Biocatalytic synthesis of chiral cyclic γ-oxoesters by sequential C-H hydroxylation, alcohol oxidation and alkene reduction

Journal:	*Green Chemistry*
Manuscript ID	GC-ART-07-2017-002215
Article Type:	Paper
Date Submitted by the Author:	21-Jul-2017

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Article type: Full paper

Website www.rsc.org/greenchem
Impact factor* 8.506

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DIPARTIMENTO DI CHIMICA, MATERIALI ED INGEGNERIA CHIMICA "Giulio NATTA"

Milano, 21st July 2017

Dear Editor, we are sending you our manuscript “Biocatalytic synthesis of chiral cyclic γ-oxoesters by sequential C-H hydroxylation, alcohol oxidation and alkene reduction” to be considered for publication as a *Full Paper* in *Green Chemistry.*

The paper describes a three-step enzymatic procedure to convert methyl and ethyl cyclopentene- and cyclohexenecarboxylates into both the enantiomers of the corresponding chiral γ-oxoesters, which are useful building blocks for the synthesis of active pharmaceutical ingredients. The second and third steps were telescoped by adding the two corresponding enzymes in a sequential mode to the reaction vessel. The biocatalysed allylic oxidation of these cycloalkenecarboxylates represents a suitable alternative to the use of traditional stoichiometric oxidants (chromium(VI)-based reagents, manganese dioxide, potassium permanganate or selenium dioxide).

While we were finalising the experimental work, a paper by Faber *et al.* (N. G. Turrini, R. C. Cioc, D. J. H. van der Niet, E. Ruijter, R. V. A. Orru, M. Hall, K. Faber, *Green Chem.,* 2017, 19, 511-518, ref. 28 in our manuscript) appeared in your Journal, describing the reduction of substrates **5a**,**b**-**6a**,**b** (*i.e.* only one step of our 3-step procedure). In that paper, a very unusual inversion of enantioselectivity between OYE1/OYE3 *vs.* OYE2 in the reduction of **5a**,**b**-**6a**,**b** was described, a remarkable observation because of the extremely high sequence homology of OYE1 and OYE2. Indeed, in our hands, no significant difference in enantioselectivity between these three enzymes was observed, as reported in the present manuscript. Therefore, we anticipated privately the results of our biotransformations to the authors (private communication to Prof. K. Faber and Prof. M. Hall). They confirmed that our data are correct by repeating some of these enzymatic reactions with OYE1 and OYE3 samples, both freshly prepared in their lab and purified to homogeneity by nickel affinity chromatography. According to them, the discrepancy between our data and the results reported in ref. 28 may be due to the fact that they used some industrial batches of OYE1 and OYE3 proteins for that work, and they are currently performing further investigations to fully understand their results. If you find it appropriate, we raise no objection against having Prof. Melanie Hall or Prof. Kurt Faber as one of the reviewers of our work.

Considering the very high selectivity of the process and the reduction of the environmental impact by substitution of toxic reagents, compared to existing methods, we are confident of the interest that this work may arise in the readers of your Journal.

Sincerely yours,

Elisabetta Brenna

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Biocatalytic synthesis of chiral cyclic γ-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

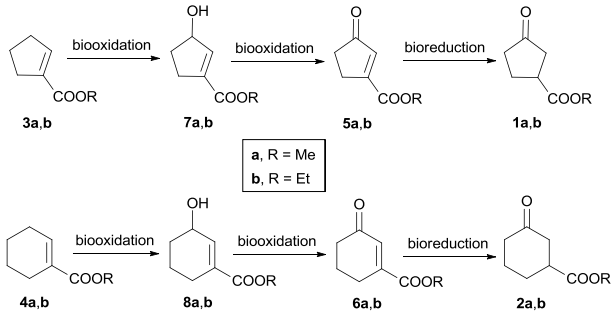
The key role played by biocatalysis in improving the sustainability of fine and bulk chemicals manufacturing is well recognized.[1] The capability of enzymes to catalyze a remarkably wide range of organic reactions with excellent chemo-, regio-, and stereoselectivity under mild conditions (temperature, pH, pressure), limiting the amount of waste and by-products, is greatly appreciated, especially in the pharmaceutical field.[2] Furthermore, the combination of multi-enzymatic reactions in a cascade sequence[3] 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.[4]

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 α,β-unsaturated ketones.[5] 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.[6] Biocatalysis offers advantageous strategies for this reaction, based on the versatile oxidative capabilities of (mono- and di-) oxygenases, peroxygenases and oxidases.[7] 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.[8]

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 γ-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**.

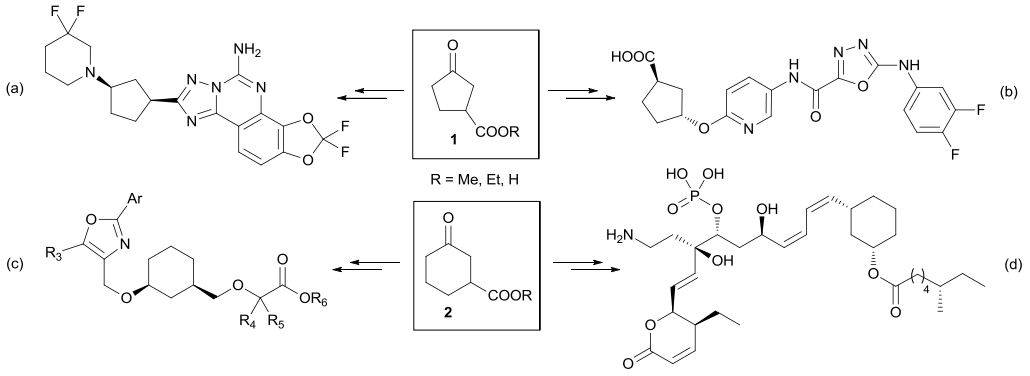


Figure 1. Structures of active pharmaceutical ingredients incorporating a 1,3-difunctionalised C_5 or C_6 ring: (a) antagonists of the A_{2A} receptor, employed for the treatment of neurological disorders;[9] (b) inhibitors of acetyl-CoA:diacylglycerol acyltransferase acvtivity, employed for the treatment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

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_2Cl_2 at 0-5°C;[13,14] (ii) *t*-butyl hydroperoxide as stoichiometric oxidant, in the presence of either 20% $Pd(OH)_2/C$ (5 mol%)[15] or dirhodium complexes[16,17] as catalysts in CH_2Cl_2 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**.[18]In a following work,[19] 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 γ-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.[20-23] 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.[24] 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 $CaCl_2$, 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.[24] 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%).

