Substrate-engineering Approach to the Stereoselective Chemo-Multienzymatic Cascade Synthesis of *Nicotiana tabacum* Lactone

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Abstract

A one-pot multistep stereoselective synthesis of each stereoisomer of *Nicotiana tabacum* lactone is reported. In the first two steps the reduction of *α*,*β*-unsaturated ketoester gives the key intermediate ethyl 4-hydroxy-3-methylpentanoate. The reduction was catalyzed by a multienzymatic system comprising an ene-reductase (ER) and an alcohol dehydrogenase (ADH). This cascade process was highly chemoselective and stereoselective. In the last step, treatment of the hydroxyester with trifluoroacetic acid gives the *γ*-lactone in a very high overall yield (up to 78%) and with an excellent stereoselectivity (*de* >94%, *ee* >98%). The access to each stereoisomer was achieved by a substrate engineering approach and by selecting a

Prelog or an *anti*-Prelog ADH. Furthermore, computational studies of the binding modes of the substrates into the catalytic site of ene-reductases have been carried out, giving an insight of the observed enantiodivergence.

Keywords

Multienzymatic cascade reaction; enereductases; alcoholdehydrogenases; lactone

1.Introduction

Introduction text It is well established that the odor perception of chiral molecules is strictly related to their stereochemistry,[1] this feature has an enormous impact in the field of fragrances, where quite often an enantiomer exhibits a completely different scent profile and/or odor threshold with respect to its antipode, and the same is true for odorous diastereoisomers.[2] Among all naturally occurring fragrances, *γ*-lactones are doubtlessly one of the most important classes of compounds, since they exhibit unique odoriferous properties.[3] For example, the 4,5-dimethyl substituted *γ*-lactone **1** is present into the sun-cured leaves of *Nicotiana tabacum* and it confers a typical and pleasant fruity character to the latter.[4] However, even in this case, either the odor profile and threshold of each stereoisomer are linked to the absolute configuration[5] (Figure 1).

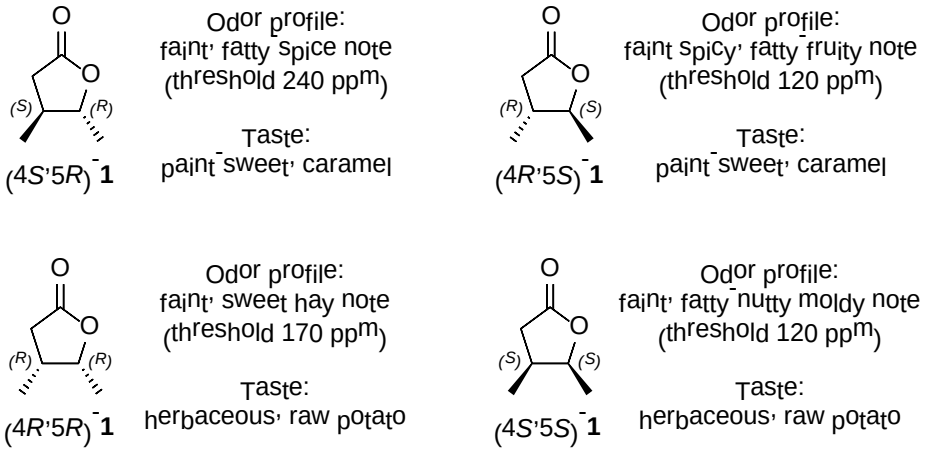
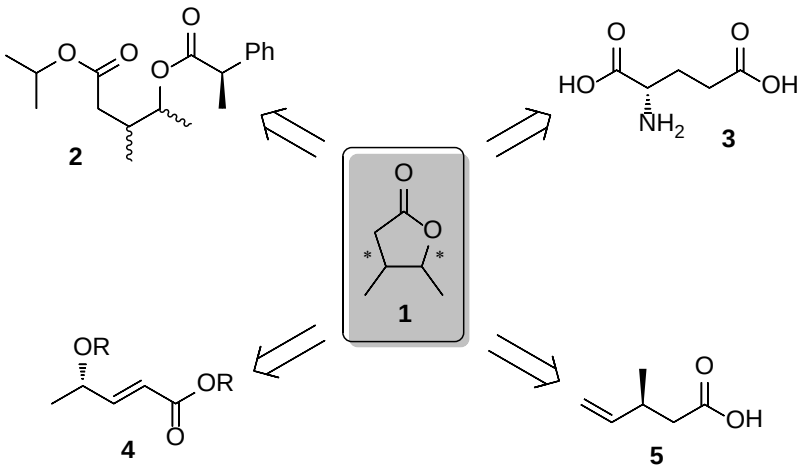


Figure 1: Structure and sensorial properties of 4,5-dimethyl-*γ*-butyrolactones **1**.

So far, several chemical methods for the stereoselective preparation of **1** have been reported in the literature (Scheme 1). A first approach was based on liquid chromatography (LC) separation of the four possible diastereoisomers of diester **2** (obtained from racemic **1** and (*R*)-2-phenylpropionyl chloride).[5] Another strategy was

based on the chiral pool, starting from (*S*)-glutamic acid **3**, and involving the Michael addition of lithium dimethylcuprate to an *α,β*-unsaturated lactone intermediate.[6] A similar route was based on the addition of Me_2CuLi to the chiral ester **4**.[7] Finally, an alternative synthesis relied on the iodolactonisation of **5**.[8]



Scheme 1: Chemical methods for the obtainment of optically enriched stereoisomers of **1**.

However, all these synthetic routes are characterized by a high number of steps (>5), low overall yields (<20%), modest up to good selectivity, complex purification processes, toxic or hazardous reagents and solvents, and impractical reaction conditions, which, altogether, make these syntheses neither “green” nor atom economic. In addition, none of them is feasible for the preparation of all the stereoisomer of **1**.

