,b** (5 g L<sup>-1</sup>, with DMSO as a cosolvent) with catalytic TEMPO<sup>+</sup> BF<sub>4</sub><sup>-</sup> and laccase Amano M120 in acetate buffer (pH 5.0) under O<sub>2</sub> atmosphere, afforded quantitative oxidation to the corresponding ketones **5a,b-6a,b** over 24 hours at 30°C.

### **Enantioselective reduction of cyclic unsaturated ketoesters 5a,b-6a,b**

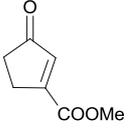
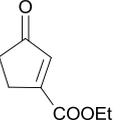
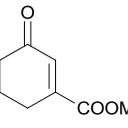
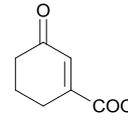
The enantioselective reduction of compounds **5a,b-6a,b** had been previously described in the literature. Reetz *et al.*<sup>27</sup> had reported that the reduction of methyl esters **5a** and **6a** mediated by YqjM afforded the (*R*)-enantiomer of saturated ketoesters **1a** and **2a** very efficiently. Moreover, while we were finalizing our experimental work on this topic, a paper by Faber *et al.*<sup>28</sup> was published, describing the enantioselective hydrogenations of substrates **5a,b-6a,b** (obtained by traditional chemical oxidation with CrO<sub>3</sub>) mediated by a panel of known ene-reductases.

We submitted the unsaturated ketoesters **5a,b-6a,b**, prepared according to the two-step oxidation protocol, to ene-reductase mediated hydrogenation, using as catalysts ene-reductases belonging to the Old Yellow Enzyme family: OYE1 (from *Saccharomyces pastorianus*), OYE2 and OYE3 (from *S. cerevisiae*), OYE2.6 (from *Pichia stipitis*), LeOPR1 (from *Solanum lycopersicum*), and YqjM (from *Bacillus subtilis*). The recycling of the catalytic NADPH cofactor was performed with

glucose dehydrogenase (GDH from *Bacillus megaterium*), using glucose as a sacrificial cosubstrate. The results of the preliminary screening are reported in Table 1.

In our hands, OYE1-3 and OYE2.6 invariably gave the same enantiomer of the reduced ketoesters **1a,b-2a,b**, while the reactions mediated by YqjM and LeOPR occurred with opposite enantioselectivity.<sup>29</sup> The absolute configuration of compounds **1a,b** and **2a**, recovered from OYE1 mediated reduction, was assigned to be (*S*) by comparison of the optical rotation data of these samples with literature values (see Experimental Section). The configuration of compound **2b**, obtained by OYE1 mediated reduction of **2b**, was established to be (*S*) by converting this sample (94% ee) into the (+)-(*S*)-enantiomer of the corresponding carboxylic acid.

**Table 1.** Ene-reductase mediated hydrogenation<sup>a</sup> of compounds **5a,b-6a,b**

	 <b>5a</b>		 <b>5b</b>		 <b>6a</b>		 <b>6b</b>	
Enzyme	c <sup>b</sup> [%]	ee <sup>c</sup> [%]	c <sup>b</sup> [%]	ee <sup>c</sup> [%]	c <sup>b</sup> [%]	ee <sup>c</sup> [%]	c <sup>b</sup> [%]	ee <sup>c</sup> [%]
OYE1	99	90 ( <i>S</i> )	99	86 ( <i>S</i> )	99	94 ( <i>S</i> )	99	96 ( <i>S</i> )
OYE2	99	82 ( <i>S</i> )	99	80 ( <i>S</i> )	99	75 ( <i>S</i> )	99	90 ( <i>S</i> )
OYE3	99	90 ( <i>S</i> )	99	71 ( <i>S</i> )	99	62 ( <i>S</i> )	99	42 ( <i>S</i> )
OYE 2.6	99	92 ( <i>S</i> )	99	87 ( <i>S</i> )	99	96 ( <i>S</i> )	99	92 ( <i>S</i> )
LeOPR1	99	88 ( <i>R</i> )	99	99 ( <i>R</i> )	99	98 ( <i>R</i> )	99	99 ( <i>R</i> )
YqjM	99	86 ( <i>R</i> )	99	86 ( <i>R</i> )	99	99 ( <i>R</i> )	99	99 ( <i>R</i> )