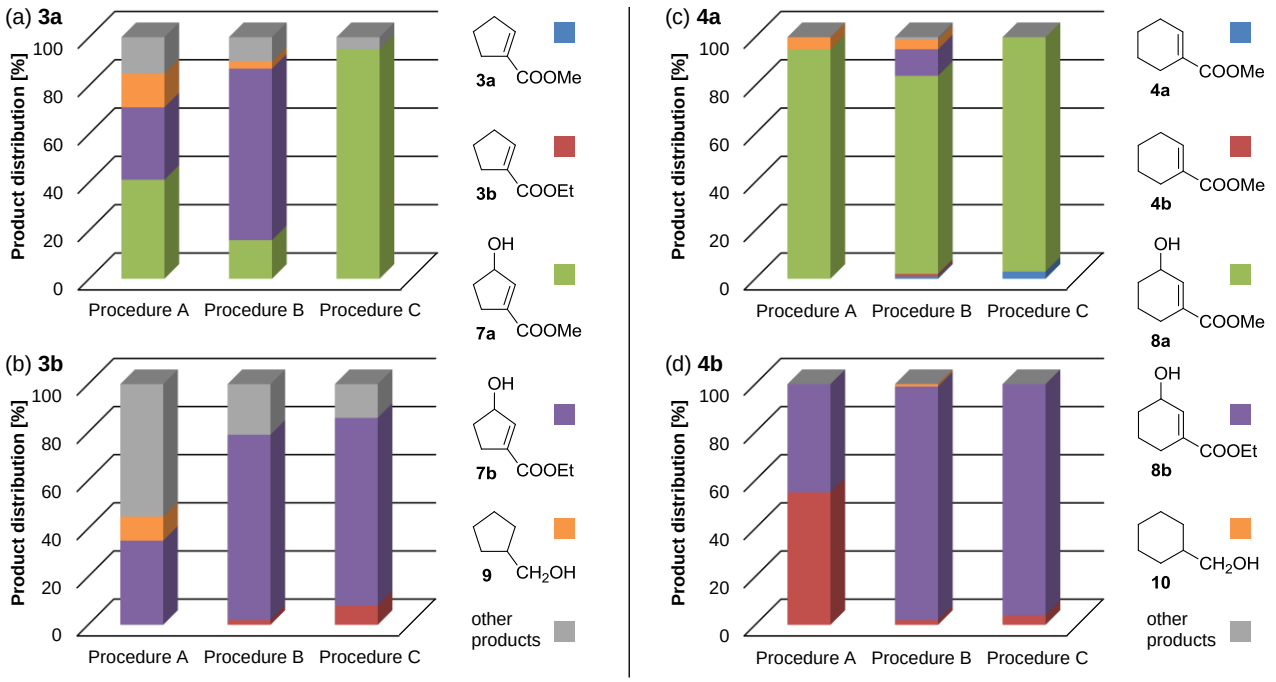


Figure 2. Results of *R. oryzae* mediated biotransformations of compounds **3a** (a), **3b** (b), **4a** (c), **4b** (d), according to the following experimental procedures. Procedure A: 5 mM substrate (DMSO as cosolvent), *R. oryzae* whole cells, 25°C; Procedure B: 5 mM substrate (DMSO as cosolvent), *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.[25] 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).[26] The treatment of allylic alcohols **7a**,**b**-**8a**,**b** (5 g L^{-1}, with DMSO as a cosolvent) with catalytic $TEMPO^{+}$ BF_4^{-} and laccase Amano M120 in acetate buffer (pH 5.0) under O_2 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

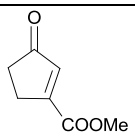
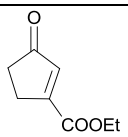
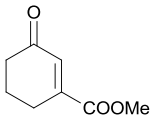
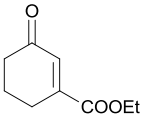
The enantioselective reduction of compounds **5a**,**b**-**6a**,**b** had been previously described in the literature. Reetz *et al.*[27] had reported that the reduction of methyl esters **5a** and **6a** mediated by YqjM afforded the (*R*)-enantiomer of saturated ketoesters **1a** and **2a** very efficiently. Moreover, while we were finalizing our experimental work on this topic, a paper by Faber *et al.*[28] was published, describing the enantioselective hydrogenations of substrates **5a**,**b**-**6a**,**b** (obtained by traditional chemical oxidation with CrO_3) 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.[29] 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[a] of compounds **5a**,**b**-**6a**,**b**

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

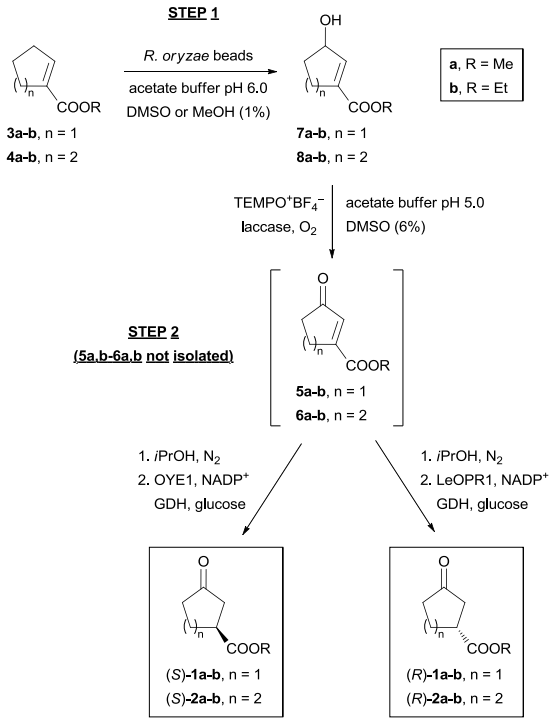
[a] 5 mM substrate, 20 mM glucose, isolated OYE, GDH, $NADP^+$, 1% DMSO, phosphate buffer pH 7.0, 30°C, 24 h; [b] conversion calculated on the basis of GC analysis of the crude mixture after 24 h (isolation yields are reported in the Experimental Section); [c] enantiomeric excess calculated on the basis 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),[30] while it is converted very poorly by LeOPR1 and YqjM,[31] and no information are available about its reduction with OYE2.6. Similarly, 3-methylcyclohexenone is converted into (*S*)-3-methylcyclohexanone by OYE1-3[32,30] and

OYE2.6,[33] and it is scarcely accepted by LeOPR1 and YqjM.[31] Furthermore, investigations performed by using OYE1[34] and OYE2.6[33] highlighted that the reduction of 3-alkylcyclohexenones is very sensitive to steric hindrance of the substituent at the β-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 γ-oxoesters of this work, the presence of either a methyl or an ethyl ester group linked to the β 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 simplifed 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 γ-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/$NADP^+$/GDH/glucose system were found to be compatible, these intermediates γ-oxoesters

were not isolated and the final C=C reduction was carried out telescopically. Firstly, in order to avoid the consumption of $NADP^+$ by reaction with $TEMPO^+$, 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 $NADP^+$ 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 γ-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 γ-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^{-1} / 150°C (1 min) / 12°C min^{-1} / 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_2, constant flow 3.7 mL min^{-1}, injector temperature 250°C, detector temperature 250°C with the following temperature programs: a) compound **1a**: 90°C / 0.7°C min^{-1} / 112°C / 90°C min^{-1} / 220°C (3 min), (*S*)-**1a** t_R = 25.1 min; (*R*)-**1a** t_R = 26.7 min; b) compound **1b**: 100°C (35 min) / 90°C min^{-1} / 220°C (3 min), (*S*)-**1b** t_R = 23.7 min; (*R*)-**1b** t_R = 26.2 min; c) compound **2a**: 100°C / 0.3°C min^{-1} / 110°C / 90°C min^{-1} / 220°C (3 min), (*R*)-**2a** t_R = 20.4 min; (*S*)-**2a** t_R = 21.6 min; d) compound **2b**: 95°C (45 min) / 90°C min^{-1} / 220°C (3 min), (*R*)-**2b** t_R = 38.5 min; (*S*)-**2b** t_R = 41.0 min. ^1H and ^{13}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^{TM}(DE3) strains harbouring a specific plasmid prepared as previously reported: pET30a-OYE1 from the original plasmid provided by Neil C. Bruce,[35] pET30a-OYE2 and pET30a-OYE3 from *S. cerevisiae* BY4741 and pKTS-GDH from *B. megaterium* DSM509;[36] 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.[28]