Anyway, a careful retrosynthetic analysis (Scheme 2) shows clearly that a shorter and more convenient route to **1** might be based on a stereospecific reduction of the C=C double bond of an *α,β*-unsaturated ketoester such as **6a** or **6b**, followed by a stereoselective reduction of the carbonyl group of the corresponding saturated ketoester **7** to give access to the hydroxyester **8** (in principle the reversed order of

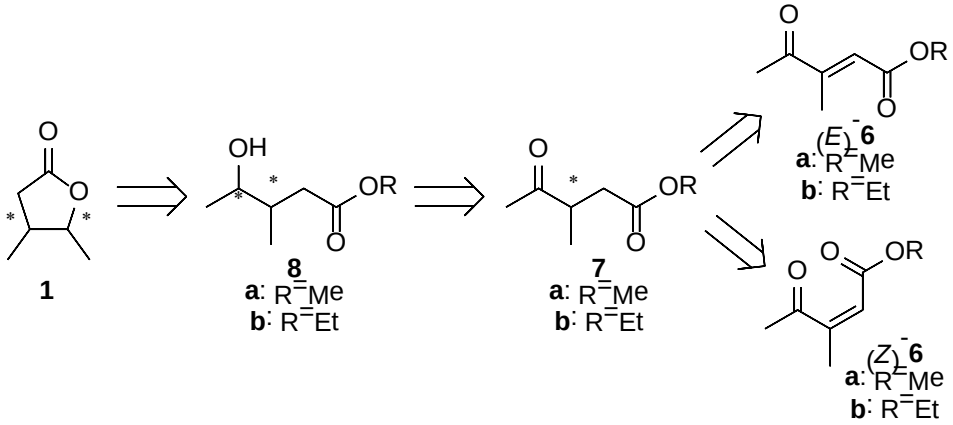
two steps could be equally feasible); finally, the cyclization of **8** would afford the lactone.

It is clear that the two reductive steps are crucial for the definition of absolute configuration of **1**, while the ring closure of the key intermediate **8** should not give particular problems. However, only whole-cell microorganisms[9] offer the unique feature to catalyze in a one-pot process the reduction of both the C=C double bond and the carbonyl group of unsaturated ketones, usually with a high stereoselectivity, but involving a troublesome workup. Specifically, the ene-reductase enzymes (ERs) catalyze the reduction of alkenes conjugated to electron-withdrawing groups (*e.g*. carbonyl groups of aldehydes and ketones or nitro groups),[10] whereas the alcohol dehydrogenase enzymes (ADHs) reduce the carbonyl group of aldehydes or ketones.[11]

Crout *et al.* have tested the bioreduction of **6b** with several yeasts from different natural sources: even in the presence of a significant biodiversity, most of screened microorganisms gave always the same diastereoisomer of alcohol **8b**, *i.e.* (3*S*,4*S*)-**8b**, with a low up to good diastereoselectivity but always in a poor yield;[12] mainly because the ADHs are not chemoselective, and therefore the alcohol **8b** was contaminated with its corresponding allylic alcohol. However, concerning this problem and other typical drawbacks of whole-cell biocatalysts, it has been shown that the combination of an overexpressed ER with an ADH gives substantial improvements in terms of productivity, yield and in certain cases of stereoselectivity.[13a-c] Moreover, the availability of several pro-*R* or pro-*S* ADHs gives the possibility to prepare more stereoisomers, usually with a very high diastereoselectivity.[13c,14]

Within our ongoing research program on the stereoselective synthesis of chiral fragrances by means of biocatalytic methods,[15] we have been interested in the

preparation of each stereoisomer of *N. tabacum* lactone **1** by coupling an ER with an ADH in a cascade system.



Scheme 2: Retrosynthesis of each stereoisomer of γ-lactone **1** based on stereospecific reduction of the C=C double bond, enantioselective reduction of the carbonyl group and ring closure.

Indeed, the choice of the starting material among the alkenes (*E*)-**6** and (*Z*)-**6** could give access to the (*R*) or (*S*) enantiomer of the saturated ketoester **7**, whereas, in the second step, the choice of a pro-*S* or pro-*R* ADH would determine the absolute configuration of the second stereogenic centre in **8**.

While our investigations were still in progress, the very same strategy was published by Pietruszka *et al.*, in a very systematic and successful study,[14b] with a one-pot three-step approach leading to **1**, thus proving the effectiveness of this strategy. As an update of their findings, in the following we report on: (i) the results we obtained by applying the same strategy with a different set of biocatalysts; (ii) the exploitation of an alternative regioisomeric substrate as a complementary approach; and (iii) the possibility of carrying out the reactions also in a cascade fashion.

2. Experimental

2.1 Sources of enzymes

The OYEs and the GDH employed were overexpressed in *E. coli* BL21(DE3) strains harbouring specific plasmids prepared according to standard molecular biology techniques: pET30a-OYE1 from the original plasmid provided by Neil C. Bruce,[25] pET30a-OYE2 and pET30a-OYE3 from *Saccharomyces cerevisiae* BY4741 and pKTS-GDH from *Bacillus megaterium* DSM509.[26]

The ADHs employed were obtained from commercial sources: CPADH (from *Candida parapsilosis*) and READH (from *Rhodococcus erythropolis*) were purchased from Jülich; BYADH (from baker's yeast), HLADH (from horse liver), PLADH (from *Parvibaculum lavamentivorans*), DRADH (from *Deinococcus radiodurans*), LKADH (from *Lactobacillus kefir*) and KRED (ketoreductase, recombinant in *E. coli*) were purchased from Sigma-Aldrich.