<sup>a</sup> 5 mM substrate, 20 mM glucose, isolated OYE, GDH, NADP<sup>+</sup>, 1% DMSO, phosphate buffer pH 7.0, 30°C, 24 h;

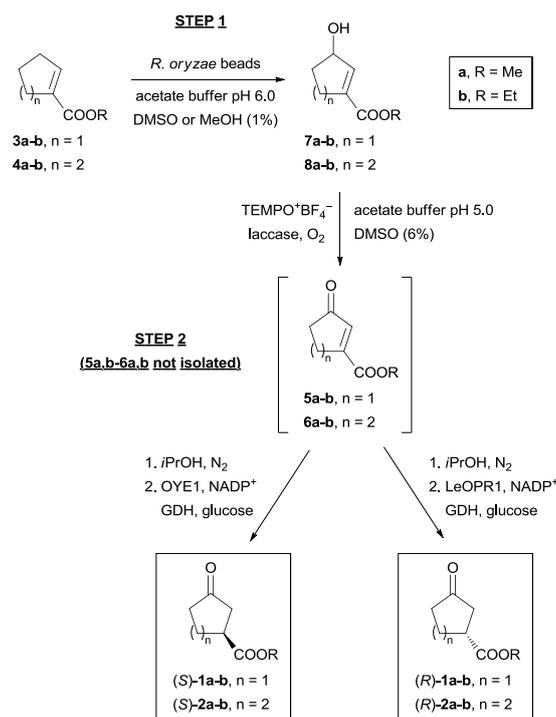
<sup>b</sup> conversion calculated on the basis of GC analysis of the crude mixture after 24 h (isolation yields are reported in the Experimental Section); <sup>c</sup> enantiomeric excess calculated on the basis of GC analysis on a chiral stationary phase (see Experimental Section).

The enantioselectivities of these cycloalkene bioreductions are in agreement with literature data for structurally related β-alkylcycloalkenones. For instance, 3-methylcyclopentenone is reduced to the (*S*)-enantiomer of the saturated ketone by OYE1-3 (>99% ee),<sup>30</sup> while it is converted very poorly by LeOPR1 and YqjM,<sup>31</sup> and no information are available about its reduction with OYE2.6. Similarly, 3-methylcyclohexenone is converted into (*S*)-3-methylcyclohexanone by OYE1-3<sup>32,30</sup> and

OYE2.6,<sup>33</sup> and it is scarcely accepted by LeOPR1 and YqjM.<sup>31</sup> Furthermore, investigations performed by using OYE1<sup>34</sup> and OYE2.6<sup>33</sup> highlighted that the reduction of 3-alkylcyclohexenones is very sensitive to steric hindrance of the substituent at the  $\beta$ -position: conversions decreased by increasing the size of the alkyl chain, and substrates with a substituent longer than a *n*-propyl chain were not accepted by the enzymes. In the transformation of the cyclic  $\gamma$ -oxoesters of this work, the presence of either a methyl or an ethyl ester group linked to the  $\beta$  olefinic carbon atom is not detrimental for conversion and enantioselectivity of the reactions mediated by the six investigated ERs.

### **Conversion of cycloalkenecarboxylates **3a,b-4a,b** into (*R*)- and (*S*)-**1a,b-2a,b****

The two oxidative steps and the alkene reduction were combined to convert cycloalkenecarboxylates **3a,b-4a,b** into (*R*)- and (*S*)-**1a,b-2a,b** (Scheme 2). The C-H allylic hydroxylation of derivatives **3a,b-4a,b** was performed according to Procedure C (30 g of *R. oryzae* beads in 120 mL of acetate buffer pH 6.0, in 300 mL flasks), with 5 mM substrate concentration. The reactions were monitored by GC/MS.



**Scheme 2.** Conversion of cycloalkenecarboxylates **3a,b-4a,b** into (*R*)- and (*S*)-**1a,b-2a,b**.

The entrapment of *R. oryzae* in calcium alginate beads considerably simplified the reaction work-up, which consisted simply in the removal of the beads by filtration and extraction of the filtrate with EtOAc. As discussed above, the use of a buffer solution instead of the fungal cultural medium greatly decreased the amounts of subproducts and metabolites in the isolated allylic alcohols. In particular, **7a** and **8a,b** were obtained in satisfactory purity and were submitted to the following step without further treatment. Only in the case of ethyl ester **3b**, the formation of 10% of saturated ketone (*S*)-**1b** was observed and column chromatography purification of allylic alcohol **7b** was thus necessary.