Entrapment of *R. oryzae*

Rhizopus oryzae CBS 112.07 was routinely maintained on MEA (malt extract agar) medium (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, peptone 2 g L^{-1}, agar 20 g L^{-1}). The fungal spores were suspended in sterile water, mixed with a sterile Na-alginate solution (40 g L^{-1}), and added dropwise to a sterile solution of $CaCl_2$ (20 g L^{-1}) 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 μg mL^{-1} kanamycin for pET30a, 100 μg mL^{-1} ampicillin for pKTS, pDJBx and 100 μg mL^{-1} ampicillin and 30 μg 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_{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) 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_i 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_i 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_i 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_2HPO_4, 1.8 mM KH_2PO_4, 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 γ-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°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°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 Na_2SO_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}H NMR ($CDCl_3$, 400 MHz):[37] δ = 6.70 (q, J = 2.1 Hz, 1H, C*H*=C), 4.97 (m, 1H, C*H*OH), 3.76 (s, 3H, COOCH_3), 2.78 - 2.66 (m, 1H, *CH*H), 2.56 - 2.32 (m, 2H, 2*CH*H), 1.85 – 1.73 (m, 1H, 2*CH*H); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 166.1, 143.0, 138.9, 77.2, 52.0, 33.2, 30.0; GC/MS (EI) t_R = 11.83 min: *m*/*z* (%) = 142 (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}H NMR ($CDCl_3$, 400 MHz): δ = 6.69 (q, J = 2.1 Hz, 1H, C*H*=C), 4.97 (m, 1H, C*H*OH), 4.21 (q, J = 7.1 Hz, COOCH_2CH$_3$), 2.80 - 2.65 (m, 1H, *CH*H), 2.55-2.30 (m, 2H, 2*CH*H), 1.86-1.75 (m, 1H, 2*CH*H), 1.30 (t. J = 7.1 Hz, COOCH$_2$*CH$_3$*; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 165.3, 142.9, 139.2, 77.3, 60.7, 33.7, 30.0, 14.3; GC/MS (EI) t_R = 13.54 min: *m*/*z* (%) = 127 (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}H NMR ($CDCl_3$, 400 MHz):[18] δ = 6.87 (m, 1H, C*H*=), 4.35 (m, 1H, C*H*OH), 3.75 (s, 3H, COO*CH$_3$*), 2.36-2.16 (m, 2H, 2*CH*H), 2.00-1.50 (m, 4H, 2 *CH$_2$*); ^{13}C NMR ($CDCl_3$, 100.6 MHz: [18] δ = 167.9, 140.3, 132.1, 65.8, 51.8, 31.0, 24.2, 19.2; GC/MS (EI) t_R = 14.85 min: *m*/*z* (%) = 156 (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}H NMR ($CDCl_3$, 400 MHz): δ = 6.87 (q, J = 2.2 Hz, 1H, C*H*=C), 4.36 (m, 1H, C*H*OH), 4.20 (q, J = 7.1 Hz, COOCH_2CH$_3$), 2.35-2.15 (m, 2H, 2*CH*H), 2.00-1.75 (m, 2H, 2*CH*H), 1.70-1.50 (m, 2H, 2*CH*H), 1.28 (t, J = 7.1 Hz, 3H, COOCH$_2$*CH$_3$*); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 167.4, 139.5, 132.9, 66.1, 60.7, 31.4, 24.4, 19.2, 14.4; GC/MS (EI) t_R = 16.44 min: *m*/*z* (%) = 170 (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 $TEMPO^+BF_4^-$ (70 μmol, 0.2 eq.) and laccase Amano M120 (50 mg). After stirring at 30°C for 24 hours under O_2 atmosphere to achieve complete conversion into intermediate oxoesters **5a,b**-**6a,b**, *i*PrOH (100 uL) was added to quench the excess of $TEMPO^+$, and N_2 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.), $NADP^+$ (35.0 μmol, 0.1 eq.), and GDH (5 U mL^{-1}). 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_2SO_4, 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); $[\alpha]_D$ = –36.2 (*c* 1.0, $CHCl_3$) [lit. ref. 38 $[\alpha]_D$ = 37 (*c* 3.5, $CHCl_3$, for (*R*)-**1a** with ee = 95%); ^{1}H NMR ($CDCl_3$, 400 MHz):[28] δ = 3.70 (s, 3H, COO*CH*$_3$), 3.11 (m, 1H, *CH*$COOCH_3$), 2.51-2.01 (m, 6H, 3*CH*$_2$); ^{13}C NMR ($CDCl_3$, 100.6 MHz):[28] δ =216.5, 174.8, 52.2, 41.2, 40.9, 37.5, 26.6; GC/MS (EI) t_R = 10.43 min: *m*/*z* (%) = 142 (M^+, 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); $[\alpha]_D$ = +35.0 (*c* 1.0, $CHCl_3$). ^{1}H and ^{13}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.[39] $[\alpha]_D$ = -22.2 (*c* 1.0, MeOH, for (*S*)-**1b** with ee = 99%); ^{1}H NMR ($CDCl_3$, 400 MHz):[28] δ = 4.18 (q, *J* = 7.1 Hz, COOC*H*$_2$CH$_3$), 3.10 (m, 1H, *CH*COOCH$_2$CH$_3$), 2.55-2.09 (m, 3 C*H*$_2$, 6H), 1.29 (t, *J* = 7.1 Hz, 3H, COOCH$_2$*CH*$_3$); ^{13}C NMR ($CDCl_3$, 100.6 MHz):[28] δ = 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^+, 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). ^{1}H and ^{13}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^{-1}, EtOH, for (*R*)-**1a** with ee = 99%); ^{1}H NMR ($CDCl_3$, 400 MHz):[28] δ = 3.70 (s, 3H, COO*CH*$_3$), 2.80 (m, 1H, *CH*COOCH$_3$), 2.55 (d, *J* = 7.9 Hz, 2H, *CH*$_2$CHCOO), 2.45 -2.25 (m, 2H, 2*CH*H), 2.20 -2.10 (m, 2H, 2*CH*H), 1.90 - 1.65 (m, 2H, 2*CH*H); ^{13}C NMR ($CDCl_3$, 100.6 MHz):[28] δ = 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^+, 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); $[\alpha]_D$ = –3.0 (*c* 1.5, MeOH). ^{1}H and ^{13}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); $[\alpha]_D$ = +2.7 (*c* 1.6, MeOH). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.15 (q, s, 2H, COOC*H*$_2$CH$_3$), 2.78 (m, 1H, *CH*COOCH$_2$CH$_3$), 2.55 (d, *J* = 7.9 Hz, 2H, *CH*$_2$CHCOO), 2.40-2.25 (m, 2H, 2*CH*H), 2.20-2.05 (m, 2H, 2*CH*H), 1.95 - 1.70 (m, 2H, 2*CH*H), 1.27 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ = 209.4, 173.8, 61.0, 43.3, 43.2, 41.0, 27.8, 24.6, 14.3; GC/MS (EI) t_R = 14.70 min: *m*/*z* (%) = 170 (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-oxocylcohexanecarboxylic acid (0.026 g, 87%): $[\alpha]_D$ = +16.5 (*c* 1, MeOH) lit. ref. 40 $[\alpha]_D$ = –17.6 (*c* 4, MeOH) for the (*R*)-enantiomer; ^{1}H NMR ($CDCl_3$, 400 MHz):[41] δ = 2.92-2.80 (m, 1H, *CH*COOH), 2.65-2.50 (m, 2H, *CH*$_2$CHCOO), 2.45-2.25 (m, 2H, 2*CH*H), 2.20-2.00 (m, 2H, 2*CH*H), 1.95-1.70 (m, 2H, 2*CH*H); GC/MS (EI) t_R = 15.45 min: *m*/*z* (%) = 142 (M^+, 33), 114 (20), 97 (92), 55 (100).