2.2 Analytical methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz) in $CDCl_3$ solution at r.t. using TMS as internal standard for ^{1}H, and $CDCl_3$ for ^{13}C. Chemical shifts are expressed in ppm relative to TMS and *J* values in Hz.

GC-MS analyses were performed on an Agilent HP 6890 gaschromatograph equipped with a 5973 mass detector and an Agilent HP-5 (30 m × 0.25 mm × 0.25 μm) column. Temperature program: 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 ketoesters **7a-b** and lactones **1** were determined by chiral GC analysis performed on a DANI HT 86.10 gaschromatograph equipped with a DAcTBSil.BetaCDX column (25 m × 0.25 mm × 0.25 μm). Temperature program: 60 °C (1 min) / 0.5 °C min^{-1} / 70 °C (1 min) / 30 °C min^{-1} / 180 °C (2 min).

Optical rotations were determined on a Dr. Kernchen Propol digital automatic polarimeter at 589 nm and are given in ° cm^3 g^{-1} dm^{-1}.

Thin layer chromatography (TLC) analyses were performed on Merck Kieselgel 60 F_{254} plates. All chromatographic separations were carried out on silica gel columns.

Protein concentrations were determined according to Bradford, using bovine serum albumine (BSA) as a standard.

*2.3 General procedure for the synthesis of α,β-unsaturated ketoesters (E)- and (Z)-**6a-b**:*

A solution of diacetyl (3.78 g) and the suitable triphenylphosphonium ylide (44.0 mmol) in CH_2Cl_2 (200 mL) was stirred at r.t. for 24 h, then concentrated under reduced pressure.[16] To the ice-cooled crude mixture was added *n*-hexane/Et_2O (150 mL, 2:1) to precipitate triphenylphosphine oxide, which was removed by filtration. The solution was then concentrated under reduced pressure affording a yellow oil (*E*/*Z* 92:8, yield 91%). The (*E*)- and (*Z*)-isomers were separated by column chromatography purification (eluent *n*-hexane/EtOAc, 7:3).

*2.3.1. Methyl (E)-3-methyl-4-oxopent-2-enoate (E)-**6a**.* 4.2 g; yield 69%; purity 98% by GC (t_R = 8.51 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.57 (q, *J* = 2.2, 1H), 3.79 (s, 3H), 2.22 (d, *J* = 2.2, 3H), 2.16 (s, 3H); ^{13}C NMR (400 MHz, $CDCl_3$): δ 199.3, 166.3, 150.5, 125.6, 51.4, 25.8, 12.8; MS: *m/z* (%) 142 $[M]^+$ (3), 127 (11), 110 (100), 99 (15).

2.3.2 Methyl (Z)-3-methyl-4-oxopent-2-enoate (Z)-6a. 0.4 g; yield 6%; purity 97% by GC (t_R = 8.10 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.69 (q, *J* = 1.4, 1H), 3.70 (s, 3H), 2.35 (s, 3H), 1.98 (d, *J* = 1.4, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.2, 165.2, 157.4, 116.1, 51.1, 27.9, 19.7; MS: *m/z* (%) 142 $[M]^+$ (2), 127 (100), 111 (18), 99 (9).

*2.3.3. Ethyl (E)-3-methyl-4-oxopent-2-enoate (E)-**6b**.* 4.8 g; yield 71% purity 97% by GC (t_R = 10.38 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.58 (q, *J* = 1.4, 1H), 4.25 (q, *J*

=7.15, 2H), 2.39 (s, 3H), 2.23 (d, *J* = 1.4, 3H), 1.40 (t, *J* = 7.15, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.4, 165.9, 150.1, 126.2, 60.4, 25.8, 13.9, 12.7; MS: *m/z* (%) 156 $[M]^+$ (2), 141 (3), 110 (100), 99 (3).

*2.3.4. Ethyl (Z)-3-methyl-4-oxopent-2-enoate (Z)-**6b**.* 0.4 g; yield 7%; purity 99% by GC (t_R = 9.82 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.68 (q, *J* = 1.70, 1H), 4.17 (q, *J* = 7.2, 2H), 2.39 (s, 3H), 1.98 (d, *J* = 1.70, 3H), 1.26 (t, *J* = 7.2, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 205.5, 164.8, 157.0, 116.7, 60.3, 28.2, 19.8, 13.8; MS: *m/z* (%) 141 $[M-15]^+$ (100), 113 (90), 99 (20), 85 (21).

*2.3.5. Methyl 3-methylene-4-oxopentanoate **10**.*

To a well stirred and an ice-cooled mixture of acetylacetone (20.0 g, 200 mmol) and K_2CO_3 (27.6 g, 200 mmol) was added ethyl 2-bromoacetate (33.4 g, 200 mmol). After 12 h the reaction mixture was filtered, and extracted with Et_2O (3 × 30 mL). The combined organic phase was washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give a yellow oil (ethyl 3-acetyl-4-oxopentanoate, **11**), used without further purification. To a stirred mixture of the latter (30.0 g, 161 mmol) and aq. formaldehyde (32 mL, 35% w/w) was added a suspension of K_2CO_3 (44.4 g, 322 mmol) in H_2O (32 mL). After 24 h the reaction mixture was poured into water (200 mL) and extracted with Et_2O (5 × 50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by bulb to bulb distillation under reduced pressure (120°C, 1 mmHg).

21.6 g; yield 86%; purity 99% by GC (t_R = 10.18 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.15 (s, 1H), 5.94 (s, 1H), 4.14 (q, *J* = 7.15, 2H), 3.29 (s, 2H), 2.37 (s, 3H), 1.25 (t, *J* = 7.15, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 197.8, 170.4, 142.3, 127.5, 60.2, 36.4, 24.8, 13.7; MS: *m/z* (%) 156 $[M]^+$ (23), 141 (46), 128 (10), 111 (100).