The allylic alcohols **7a,b-8a,b** were converted directly by laccase/TEMPO oxidation into  $\gamma$ -oxoesters **5a,b-6a,b** (total reaction volume 10 mL, substrate concentration 35 mM) affording complete conversion. Since the reaction conditions for the laccase/TEMPO system and for the OYE/NADP<sup>+</sup>/GDH/glucose system were found to be compatible, these intermediates  $\gamma$ -oxoesters

were not isolated and the final C=C reduction was carried out telescopically. Firstly, in order to avoid the consumption of NADP<sup>+</sup> by reaction with TEMPO<sup>+</sup>, after the oxidation the excess of molecular oxygen dissolved in the reaction medium was removed by bubbling nitrogen, and isopropanol was added to reduce the residual oxoammonium cation. Then, the suitable ene-reductases, together with NADP<sup>+</sup> and the cofactor regenerating system (GDH/glucose), were added to the reaction mixtures for the final reduction, which occurred quantitatively in all cases. Both the enantiomers of cyclic  $\gamma$ -oxoesters **1a,b-2a,b** were obtained in 47-68% overall isolation yields from the starting cycloalkenecarboxylates, without any chromatographic separation (except for intermediate **7b**).

## Conclusions

The two-step oxidation procedure, using *R. oryzae* resting cells entrapped in alginate beads and the laccase/TEMPO system, represents a sustainable enzymatic synthetic approach to unsaturated cyclic oxoesters **5a,b-6a,b**, showing lower environmental burden and higher efficiency than classical chemical oxidations. The biohydroxylation step is characterised by high regioselectivity with limited formation of side-products, such as regioisomeric allyl alcohols and epoxides (commonly occurring in chemical oxidation procedures, and causing a decrease of the overall yield of the desired products). The use of beads suspended in buffer solution makes the reaction work-up much easier: the fungal catalyst is removed by filtration, and the allylic alcohols are isolated in high purity (except for **7b**, that required column chromatography) without further purification before the following oxidation step, thus minimizing solvents/energy requirements of the process. The possibility to combine the laccase/TEMPO oxidation with the alkene bioreduction in a sequential cascade increases the value of the procedure. The availability of ene-reductases characterized by broad substrate tolerance for cyclic  $\gamma$ -oxoesters **4a,b-5a,b** and showing high and opposite

enantioselectivity, enabled the preparation of both the enantiomers of the corresponding saturated derivatives of satisfactory purity for API synthesis. Finally, the procedure is performed in water (with minimal amounts of DMSO or methanol as cosolvents) and the non-toxic waste generated during the whole process can be easily disposed of, in contrast with the traditional chemical procedures.

## Keywords

biocatalysis; oxidoreductases; enantioselectivity, ene-reductases, laccases

## Experimental

### General

GC-MS analyses were performed using a HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent). The following temperature program was employed: 60°C (1 min) / 6°C min<sup>-1</sup> / 150°C (1 min) / 12°C min<sup>-1</sup> / 280°C (5 min). The enantiomeric excess values of compounds **1a,b-2a,b** were determined by GC analysis. Chiral GC analyses were performed on a MEGA-DEX DAC-Beta column (25 m × 0.25 mm × 0.25 μm, Mega), carrier gas H<sub>2</sub>, constant flow 3.7 mL min<sup>-1</sup>, injector temperature 250°C, detector temperature 250°C with the following temperature programs: a) compound **1a**: 90°C / 0.7°C min<sup>-1</sup> / 112°C / 90°C min<sup>-1</sup> / 220°C (3 min), (*S*)-**1a** t<sub>R</sub> = 25.1 min; (*R*)-**1a** t<sub>R</sub> = 26.7 min; b) compound **1b**: 100°C (35 min) / 90°C min<sup>-1</sup> / 220°C (3 min), (*S*)-**1b** t<sub>R</sub> = 23.7 min; (*R*)-**1b** t<sub>R</sub> = 26.2 min; c) compound **2a**: 100°C / 0.3°C min<sup>-1</sup> / 110°C / 90°C min<sup>-1</sup> / 220°C (3 min), (*R*)-**2a** t<sub>R</sub> = 20.4 min; (*S*)-**2a** t<sub>R</sub> = 21.6 min; d) compound **2b**: 95°C (45 min) / 90°C min<sup>-1</sup> / 220°C (3 min), (*R*)-**2b** t<sub>R</sub> = 38.5 min; (*S*)-**2b** t<sub>R</sub> = 41.0 min. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a 400 or 500 MHz spectrometer, and the chemical shift scale was based on internal

tetramethylsilane. TLC analyses were performed on Merck Kieselgel 60 F254 plates. All the chromatographic separations were carried out on silica gel columns.