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

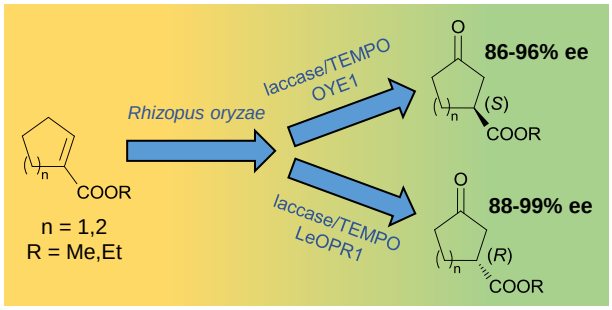
From **8b** (0.060 g, 0.36 mmol), after TEMPO/laccase oxidation and LeOPR1-mediated reduction, compound (*R*)-**2b** (0.046 g, 75%) was obtained: ee = 99% (chiral GC); $[\alpha]_D$ = - 2.9 (*c* 1.7, MeOH). ^{1}H and ^{13}C NMR spectra were in agreement with those of the (*S*)-enantiomer.

Acknowledgement

This work was supported by Fondazione Cariplo (grant no. 2014-0568). Prof. F. Molinari, and Dr. D. Romano (University of Milan, Department of Food, Environmental and Nutritional Science) are kindly acknowledged for making available the fungal strains. Prof J. D. Stewart (University of Florida) is acknowledged for the kind gift of OYE2.6 and LeOPR1 expression constructs, and Prof. M. Hall (University of Graz) for that of YqjM. Mr. Davide De Simeis and Giacomo Toselli are kindly acknowledged for helpful technical assistance.

Graphical Abstract

The conversion of C_5 and C_6 cycloalkenecarboxylates into both enantiomers of the corresponding cyclic γ-oxoesters is performed at room temperature using three enzymes, two isolation steps, and no environmentally-unfriendly oxidants.



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29. In contrast with previously published data (ref. 28), no inversion of enantioselectivity was observed with OYE1 and 3 with respect to OYE2.

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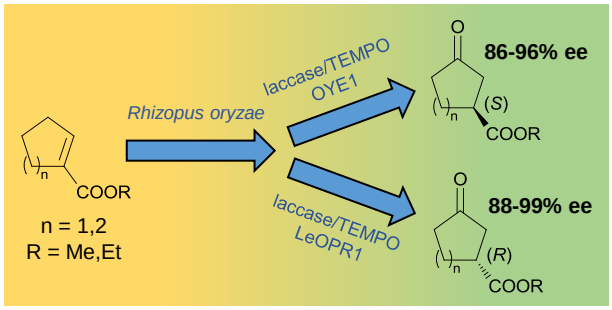
Graphical Abstract

The conversion of C_5 and C_6 cycloalkenecarboxylates into both enantiomers of the corresponding cyclic γ-oxoesters is performed at room temperature using three enzymes, two isolation steps, and no environmentally-unfriendly oxidants.



Biocatalytic synthesis of chiral cyclic γ-oxoesters by sequential C-H hydroxylation, alcohol oxidation and alkene reduction

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ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

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General methods

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^{-1} / 150°C (1 min) / 12°C min^{-1} / 280°C (5 min). ^{1}H and ^{13}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. Chiral GC analyses of compounds **7a,b**-**8a,b** as acetyl derivatives (obtained by treatment with acetic anhydride in pyridine) were performed on a Chirasil DEX CB (25m × 0.25mm × 0.25 μm, Chrompack) column, installed on HP 6890 gas chromatographs, with the following temperature program: 80°C / 1.0°C min^{-1} / 118°C (1 min) / 90°C min^{-1} / 220°C (5 min): compound **7a** t_R minor enantiomer = 20.7 min, t_R major enantiomer = 22.2 min; compound **7b** t_R minor enantiomer = 25.6 min, t_R major enantiomer = 27.3 min; compound **8a** t_R minor enantiomer = 29.8 min, t_R major enantiomer = 30.2 min; compound **8b** t_R minor enantiomer = 35.3 min, t_R major enantiomer = 35.9 min.

Chemical synthesis of 1a,b-8a,b

Methyl cyclopentenecarboxylate (**3a**) and methyl cyclohexenecarboxylate (**4a**) were commercial products. The corresponding ethyl esters (**3b** and **4b**) were prepared by transesterification with ethanol and catalytic sulfuric acid. Methyl and ethyl esters of compounds **4**-**7**, to be used as reference standards, were prepared according to literature procedures:

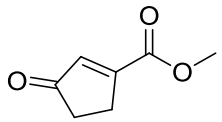
(i) oxidation of **3a,b**-**4a,b** with chromium(VI) oxide, with acetic anhydride and acetic acid in CH_2Cl_2 at 0-5°C;[1,2]

(ii) reduction of **5a,b**-**6a,b** with $NaBH_4$ and cerium(III) chloride heptahydrate in MeOH at 0°C;[3] racemic compounds **1a,b**-**2a,b** were prepared by hydrogenation of derivatives **5a,b**-**6a,b** in the presence of Pd/C in EtOAc solution.

Conversion of allylic alcohols 7a,b-8a,b into derivatives 5a,b-6a,b

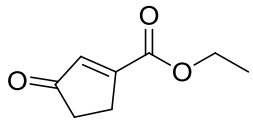
A solution of crude allylic alcohols **7a,b**-**8a,b**, obtained by *R. oryzae* mediated hydroxylation (procedure C, main text), in DMSO (0.42 mL, 500 mM) was added to an acetate buffer (6 mL, 50 mM, pH 5), containing $TEMPO^+BF_4^-$ (42 μmol, 0.2 eq.) and laccase Amano M120 (30 mg). After stirring at 30 °C for 24 h under O_2 atmosphere, the reaction mixture was extracted with EtOAc, and the combined organic solutions were dried over anhydrous Na_2SO_4, to give derivatives **5a,b**-**6a,b**, which were submitted to purification by column chromatography (silica gel), eluting with *n*-hexane and increasing amounts of EtOAc, to afford pure compounds for analytical characterisation.