*2.4 General procedure for the synthesis of saturated ketoesters **7a-b**:*

To a solution of the suitable α,β-unsaturated ketoester (*E*)-**6a-b** (0.6 mmol) in EtOAc (20 mL) was added Pd/C (20 mg) and the mixture was stirred under a H_2 atmosphere until completion of the reaction. Afterwards, the mixture was filtered on a celite pad and concentrated under reduced pressure to give the saturated ketoesters as a clear colorless oil.

*2.4.1. Methyl 3-methyl-4-oxopentanoate **7a**.* 90 mg; yield 96%; purity 99% by GC (t_R = 7.44 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.67 (s, 3H), 3.05 (m, 1H), 2.78 (dd, *J* =8.2, 16.7, 1H), 2.32 (dd, *J* =5.5, 16.7, 1H), 2.23 (s, 3H), 1.17 (d, *J* = 7.30, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 210.3, 172.5, 51.5, 42.7, 36.5, 28.1, 16.3; MS: *m/z* (%) 144 $[M]^+$ (11), 129 (33), 113 (100), 102 (63).

*2.4.2. Ethyl 3-methyl-4-oxopentanoate **7b**.* 90 mg; yield 98%; purity 99% by GC (t_R = 9.11 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.12 (q, *J* =7.2, 2H), 3.09 (m, 1H), 2.74 (dd, *J* =8.2, 16.9, 1H), 2.3 (dd, *J* =5.50, 16.9, 1H), 2.21 (s, 3H), 1.24 (t, *J* =7.2 , 3H), 1.15 (d, *J* = 7.17, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 210.6, 172.2, 60.5, 42.7, 36.9, 28.3, 16.4, 14.1; MS: *m/z* (%) 158 $[M]^+$ (7), 143 (15), 113 (100), 101 (35).

*2.5 General procedure for the synthesis of allylic alcohols (E)-**9a-b**:*

To an ice-cooled solution of the suitable *α,β*-unsaturated ketoester (*E*)-**6a-b** (0.6 mmol) in EtOH (30 mL) was slowly added $NaBH_4$ (0.6 mmol). The ice bath was then removed and the solution was stirred at r.t. for 1 h. Most of the EtOH was removed under reduced pressure, then the reaction mixture was quenched with ice water, acidified with dilute HCl and extracted with EtOAc (3 × 30 mL). The organic layer was washed with water and brine, dried over Na_2SO_4 and then concentrated under reduced pressure to give a yellow oil.

*2.5.1. Methyl (E)-4-hydroxy-3-methylpent-2-enoate (E)-**9a**.* 80 mg; yield 94%; purity 99% by GC (t_R = 10.45 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.14 (q, *J* = 1.4, 1H), 4.30 (q, *J* = 6.5, 1H), 3.88 (s, 3H), 2.30 (d, *J* =1.4, 3H), 1.49 (d, *J* = 6.5, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.3, 161.4, 113.6, 72.3, 50.9, 21.7, 14.8; MS: *m/z* (%) 126 $[M-18]^+$ (25), 111 (195), 101 (100), 95 (20).

*2.5.2. Ethyl (E)-4-hydroxy-3-methylpent-2-enoate (E)-**9b**.* 90 mg; yield 97%; purity 99% by GC (t_R = 12.05 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.96 (q, *J* = 1.3, 1H), 4.26 (q, *J* = 6.1, 1H), 4.17 (q, *J* = 7.2, 2H), 2.13 (d, *J* = 1.3, 3H), 1.32 (d, *J* = 6.14, 3H), 1.27 (t, *J* = 7.2, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 161.1, 114.0, 72.2, 59.7, 21.6, 14.8, 14.2; MS: *m/z* (%) 140 $[M-18]^+$ (22), 129 (5), 115 (100), 111 (36).

2.6 Synthesis of standard for chiral GC analysis Ethyl

*2.6.1. 4-hydroxy-3-methylpentanoate **8b**.*

To a solution of the suitable allylic alcohol (*E*)-**9b** (0.6 mmol) in EtOAc (20 mL) and NEt_3 (50 μL) a catalytic amount of Pd/C was added, cooling in an ice-water bath. The mixture was stirred under an H_2 atmosphere until completion of the reaction, then the latter was filtered on a celite pad. Concentration under reduced pressure gave the saturated alcohol as a yellowish oil.[27]

90 mg; yield 97%; purity 94% by GC (t_R *anti* = 10.12 min and t_R *syn* = 10.32 min); ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.13 (q, *J* = 7.14, 2H), 3.8 (m, 1H), 3.6 (m, 1H), 2.53 (m, 1H), 2.48 (m, 1H), 2.23 (m, 1H), 2.19 (m, 1H), 2.08 (m, 1H), 1.98 (m, 1H), 1.26 (t, *J* = 7.1, 3H), 1.20 (d, *J* =6.4, 3H), 1.16 (d, *J* =6.4, 3H), 0.98 (m, 3H), 0.95 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 79.5, 60.4, 37.6, 36.8, 33.4, 15.3, 13.8; MS: *m/z* (%) 145 $[M-15]^+$ (15), 116 (100), 101 (52), 97 (23).

2.6.2. 4,5-Dimethyl-γ-butyrolactone ***1.***

To a solution of saturated alcohol **8b** (50 mg, 0.34 mmol) in Et_2O (2.5 mL), was added TFA (25 µL of a 1% v/v sol. in Et_2O). After 12 h, the solution was washed with brine (1 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give a yellow oil in quantitative yield.