### **Strains and plasmids**

*Colletotrichum lini* CBS 112.21, *Rhizopus oryzae* Mucl 28423, *Aspergillus niger* (Microbiologia Industriale Milano) and *Alternaria tenuis* (Microbiologia Agraria, Alimentare, Ecologica) were obtained from the fungal collection of the Department of Food, Environmental and Nutritional Science (DeFENS), University of Milan (Prof. F. Molinari and Dr. D. Romano). Laccase Amano M120 was employed. Ene-reductases (OYE1-3, OYE2.6, and LeOPR1) and glucose dehydrogenase (GDH) were overproduced in *E. coli* BL21(DE3) or *E. coli* Rosetta<sup>TM</sup>(DE3) strains harbouring a specific plasmid prepared as previously reported: pET30a-OYE1 from the original plasmid provided by Neil C. Bruce,<sup>35</sup> pET30a-OYE2 and pET30a-OYE3 from *S. cerevisiae* BY4741 and pKTS-GDH from *B. megaterium* DSM509,<sup>36</sup> pDJBx-OYE2.6 and pDJBx-LeOPR1 from the original plasmids provided by Prof. Jon D. Stewart. As for YqjM, the original plasmid provided by Prof. M. Hall was used directly as provided.<sup>28</sup>

### **Entrapment of *R. oryzae***

*Rhizopus oryzae* CBS 112.07 was routinely maintained on MEA (malt extract agar) medium (malt extract 20 g L<sup>-1</sup>, glucose 20 g L<sup>-1</sup>, peptone 2 g L<sup>-1</sup>, agar 20 g L<sup>-1</sup>). The fungal spores were suspended in sterile water, mixed with a sterile Na-alginate solution (40 g L<sup>-1</sup>), and added dropwise to a sterile solution of CaCl<sub>2</sub> (20 g L<sup>-1</sup>) under mild stirring, to form cell beads with the average diameter of 3.5 mm. The beads were filtered, and pre-cultured in a flask (ca. 10 g of beads per 40 mL of MEA medium) at 25°C on a rotary shaker at 160 rpm for 2 days, to form a shell of mycelia on the outer surface. For Procedure B, the beads were employed as a suspension in this culture

medium. For Procedure C the beads were recovered by filtration, washed with sterile water and suspended in acetate buffer (20 mM, pH 6.0).

### **Overproduction of enzymes in *E. coli* BL21(DE3)**

LB medium (5 mL) containing the appropriate antibiotic (50  $\mu\text{g mL}^{-1}$  kanamycin for pET30a, 100  $\mu\text{g mL}^{-1}$  ampicillin for pKTS, pDJBx and 100  $\mu\text{g mL}^{-1}$  ampicillin and 30  $\mu\text{g mL}^{-1}$  chloramphenicol for YqjM) was inoculated with a single colony from a fresh plate and grown for 8 h at 37°C and 220 rpm. This starter culture was used to inoculate 500 mL LB medium (TB medium in the case of YqjM). The latter culture was shaken at 37°C and 220 rpm until OD<sub>600</sub> reached 0.4-0.5, then enzyme expression was induced by the addition of 0.1 mM IPTG (50  $\text{ng mL}^{-1}$  anhydrotetracycline was also added in the case of the pKTS-GDH plasmid) at 30°C and 160 rpm.

In the case of OYE1-3, GDH and YqjM, 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 KP<sub>i</sub> buffer pH 7.0, 300 mM NaCl, 10 mM imidazole) and disrupted by sonication (Omni Ruptor 250 ultrasonic homogeniser, five sonication cycles, 15 s each, 50% duty). YqjM was employed as cell-free extract, whereas for OYE1-3 and GDH the cell-free extract, after centrifugation (20000 g, 20 min, 4°C), was chromatographed on IMAC stationary phase (Ni-Sepharose Fast Flow, GE Healthcare) with a mobile phase composed of 20 mM KP<sub>i</sub> 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 KP<sub>i</sub> buffer pH 7.0 (12 h each, 4°C) to remove imidazole and salts. Purified protein aliquots were stored frozen at -80°C.