Methyl 3-oxocyclopent-1-enecarboxylate (5a)



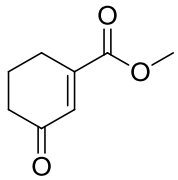
From compound **8a** (0.030 g, 0.21 mmol) derivative **5a** was obtained (0.026 g, 89%): ^{1}H NMR ($CDCl_3$, 400 MHz):[4] δ = 6.76 (t, J = 2.1 Hz, 1H, C*H*=), 3.87 (s, 3H, COO*CH*$_3$), 2.88 (m, 2H, CC*H*$_2$), 2.56 (m, 2H, COC*H*$_2$); ^{13}C NMR ($CDCl_3$, 100.6 MHz):[4] δ = 209.1, 164.9, 163.9, 138.4, 52.7, 35.7, 27.6; GC/MS (EI) t_R = 11.20 min: *m*/*z* (%) = 140 (M^+, 69), 112 (75), 109 (38), 53 (100).

Ethyl 3-oxocyclopent-1-enecarboxylate (5b)



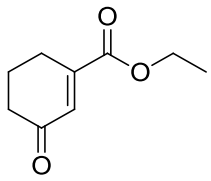
From compound **7b** (0.033 g, 0.21 mmol) derivative **5b** was obtained (0.030 g, 92%): ^{1}H NMR ($CDCl_3$, 400 MHz): [4] δ = 6.75 (t, J = 2.1 Hz, 1H, C*H*=), 4.32 (q, J = 7.1 Hz, 2H, COOC*H*$_2$CH$_3$), 2.85 (m, 2H, CC*H*$_2$), 2.53 (m, 2H, COC*H*$_2$), 1.35 (t, J = 7.1 Hz, 3H, COOCH$_2$*CH*$_3$); ^{13}C NMR ($CDCl_3$, 100.6 MHz): [4] δ = 209.1, 164.4, 138.1, 61.7, 35.7, 27.6, 14.2; GC/MS (EI) t_R = 12.92 min: *m*/*z* (%) = 154 (M^+, 44), 126 (50), 109 (44), 98 (100).

Methyl 3-oxocyclohex-1-enecarboxylate (6a)



From compound **8a** (0.033 g, 0.21 mmol) derivative **6a** was obtained (0.028 g, 87%):^{1}H NMR ($CDCl_3$, 400 MHz): [4] δ = 6.73 (t, *J* = 1.9 Hz, 1H, C*H*=), 3.83 (s, 3H, COO*CH*$_3$), 2.58 (m, 2H, COC*H*$_2$), 2.44 (m, 2H, CC*H*$_2$), 2.06 (m, 2H, $CH_2CH_2CH_2$); ^{13}C NMR ($CDCl_3$, 100.6 MHz): [4] δ = 200.0, 167.0, 148.8, 133.0, 52.6, 37.7, 24.9, 22.2; GC/MS (EI) t_R = 13.78 min: *m*/*z* (%) = 154 (M^+, 60), 126 (100), 98 (80).

Ethyl 3-oxocyclopent-1-enecarboxylate (6b)



From compound **8b** (0.036 g, 021 mmol) derivative **6b** was obtained (0.032 g, 91%): ^{1}H NMR ($CDCl_3$, 400 MHz): [4] δ = 6.74 (t, *J* = 1.9 Hz, 1H, C*H*=), 4.28 (q, *J* = 7.1 Hz, COOC*H*$_2$CH$_3$), 2.58 (m, C*H*$_2$CO), 2.45 (m, 2H, C*H*$_2$C), 2.06 (m, $CH_2CH_2CH_2$), 1.33 (t, *J* = 7.1 Hz, $COOCH_2CH_3$); ^{13}C NMR ($CDCl_3$, 100.6 MHz):[4] δ = 200.2, 166.2, 149.3, 133.0, 61.8, 37.8, 25.0, 22.3, 14.2; GC/MS (EI) t_R = 15.51 min: *m*/*z* (%) = 168 (M^+, 26), 140 (26), 133 (11), 123 (16), 112 (100).

General procedures for *R. oryzae* mediated hydroxylations of substrates 3a,b-4a,b

Procedure A

Rhizopus oryzae CBS 112.07 was cultured in 500 mL Erlenmeyer flasks containing 100 mL of MEA (malt extract agar) medium (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, peptone 2 g L^{-1}, agar 20 g L^{-1}), and incubated at 25°C on a rotary shaker at a rotational speed of 160 rpm. Suspensions of spores (1.6×10^4 / mL) were used as inoculum. After 2 days of pre-growth, a solution of the substrate in DMSO (1 mL, 500 mM) was added. For each substrate, three biological replicates were run. The reaction was monitored by GC/MS: 1 mL samples, taken at specified intervals (usually every 24 h), were extracted with EtOAc (500 μL), the organic phase was dried over anhydrous Na_2SO_4 and analysed by means of GC/MS after acetylation with acetic anhydride and pyridine.

Procedure B

R. oryzae beads, prepared according to the procedure described in the Experimental Section (see Entrapment of *R. oryzae*), were pre-cultured in a flask (ca. 10 g of beads per 40 mL of MEA medium in a 100 mL flask) at 25°C on a rotary shaker at a rotational speed of 160 rpm. After 2 days of pre-growth, a solution of the substrate in DMSO (400 μL, 500 mM) was added. For each substrate, three biological replicates were run. The reaction was monitored by GC/MS: 1 mL samples, taken at specified intervals (usually every 24 h), were extracted with EtOAc (500 μL), the organic phase was dried over anhydrous Na_2SO_4 and analysed by means of GC/MS after acetylation with acetic anhydride and pyridine.