Data of *trans*-**1**: GC t_R = 6.80 min; chiral GC t_R (4*S*,5*R*) = 10.25 min, t_R (4*R*,5*S*) = 11.33 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.13 (m, 1H), 2.66 (m, 1H), 2.22 (m, 1H), 2.17 (m, 1H), 1.40 (d, *J* =6.1, 3H), 1.14(d, *J* =6.1, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.3, 83.4, 38.2, 37.2, 19.1, 16.7; MS: *m/z* (%) 114 $[M]^+$ (22), 99 (43), 70 (100), 55 (96).

Data of *cis*-**1**: GC t_R = 7.64 min; chiral GC: t_R (4*R*,5*R*) = 14.66 min, t_R (4*S*,5*S*) = 15.27 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.65 (m, 1H), 2.66 (dd, *J* =8.9, 16.70, 1H), 2.60 (m, 1H), 2.19 (dd, *J* =5.1, 16.7, 1H), 1.29 (d, *J* =6.5, 3H), 1.0 (d, *J* =6.8, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.7, 79.6, 36.8, 33.4, 15.3, 13.8; MS: *m/z* (%) 114 $[M]^+$ (22), 99 (43), 70 (100), 55 (96).

2.7 General procedures for the biotransformations

2.7.1. ER mediated reductions (screening scale). The substrate (*E*)-**6a-b**, (*Z*)-**6a-b** or **10** (5 µmol) dissolved in DMSO (10 µL), was added to a solution of glucose (20 mM, 4 eq. with respect to the substrate), $NADP^+$ (0.1 mM), GDH (4 U mL^{-1}) and OYE1-3 (0.4-40 µg mL^{-1}) in KP_i buffer (1.0 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30°C), then the aqueous phase was extracted with EtOAc (2 × 300 µL), centrifuging after each extraction (15000 *g*, 1.5 min). The combined organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure.

2.7.2. ER mediated reductions (preparative scale). The substrate (*E*)-**6a**, (*E*)-**6b**, or **10** (50 mg) dissolved in DMSO (300 µL), was added to a solution of glucose (4 eq.

with respect to the substrate, ab. 250 mg), $NADP^+$ (0.05 mM), GDH (4 U mL^{-1}) and OYE2 (40 μg mL^{-1}) in KP_i buffer (15 mL, 50 mM, pH 7.0). The mixture was stirred for 12 h in an orbital shaker (160 rpm, 30°C), then the aqueous phase was extracted with EtOAc (3 × 5 mL), centrifuging after each extraction (3000 *g*, 5 min). The combined organic solutions were dried over Na_2SO_4 and concentrated under reduced pressure, yielding the corresponding saturated ketoester **7a-b**, in almost quantitative yield.

From substrate (*E*)-**6a**, the saturated ketone (*S*)-**7a** was obtained: >99% conv., 96% *ee*, $[\alpha]_D$ = –70.1 (*c* 1.5, $CHCl_3$) *vs.* lit. 95% *ee*, $[\alpha]_D$ = –54 (*c* 0.4, THF).[28]

From substrate (*E*)-**6b**, the saturated ketone (*S*)-**7b** was obtained: >99% conv., 97% *ee*, $[\alpha]_D$ = –52 (*c* 1.5, $CHCl_3$) *vs.* lit. 98% *ee*, $[\alpha]_D$ = –47 (*c* 1.1, MeOH).[12]

From substrate **10**, the saturated ketone (*R*)-**7b** was obtained: >99% conv., >99% *ee*, $[\alpha]_D$ = +48.5 (*c* 1.5, $CHCl_3$).

2.7.3. ADH mediated reductions (screening scale). The substrate (*S*)-**7a-b** or (*R*)-**7a-b**, (5 μmol) dissolved in DMSO (10 μL), was added to a solution of glucose (20 mM, 4 eq. with respect to the substrate), NAD^+ (0.1 mM), $NADP^+$ (0.1 mM), GDH (4 U mL^{-1}) and ADH (100 μg mL^{-1}) in KP_i buffer (1.0 mL, 50 mM, pH 7.0). The mixture was treated as described for the OYE mediated reduction (screening scale). For the chemoselectivity experiments, instead of a single substrate, a mixture of the unsaturated ketoester (2.5 μmol) and the corresponding saturated ketoester (2.5 μmol) dissolved in DMSO (10 μL) was employed.

2.7.4. Chemoenzymatic cascade synthesis of the four stereoisomers of 1 (preparative scale). The substrate (*E*)-**6b** or **10** (275 mg, 1.76 mmol) dissolved in DMSO (1.0 mL), was added to a solution of glucose (1.27 g, 7.04 mmol, 4 eq. with respect to the substrate), NAD^+ (0.05 mM), $NADP^+$ (0.05 mM), GDH (4 U mL^{-1}), OYE2 (40 μg mL^{-1}) and the suitable ADH (100 μg mL^{-1}) in KP_i buffer (75 mL, 50 mM,

pH 7.0). The mixture was stirred for 36 h in an orbital shaker (160 rpm, 30°C), then the aqueous phase was extracted with Et_2O (3 × 10 mL), centrifuging after each extraction (3000 *g*, 5 min). The combined organic solutions were dried over Na_2SO_4 and filtered. Trifluoroacetic acid (50 µL of a 1% v/v sol. in Et_2O) was added and the solution was stirred at room temperature for 12 h, then carefully concentrated under reduced pressure, yielding the corresponding stereoisomer of lactone **1**.