In the case of OYE2.6, and LeOPR1, produced as GST-fusion proteins, cell pellets were resuspended in cold sepharose binding buffer (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) and lysed by sonication (Omni Ruptor 250 ultrasonic homogeniser, five sonication cycles, 15 s each, 50% duty). The cell-free extract was centrifuged (12000 rpm, 40 min,

4°C). The resulting supernatant was passed through Glutathione Sepharose 4 Fast Flow (GE Healthcare), with PBS buffer as the mobile phase. Once the absorbance (280 nm) returned to a baseline reading, the desired protein was eluted by adding a reduced glutathione (GSH) buffer solution (10 mM  $\gamma$ -L-glutamyl-L-cysteinylglycine, 50 mM Tris-HCl, pH 8.0). Protein elution was monitored at 280 nm and the fractions were collected according to the chromatogram. Purified protein aliquots were stored frozen at  $-80^{\circ}\text{C}$ .

### **General procedure for the multi-enzymatic conversion of 3a,b-4a,b into 1a,b-2a,b**

#### *Allylic hydroxylation of substrates 3a,b-4a,b by R. oryzae (Procedure C)*

Pre-cultivated *R. oryzae* beads (corresponding to ca. 30 g of those prepared according to the entrapment procedure) were suspended in acetate buffer (120 mL, 20 mM, pH 6.0) in a 300 mL flask, and a solution of the substrate (1.2 mL, 500 mM) in MeOH (for compound **3a**) or DMSO (for compounds **3b-4a,b**) was added. The flask was shaken at 160 rpm at  $25^{\circ}\text{C}$ , and the reaction was monitored by GC/MS analysis. After consumption of the starting substrate, the beads were filtered, and the aqueous solution was extracted with EtOAc. The organic phase was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and the residue was submitted to the following oxidation step without any further purification.

#### **Methyl 3-hydroxycyclopent-1-ene-1-carboxylate (7a)**

From **3a** (0.076 g, 0.60 mmol), according to Procedure C after 5 days, derivative **7a** was obtained (0.074 mg, 87%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):<sup>37</sup>  $\delta$  = 6.70 (q,  $J$  = 2.1 Hz, 1H,  $\text{CH}=\text{C}$ ), 4.97 (m, 1H,  $\text{CHOH}$ ), 3.76 (s, 3H,  $\text{COOCH}_3$ ), 2.78 - 2.66 (m, 1H,  $\text{CHH}$ ), 2.56 - 2.32 (m, 2H,  $2\text{CHH}$ ), 1.85 - 1.73 (m, 1H,  $2\text{CHH}$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.6 MHz):  $\delta$  = 166.1, 143.0, 138.9, 77.2, 52.0, 33.2, 30.0; GC/MS (EI)  $t_{\text{R}}$  = 11.83 min:  $m/z$  (%) = 142 ( $\text{M}^+$ , 5), 127 (53), 81 (100).

### **Ethyl 3-hydroxycyclopent-1-ene-1-carboxylate (7b)**

From **3b** (0.084 g, 0.60 mmol), according to Procedure C after 2 days, derivative **7b** was recovered after purification by column chromatography (0.061 g, 65%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  = 6.69 (q,  $J$  = 2.1 Hz, 1H,  $\text{CH}=\text{C}$ ), 4.97 (m, 1H,  $\text{CHOH}$ ), 4.21 (q,  $J$  = 7.1 Hz,  $\text{COOCH}_2\text{CH}_3$ ), 2.80 - 2.65 (m, 1H,  $\text{CHH}$ ), 2.55-2.30 (m, 2H,  $2\text{CHH}$ ), 1.86-1.75 (m, 1H,  $2\text{CHH}$ ), 1.30 (t,  $J$  = 7.1 Hz,  $\text{COOCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.6 MHz):  $\delta$  = 165.3, 142.9, 139.2, 77.3, 60.7, 33.7, 30.0, 14.3; GC/MS (EI)  $t_{\text{R}}$  = 13.54 min:  $m/z$  (%) = 127 ( $\text{M}^+ - 29$ , 75), 111 (38), 83 (100).