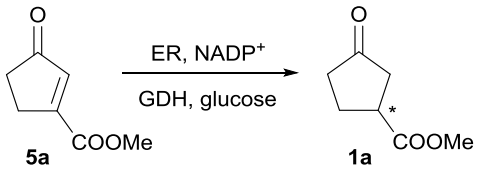
Procedure C

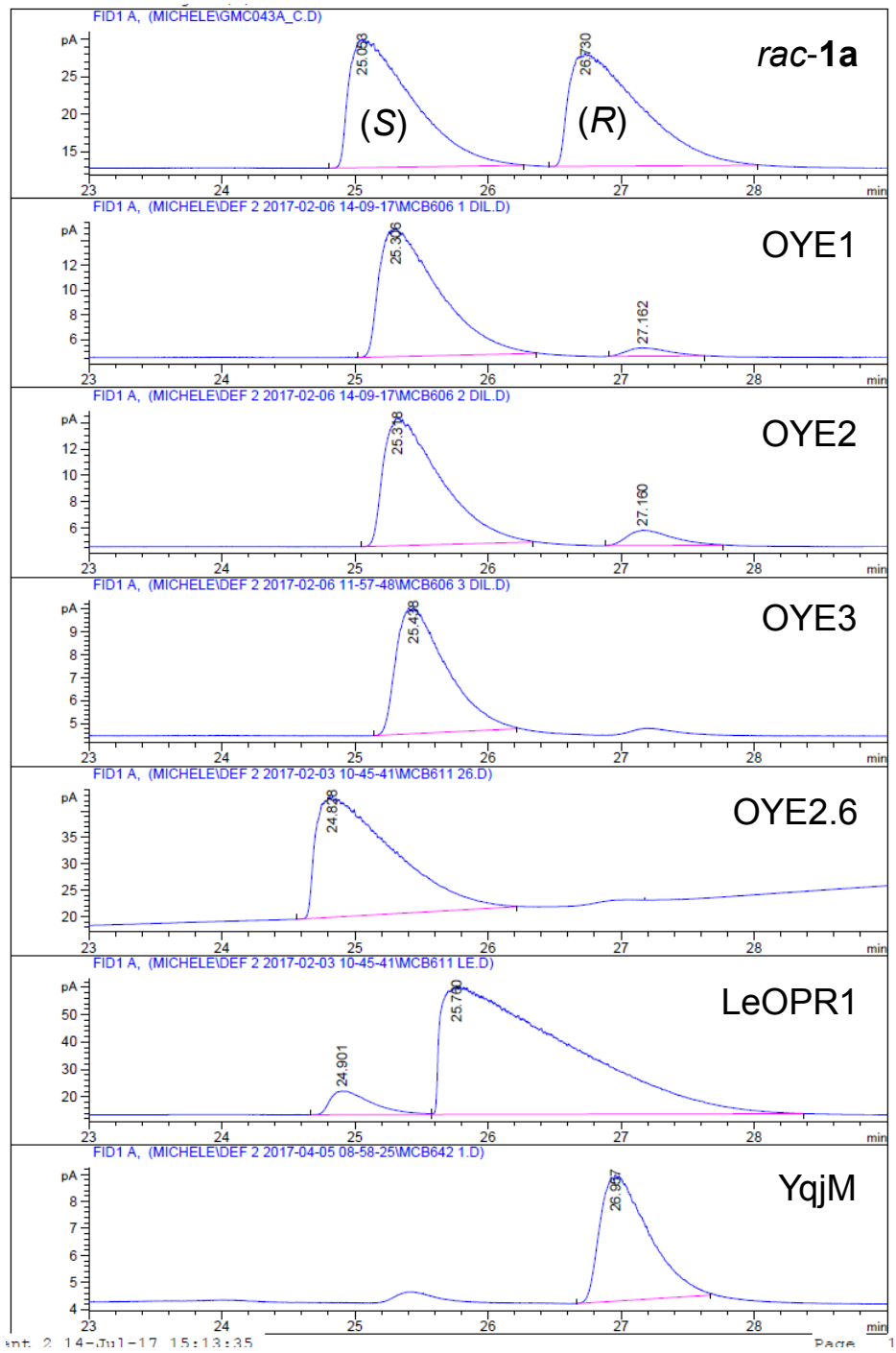
Pre-cultivated *R. oryzae* beads (corresponding to ca. 10 g of immobilised spore beads) were suspended in of acetate buffer (40 mL, 20 mM, pH 6.0) in a 100 mL flask, and a solution of the substrate (400 μL, 500 mM) in MeOH (for compound **3a**) or DMSO (for compounds **3b-4a,b**) was added. The flask was shaken at 160 rpm at 25°C. For each substrate, three biological replicates were run. The reaction was monitored by GC/MS: 1 mL samples, taken at specified intervals (usually every 24 h), were extracted with EtOAc (500 μL), the organic phase was dried over anhydrous Na_2SO_4 and analysed by means of GC/MS after acetylation with acetic anhydride and pyridine.

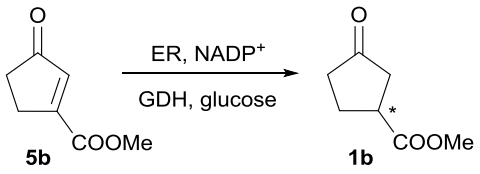
General procedure for ER mediated hydrogenations of substrates 5a,b-6a,b (screening)

A solution of the substrate in DMSO (10 μL, 500 mM) was added to a potassium phosphate buffer solution (1.0 mL, 50 mM, pH 7.0) containing glucose (20 μmol), $NADP^+$ (0.1 μmol), GDH (4 U) and the required purified or cell-free extract OYE (80-120 μg). The mixture was incubated for 24 h in an orbital shaker (160 rpm, 30°C). The solution was extracted with EtOAc (2 × 250 μL), centrifuging after each extraction (15000 *g*, 1.5 min), and the combined organic solutions were dried over anhydrous Na_2SO_4. Two replicates were performed for each biotransformation: no significant differences (less than 5%) were observed for conversion and enantiomeric excess values.