From substrate (*E*)-**6b** with OYE2 and READH, the lactone (4*S*,5*S*)-**1** was obtained: 78% yield (156 mg), 98% *de*, $[\alpha]_D = -52.4$ (*c* 1.3, $CHCl_3$) *vs.* lit. *ee* >99%, $[\alpha]_D = -53.4$ (*c* 1.1, $CHCl_3$).[29]

From substrate (*E*)-**6b** with OYE2 and KRED, the lactone (4*S*,5*R*)-**1** was obtained: 82% yield (164 mg), 96% *de*, $[\alpha]_D = +58.4$ (*c* 1.3, $CHCl_3$) *vs.* lit. *ee* >99%, $[\alpha]_D = +61.4$ (*c* 1.0, $CHCl_3$).[14b]

From substrate **10** with OYE2 and READH, the lactone (4*R*,5*S*)-**1** was obtained: 80% yield (160 mg), 98% *de*, $[\alpha]_D = -59.1$ (*c* 1.5, $CHCl_3$) *vs.* $[\alpha]_D = -60.0$ (*c* 0.08, $CHCl_3$). [30]

From substrate **10** with OYE2 and KRED, the lactone (4*R*,5*R*)-**1** was obtained: 83% yield (166 mg), 94% *de*, $[\alpha]_D = +53.6$ (*c* 1.1, $CHCl_3$), *vs.* lit. *ee* >99%, $[\alpha]_D = +54.2$ (*c* 1.0, $CHCl_3$).[14b]

2.8. Computational studies

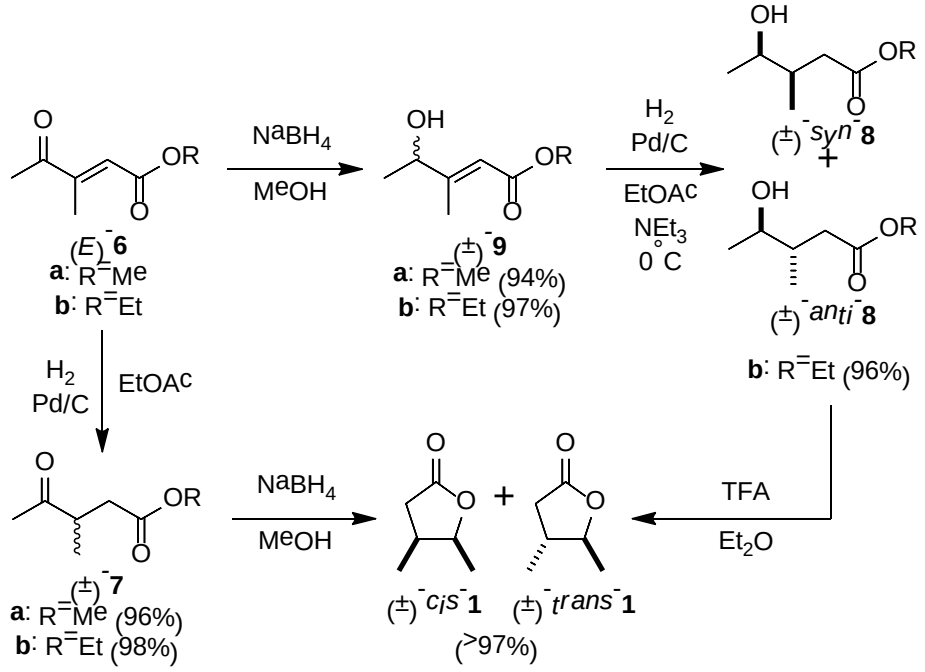
The crystal structure of OYE1 was obtained from the PDB database (1OYB.pdb). This OYE1 complex contained both the oxidized flavin cofactor (FMN) and the ligand HBA (*p*-hydroxybenzaldehyde). The cofactor FMN was further converted to its reduced form $FMNH_2$ and the resulting complex was then optimized with YASARA,[31] using the AMBER03 force field[32] for the minimization. The ligands were optimized after a MC/MM conformational search with Spartan ‘08 with the MMFF94 force field. [33] Docking was performed using AutoDock 4.2 using the default docking parameters supplied with AutoDock,[34] and point charges assigned according to the AMBER03

force field. The setup was done with the YASARA molecular modelling program.[31] The simulation cell was built 8.0 Å around the original HBA ligand. The poses were then clustered according to a rmsd < 5.0Å. The poses were then viewed using PyMOL.[35]

3. Results and Discussion

Both (*E*) and (*Z*) diastereoisomers of ketoesters **6a-b** were obtained by Wittig olefination of diacetyl with the suitable triphenylphosponium ylide,[16] in a yield of 82% (*E*/*Z*, 92:8, by ^{1}H NMR). They were easily separated by column chromatography.

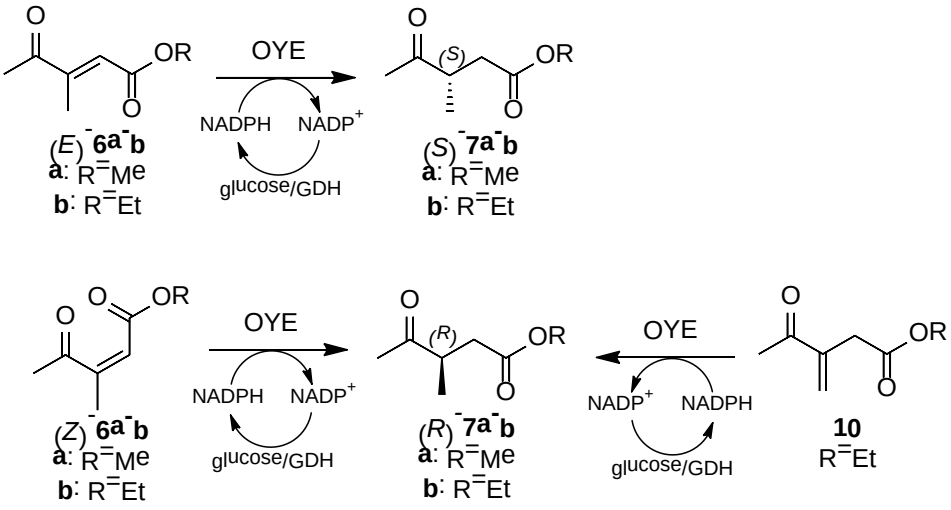
The racemic mixtures of the intermediates and the final product **1** were synthesized as shown in Scheme 3, as a reference for analytical methods for the determination of *ee*s and *de*s (more details are given in the Supporting Information). The methyl hydroxyester **8a** was not isolated since, during the hydrogenation a large amount of the latter cyclized to give **1**, even if the reaction was carried out in presence of a catalytic amount of NEt_3 and at 0°C.[17]



Scheme 3. Synthesis of racemic compounds **7-9** and **1** for analytical purposes.