### **Methyl 3-hydroxycyclohex-1-enecarboxylate (8a)**

From **4a** (0.084 g, 0.60 mmol), according to Procedure C after 3 days, derivative **8a** was obtained (0.082 g, 88%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $^{18}\delta$  = 6.87 (m, 1H,  $\text{CH}=\text{C}$ ), 4.35 (m, 1H,  $\text{CHOH}$ ), 3.75 (s, 3H,  $\text{COOCH}_3$ ), 2.36-2.16 (m, 2H,  $2\text{CHH}$ ), 2.00-1.50 (m, 4H,  $2\text{CH}_2$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.6 MHz):  $^{18}\delta$  = 167.9, 140.3, 132.1, 65.8, 51.8, 31.0, 24.2, 19.2; GC/MS (EI)  $t_{\text{R}}$  = 14.85 min:  $m/z$  (%) = 156 ( $\text{M}^+$ , 12), 141 (71), 124 (65), 97 (100).

### **Ethyl 3-hydroxycyclohex-1-enecarboxylate (8b)**

From **4b** (0.092 g, 0.60 mmol), according to Procedure C after 3 days, derivative **8b** was obtained (0.088 g, 86%):  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  = 6.87 (q,  $J$  = 2.2 Hz, 1H,  $\text{CH}=\text{C}$ ), 4.36 (m, 1H,  $\text{CHOH}$ ), 4.20 (q,  $J$  = 7.1 Hz,  $\text{COOCH}_2\text{CH}_3$ ), 2.35-2.15 (m, 2H,  $2\text{CHH}$ ), 2.00-1.75 (m, 2H,  $2\text{CHH}$ ), 1.70-1.50 (m, 2H,  $2\text{CHH}$ ), 1.28 (t,  $J$  = 7.1 Hz, 3H,  $\text{COOCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.6 MHz):  $\delta$  = 167.4, 139.5, 132.9, 66.1, 60.7, 31.4, 24.4, 19.2, 14.4; GC/MS (EI)  $t_{\text{R}}$  = 16.44 min:  $m/z$  (%) = 170 ( $\text{M}^+$ , 4), 141 (100), 124 (78), 114 (22), 97 (100).

*One-pot sequential two-step conversion of allylic alcohols 7a,b-8a,b into (S)- and (R)-1a,b-2a,b*

A solution of crude allylic alcohols **7a,b-8a,b** in DMSO (0.70 mL, 500 mM) was added to an acetate buffer (10 mL, 50 mM, pH 5.0), containing TEMPO<sup>+</sup>BF<sub>4</sub><sup>-</sup> (70 μmol, 0.2 eq.) and laccase Amano M120 (50 mg). After stirring at 30°C for 24 hours under O<sub>2</sub> atmosphere to achieve complete conversion into intermediate oxoesters **5a,b-6a,b**, *i*PrOH (100 μL) was added to quench the excess of TEMPO<sup>+</sup>, and N<sub>2</sub> was bubbled through the solution to remove dissolved oxygen. The required OYE (1-2 mg) was then added, together with glucose (1.40 mmol, 4 eq.), NADP<sup>+</sup> (35.0 μmol, 0.1 eq.), and GDH (5 U mL<sup>-1</sup>). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30°C). The solution was extracted with EtOAc, centrifuging after each extraction (15000 g, 1.5 min), and the combined organic solutions were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, to give either (*S*)- or (*R*)-**1a,b-2a,b**.

#### **(*S*)-Methyl 3-oxocyclopentanecarboxylate ((*S*)-1a)**

From **7a** (0.050 g, 0.35 mmol), after TEMPO/laccase oxidation and OYE1-mediated reduction, compound (*S*)-**1a** (0.037g, 75%) was obtained: ee = 90% (chiral GC); [α]<sub>D</sub> = -36.2 (*c* 1.0, CHCl<sub>3</sub>) [lit. ref. 38 [α]<sub>D</sub> = 37 (*c* 3.5, CHCl<sub>3</sub>, for (*R*)-**1a** with ee = 95%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):<sup>28</sup> δ = 3.70 (s, 3H, COOCH<sub>3</sub>), 3.11 (m, 1H, CHCOOCH<sub>3</sub>), 2.51-2.01 (m, 6H, 3CH<sub>2</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100.6 MHz):<sup>28</sup> δ = 216.5, 174.8, 52.2, 41.2, 40.9, 37.5, 26.6; GC/MS (EI) t<sub>R</sub> = 10.43 min: *m/z* (%) = 142 (M<sup>+</sup>, 13), 114 (75), 55 (100).