Representative GC chromatograms



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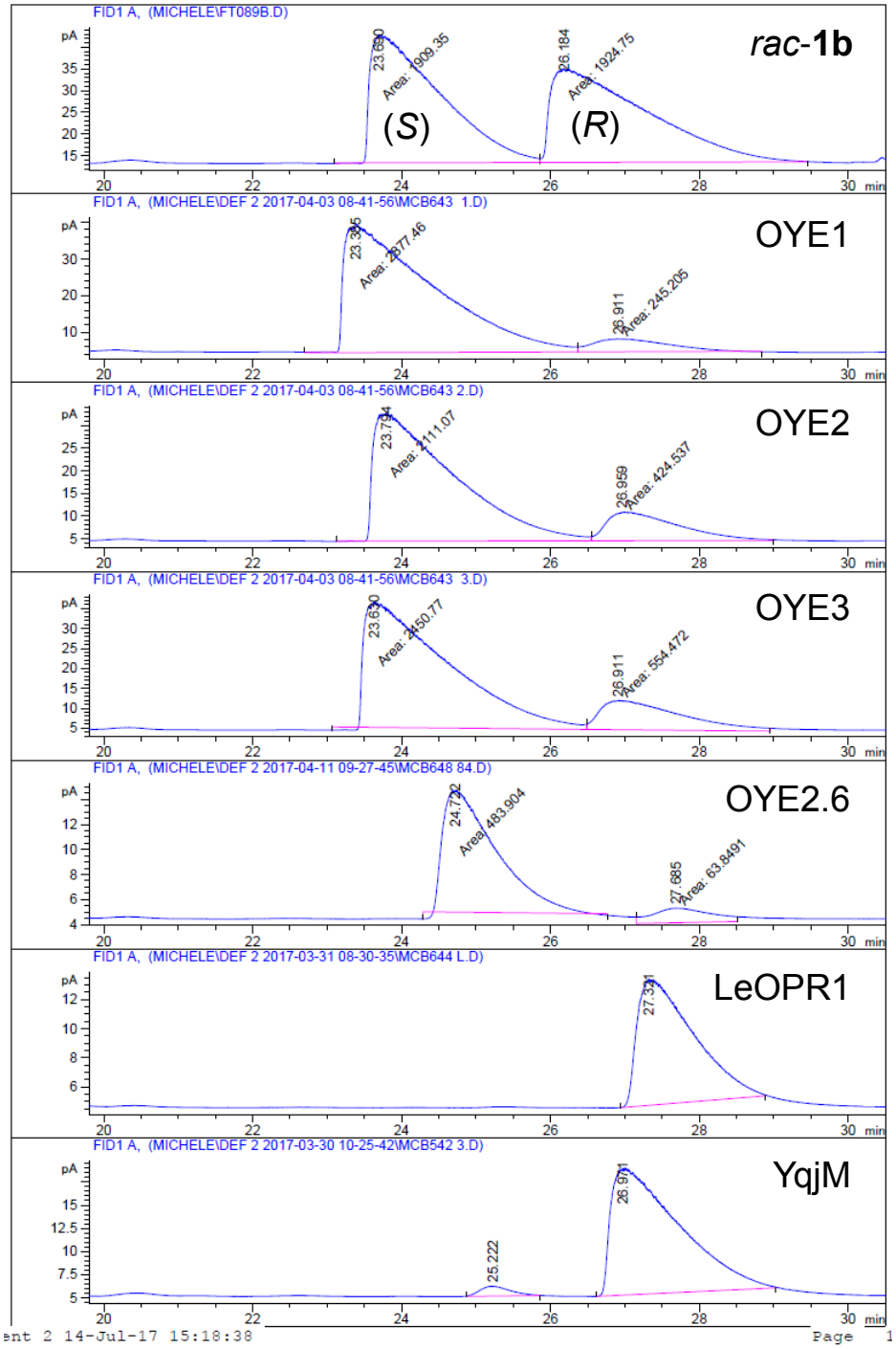
ER, NADP+
GDH, glucose
O
COOMe
5b
*
COOMe
1b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rac-1b
(S)
(R)
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 1.D)
OYE1
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 2.D)
OYE2
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 3.D)
OYE3
FID1 A, (MICHELE\DEF 2 2017-04-11 09-27-45\MCB648 84.D)
OYE2.6
FID1 A, (MICHELE\DEF 2 2017-03-31 08-30-35\MCB644 L.D)
LeOPR1
FID1 A, (MICHELE\DEF 2 2017-03-30 10-25-42\MCB542 3.D)
YqjM
ent 2 14-Jul-17 15:18:38
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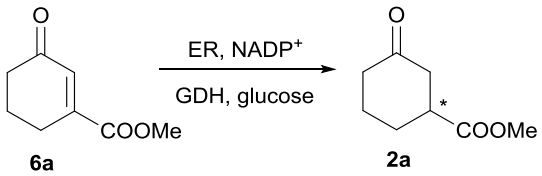
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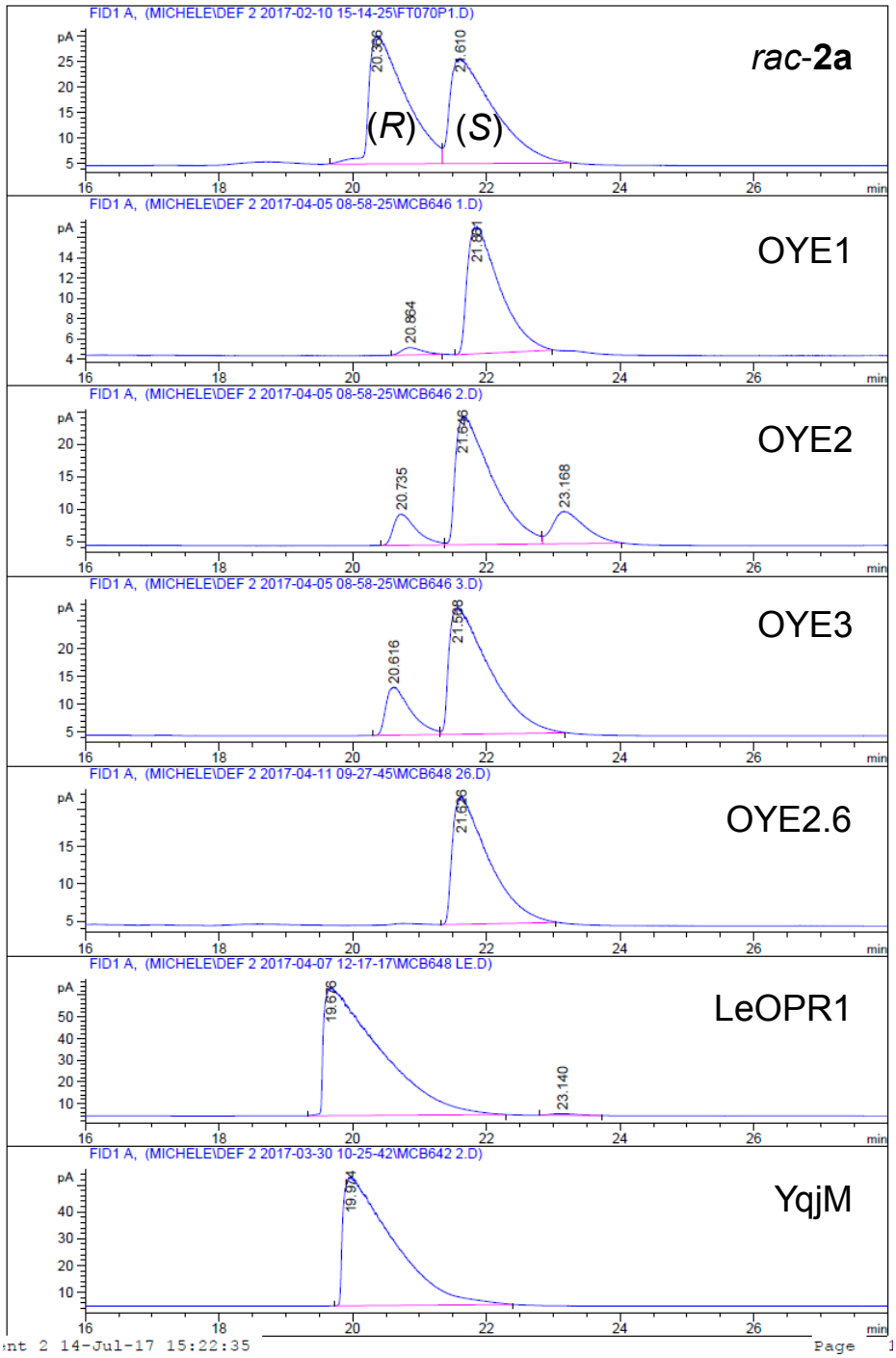
GDH, glucose
COOMe
6a
O
*
COOMe
2a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21.610
(R)
(S)
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21.646
23.168
FID1 A, (MICHELE\DEF 2 2017-04-05 08-58-25\MCB646 3.D)
OYE3
20.616
21.598
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OYE2.6
21.626
FID1 A, (MICHELE\DEF 2 2017-04-07 12-17-17\MCB648 LE.D)
LeOPR1
19.676
23.140
FID1 A, (MICHELE\DEF 2 2017-03-30 10-25-42\MCB642 2.D)
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pA
min
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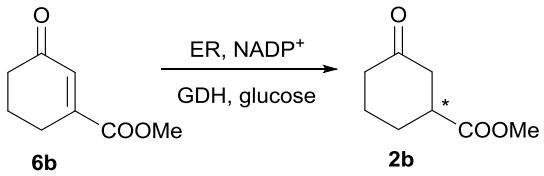
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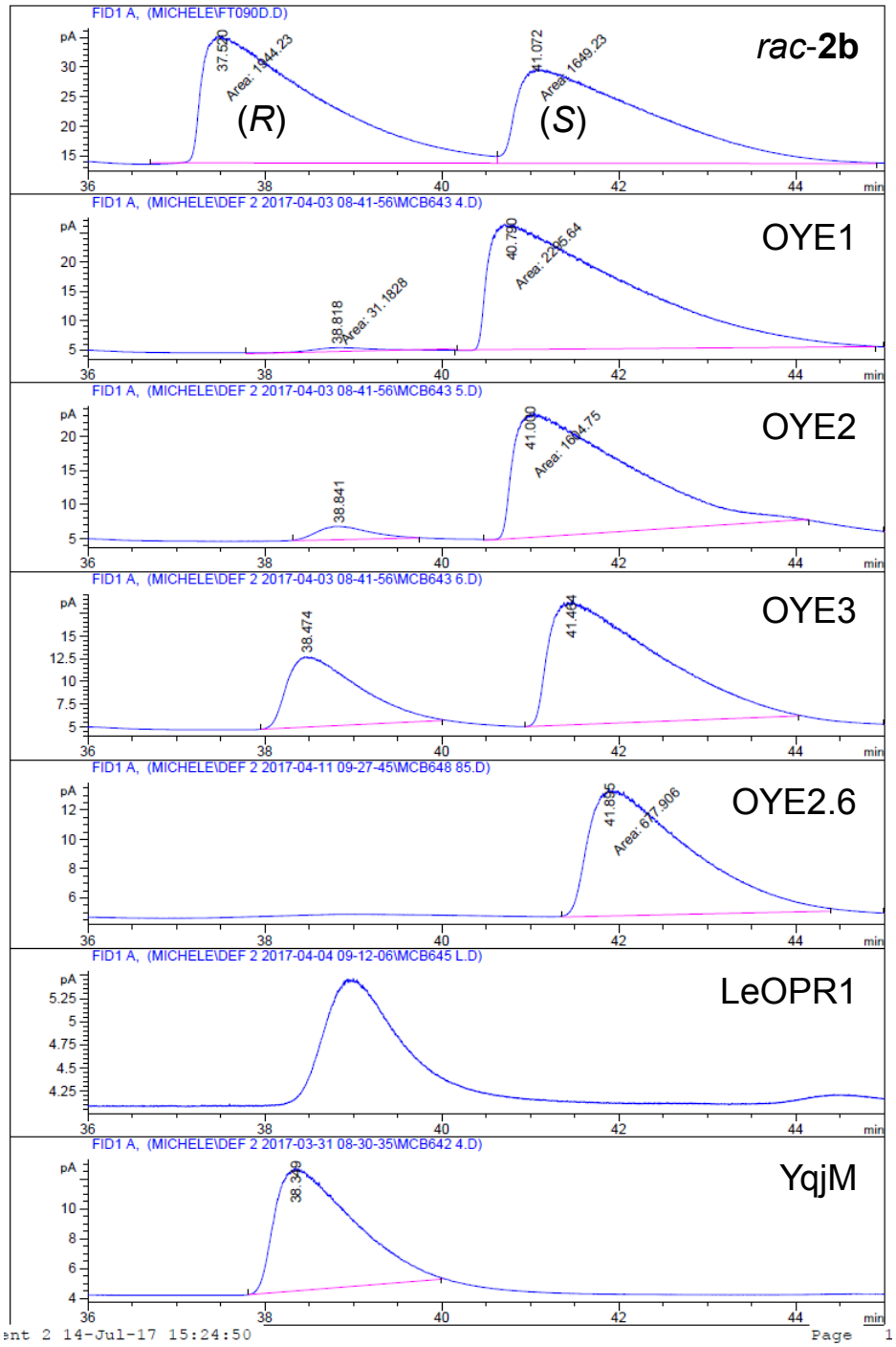
GDH, glucose
COOMe
6b
O
*
COOMe
2b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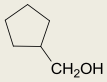
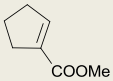
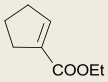
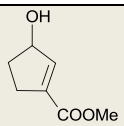
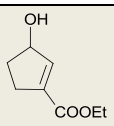
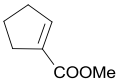
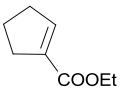
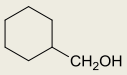
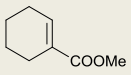
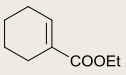
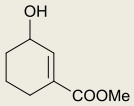
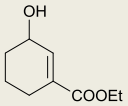
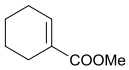
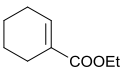
rac-2b
(R)
(S)
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 4.D)
OYE1
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 5.D)
OYE2
FID1 A, (MICHELE\DEF 2 2017-04-03 08-41-56\MCB643 6.D)
OYE3
FID1 A, (MICHELE\DEF 2 2017-04-11 09-27-45\MCB648 85.D)
OYE2.6
FID1 A, (MICHELE\DEF 2 2017-04-04 09-12-06\MCB645 L.D)
LeOPR1
FID1 A, (MICHELE\DEF 2 2017-03-31 08-30-35\MCB642 4.D)
YqjM
pA
min
ent 2 14-Jul-17 15:24:50
Page 1