According to our original strategy (Scheme 2), the same followed by Pietruszka *et al.,*[14b] the most obvious choice for a biocatalytic system would be the combination of an ER for the saturation of the C=C bond, and an ADH for the reduction of the C=O group. If the two steps are carried out telescopically, *i.e.* adding the ADH only when the C=C reduction is complete, there is no need to find an ADH that is chemoselective for the saturated ketone against the unsaturated one.

For the ER mediated step, we employed the Old Yellow Enzymes from *Saccharomyces carlsbergensis* (OYE1) and *S. cerevisiae* (OYE2 and OYE3). The regeneration of the NADPH cofactor was carried out using a glucose dehydrogenase (GDH, from *Bacillus megaterium*), with glucose as the sacrificial cosubstrate. The reduction of the α,β-unsaturated ketoester (*E*)-**6a** by these three ERs (Scheme 4), using our typical enzymatic concentration of 40 μg mL^{-1}, always afforded ketoester (*S*)-**7a** in a quantitative yield. OYE1 and OYE3 afforded an excellent *ee* of 99%, while OYE2 only a slightly worse *ee* of 96% (Table 1).



Scheme 4: OYE mediated reduction of *α,β*-unsaturated ketoesters **6a-b** and **10**.

An optimization of enzyme concentration showed that even with one tenth of the typical amount of OYE (4 µg mL^{-1}), it is still possible to achieve quantitative conversions over 24 hours reaction time, surprisingly conversions of 20-60% were still observed, even pushing the dilution to an exceptionally unusual concentration of 0.4 µg mL^{-1} (see Table S1 in the Supporting Information).

Thus, the reductions of other substrates (*Z*)-**6a**, (*E*)-**6b** and (*Z*)-**6b** were carried out using a concentration of 4 µg mL^{-1}, always achieving complete consumption of the starting material. Conversions and *ee*s are reported in Table 1.

It is clearly apparent that substrates such as **6a-b**, due to the modest steric hindrance of its substituents and the highly activated nature of their C=C bond, are very good substrates for OYEs and are converted very efficiently.

Table 1: OYE mediated reduction of *α*,*β*-unsaturated ketoesters **6a-b** and **10**.

Substrate	Product	OYE1		OYE2		OYE3	
		Conv. (%)[a]	*ee* (%)[b]	Conv. (%)[a]	*ee* (%)[b]	Conv. (%)[a]	*ee* (%)[b]
(*E*)-**6a**	(*S*)-**7a**	>99	99	>99	96	>99	99
(*Z*)-**6a**	(*R*)-**7a**	>99	80	>99	80	>99	70
(*E*)-**6b**	(*S*)-**7b**	>99	96	>99	98	>99	98
(*Z*)-**6b**	(*R*)-**7b**	>99	64	>99	70	>99	52
10	(*R*)-**7b**	>99	99	>99	99	>99	99

Expt. cond.: 2.0 g L^{-1} substrate, 4 µg mL^{-1} OYE, 4 U mL^{-1} GDH, 4 eq. glucose, 0.1 mM $NADP^+$, 50 mM KP_i buffer pH 7.0, 30°C, 12 h; a) by GC-MS; b) by GC.

For the bioreduction of substrates (*E*)- and (*Z*)-**6b**, it is interesting to compare the performance of OYE1-3 with that of the OYE homolog YqjM.[14b] Indeed, while for (*E*)-**6b** the results are quite similar (high yields and excellent *ee*s), in contrast, with (*Z*)-**6b**, OYE1-3 afforded the (*R*)-ketoester still in a quantitative yield but with an unsatisfactory optical purity (*ee*s <70%), while YqjM was much superior in terms of stereoselectivity (*ee* >98%) at the expense of a substantially lower conversion.

Therefore, we attempted a different approach to (*R*)-**7b**. In previous studies on substrate specificity of ERs, it was found that the enantiocomplementarity can be achieved not only by employing different diastereoisomers,[18] but also designing a suitable constitutional isomer with the C=C bond in a different position, such that the saturated product would be the same. As an example, *α*-methylcinnamaldehyde and 2-benzylacrylaldehyde afford the opposite enantiomers of 2-methyl-3-phenyl-1-propanol.[19]

In this case, the ketoester **10** was the only alternative option, which, comparing to (*Z*)-**6a-b**, was easily prepared on a multigram scale by a chromatography-free procedure.[20]

This approach resulted, once again, very effective: the reduction of **10** gave the antipode (*R*)-**7b** (Scheme 4) in a quantitative yield and with excellent *ee*s (Table 1).

Since it is well established that the mechanism of OYEs catalyzed reductions proceeds with a formal *anti* addition of H_2,[21] and that the ketone group of these substrates binds to the catalytic site through a bifurcated H-bond with the Asn194 and His191 residues,[14b] this result can be explained assuming that the substrates (*E*)-**6a-b** and **10** are oriented into the catalytic site as shown in Figure 2, in such a way that the reductions are enantiodivergent.