#### **(*R*)-Methyl 3-oxocyclopentanecarboxylate ((*R*)-1a)**

From **7a** (0.050 g, 0.35 mmol), after TEMPO/laccase oxidation and LeOPR1-mediated reduction, compound (*R*)-**1a** (g, %) was obtained: ee = 88% (chiral GC); [α]<sub>D</sub> = +35.0 (*c* 1.0, CHCl<sub>3</sub>). <sup>1</sup>H and <sup>13</sup>C NMR spectra were in agreement with those of the (*S*)-enantiomer.

#### **(*S*)-Ethyl 3-oxocyclopentanecarboxylate ((*S*)-1b)**

From **7b** (0.054 g, 0.35 mmol), after TEMPO/laccase oxidation and OYE1-mediated reduction, compound (*S*)-**1b** (0.040 g, 73%) was obtained: ee = 86% (chiral GC);  $[\alpha]_D = -18.8$  (*c* 1.5, MeOH) [lit.<sup>39</sup>  $[\alpha]_D = -22.2$  (*c* 1.0, MeOH, for (*S*)-**1b** with ee = 99%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):<sup>28</sup>  $\delta = 4.18$  (q, *J* = 7.1 Hz, COOCH<sub>2</sub>CH<sub>3</sub>), 3.10 (m, 1H, CHCOOCH<sub>2</sub>CH<sub>3</sub>), 2.55-2.09 (m, 3 CH<sub>2</sub>, 6H), 1.29 (t, *J* = 7.1 Hz, 3H, COOCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100.6 MHz):<sup>28</sup>  $\delta = 216.6, 174.3, 61.0, 41.2, 41.0, 37.5, 26.7, 14.3$ ; GC/MS (EI)  $t_R = 12.18$  min: *m/z* (%) = 156 (M<sup>+</sup>, 5), 128 (50), 111 (25), 100 (75), 55 (100).

#### **(R)-Ethyl 3-oxocyclopentanecarboxylate ((R)-1b)**

From **7b** (0.058 g, 0.38 mmol), after TEMPO/laccase oxidation and LeOPR1-mediated reduction, compound (*R*)-**1b** (0.041 g, 70%) was obtained: ee = 99% (chiral GC);  $[\alpha]_D = +21.7$  (*c* 1.0, MeOH). <sup>1</sup>H and <sup>13</sup>C NMR spectra were in agreement with those of the (*S*)-enantiomer.

#### **(S)-Methyl 3-oxocyclohexanecarboxylate ((S)-2a)**

From **8a** (0.052 g, 0.34 mmol), after TEMPO/laccase oxidation and OYE1-mediated reduction, compound (*S*)-**2a** (0.041 g, 77%) was obtained: ee = 94% (chiral GC);  $[\alpha]_D = +2.7$  (*c* 2.0, EtOH) [lit. ref. 27  $[\alpha]_D = -3.2$  (*c* 0.135 g mL<sup>-1</sup>, EtOH, for (*R*)-**1a** with ee = 99%); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):<sup>28</sup>  $\delta = 3.70$  (s, 3H, COOCH<sub>3</sub>), 2.80 (m, 1H, CHCOOCH<sub>3</sub>), 2.55 (d, *J* = 7.9 Hz, 2H, CH<sub>2</sub>CHCOO), 2.45 -2.25 (m, 2H, 2CHH), 2.20 -2.10 (m, 2H, 2CHH), 1.90 - 1.65 (m, 2H, 2CHH); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100.6 MHz):<sup>28</sup>  $\delta = 209.3, 174.2, 52.2, 43.20, 43.19, 41.0, 27.8, 24.6$ ; GC/MS (EI)  $t_R = 13.02$  min: *m/z* (%) = 156 (M<sup>+</sup>, 21), 124 (11), 113 (32), 97 (100).