Table S1. Results of *R. oryzae* mediated biotransformations of compounds **3a,b**-**4a,b**.

Substrate	Procedure (reaction time)	 **9**	 **3a**	 **3b**	 **7a**	 **7b**	Other products
 **3a**	A (5 days)	14	-	-	41 (60% ee)[c]	30 (46% ee)[c]	15[d]
	B (4 days)	3	-	-	16 (48% ee)	71 (46% ee)	10
	C (5 days)	-	-	-	95 (75% ee)	-	5
 **3b**	A (5 days)	10	-	-	-	35 (45% ee)	55[e]
	B (2 days)	-	-	2	-	77 (44% ee)	21[f]
	C (2 days)	-	-	8	-	78 (35% ee)	14[g]
Substrate	**Procedure (reaction time)**	 **10**	 **4a**	 **4b**	 **8a**	 **8b**	**Other products**
 **4a**	A (5 days)	5	-	-	95 (72% ee)[c]	-	-
	B (4 days)	4	1	1	82 (74% ee)	11 (83% ee)	1
	C (3 days)	-	3	-	97 (70% ee)	-	-
 **4b**	A (5 days)	-	-	55	-	45 (86 % ee)[c]	-
	B (4 days)	1	-	2	-	97 (60% ee)	-
	C (3 days)	-	-	4	-	96 (60% ee)	

[a] Procedure A: 5 mM substrate in reaction medium (DMSO as cosolvent), *R. oryzae* whole cells, 25°C; Procedure B: 5 mM substrate in reaction medium (DMSO as cosolvent), *R. oryzae* beads in culture medium, 25°C; Procedure C: 5 mM substrate in reaction medium (MeOH or DMSO as cosolvent), *R. oryzae* beads in acetate buffer pH 6.0, 25°C.

[b] Conversion calculated on the basis of GC analysis of the crude mixture, acetylated with acetic anhydride and pyridine.

[c] Enantiomeric excess calculated on the basis of GC analysis (see Experimental Section) of the corresponding acetyl derivatives on a chiral stationary phase.

[d] Unknown products.

[e] 10% of unknown compounds + 45% of ethyl 3-hydroxycyclopentane-1-carboxylate.

[f] 11% of unknown compounds + 10% of ethyl 3-hydroxycyclopentane-1-carboxylate.

[g] 10% of unknown compounds + 4% of ethyl 3-hydroxycyclopentane-1-carboxylate.

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