#### **(R)-Methyl 3-oxocyclohexanecarboxylate ((R)-2a)**

From **8a** (0.054 g, 0.35 mmol), after TEMPO/laccase oxidation and LeOPR1-mediated reduction, compound (*R*)-**2a** (0.038 g, 69%) was obtained: ee = 98% (chiral GC);  $[\alpha]_{\text{D}} = -3.0$  (*c* 1.5, MeOH).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were in agreement with those of the (*S*)-enantiomer.

#### (*S*)-Ethyl 3-oxocyclohexanecarboxylate ((*S*)-**2b**)

From **8b** (0.055 g, 0.33 mmol), after TEMPO/laccase oxidation and OYE1-mediated reduction, compound (+)-**2b** (0.042 g, 74%) was obtained: ee = 96% (chiral GC);  $[\alpha]_{\text{D}} = +2.7$  (*c* 1.6, MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta = 4.15$  (q, s, 2H,  $\text{COOCH}_2\text{CH}_3$ ), 2.78 (m, 1H,  $\text{CHCOOCH}_2\text{CH}_3$ ), 2.55 (d, *J* = 7.9 Hz, 2H,  $\text{CH}_2\text{CHCOO}$ ), 2.40-2.25 (m, 2H, 2*CHH*), 2.20-2.05 (m, 2H, 2*CHH*), 1.95 - 1.70 (m, 2H, 2*CHH*), 1.27 (t, *J* = 7.1 Hz, 3H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100.6 MHz):  $\delta = 209.4$ , 173.8, 61.0, 43.3, 43.2, 41.0, 27.8, 24.6, 14.3; GC/MS (EI)  $t_{\text{R}} = 14.70$  min: *m/z* (%) = 170 ( $\text{M}^+$ , 7), 142 (5), 128 (12), 125 (9), 113 (6), 102 (6), 97 (100).

Compound (+)-**2b** (0.035 g, 0.21 mmol) was dissolved in MeOH (2 mL) and treated with a solution of LiOH in water (4 mL, 1 M). After stirring at room temperature for 6 h, the solution was concentrated under reduced pressure, acidified with 10% HCl and extracted with EtOAc. The solvent was removed *in vacuo* to afford (+)-(*S*)-3-oxocyclohexanecarboxylic acid (0.026 g, 87%):  $[\alpha]_{\text{D}} = +16.5$  (*c* 1, MeOH) lit. ref. 40  $[\alpha]_{\text{D}} = -17.6$  (*c* 4, MeOH) for the (*R*)-enantiomer;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):<sup>41</sup>  $\delta = 2.92$ -2.80 (m, 1H,  $\text{CHCOOH}$ ), 2.65-2.50 (m, 2H,  $\text{CH}_2\text{CHCOO}$ ), 2.45-2.25 (m, 2H, 2*CHH*), 2.20-2.00 (m, 2H, 2*CHH*), 1.95-1.70 (m, 2H, 2*CHH*); GC/MS (EI)  $t_{\text{R}} = 15.45$  min: *m/z* (%) = 142 ( $\text{M}^+$ , 33), 114 (20), 97 (92), 55 (100).

#### (*R*)-Ethyl 3-oxocyclohexanecarboxylate ((*R*)-**2b**)

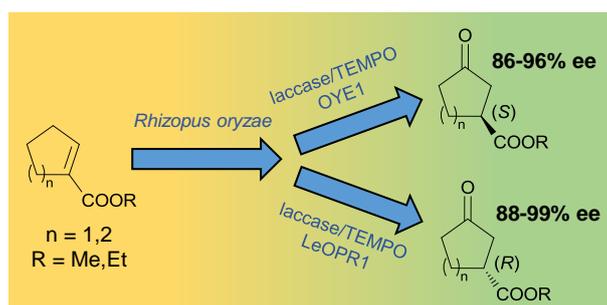
From **8b** (0.060 g, 0.36 mmol), after TEMPO/laccase oxidation and LeOPR1-mediated reduction, compound (*R*)-**2b** (0.046 g, 75%) was obtained: ee = 99% (chiral GC);  $[\alpha]_{\text{D}} = -2.9$  (*c* 1.7, MeOH).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were in agreement with those of the (*S*)-enantiomer.

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## Graphical Abstract

The conversion of C<sub>5</sub> and C<sub>6</sub> cycloalkenecarboxylates into both enantiomers of the corresponding cyclic  $\gamma$ -oxoesters is performed at room temperature using three enzymes, two isolation steps, and no environmentally-unfriendly oxidants.



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