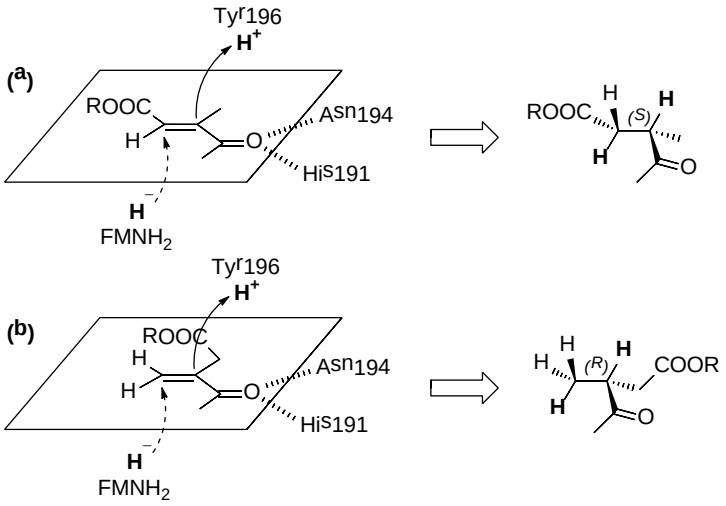


Figure 2: Binding modes of (*E*)-**6a-b** (a) and **10** (b) to the active site of OYE1-3.

In order to prove and clarify this substrate-induced switch in enantioselectivity, we also performed docking studies of the ketoester substrates to the active site of OYE1. The binding mode of (*E*)-**6b** (Figure 3a, substrate shown in orange) was found perfectly consistent with the model shown in Figure 2a. The binding mode of **10** (Figure 3b, substrate shown in purple), instead, is in accordance with the model of Figure 2b for the position of the C=C bond and for the stereochemistry of the addition, which leads to the (*R*)-enantiomer of the product, but shows that the preferential conformation is likely the *s*-cis (rather than the more common *s*-trans, as in Figure 2b).[17] Another interesting finding from these docking simulations is the presence of an additional hydrogen bond between Tyr375 and the oxygen atom of the ester functionality bearing the alkyl chain. This additional interaction (observed with both (*E*)-**6b** and **10**, Figure 3) could contribute to a tighter binding and might partially explain the very high conversion rates of this family of ketoesters.

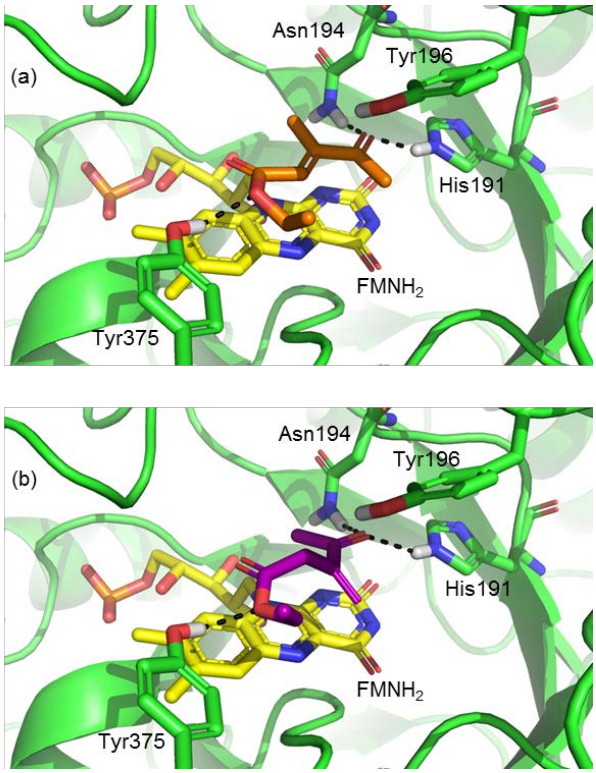
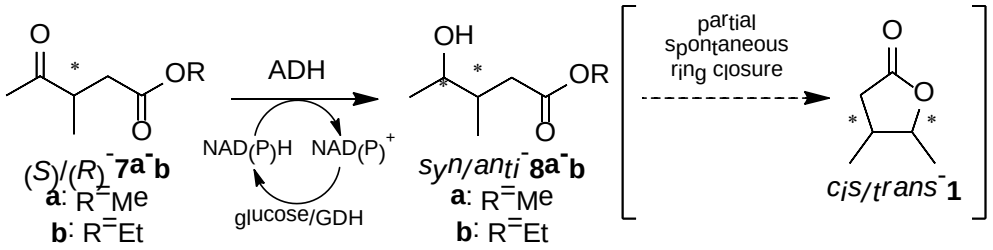


Figure 3: Docking of (*E*)-**6b** (a) and **10** (b) to the active site of OYE1.

The biotransformations of (*E*)-**6b** and **10** with OYE2 were scaled up to afford (*S*)-**7b** (*ee* 98%) and (*R*)-**7b** (*ee* 99%), respectively, on a multi-mg scale, achieving similar results to those of the screening. Both absolute stereochemistry and optical purity were confirmed by comparing the optical rotatory power values with those reported in the literature.

Concerning the ADH catalyzed reduction, as a panel of biocatalysts we selected eight commercially available dehydrogenases (recombinant and/or purified) from bacterial, fungal and animal origin (full list given in the Experimental Section). The reduction of the carbonyl group of each enantiomer of **7b**, with this panel of recombinant ADHs was tested using again the glucose/GDH system for cofactor regeneration (Scheme 5). The use of the same cofactor regeneration system is clearly advantageous for the combination of the two steps in one pot. The results are shown in Table 2 (very similar results were obtained with **7a**, shown in Table S2 in the Supporting Information). The interpretation of these results is made more difficult

by the observation that hydroxyesters **8a-b** partially cyclize under the biotransformation conditions (*anti*-**8** gives *trans*-**1**, *syn*-**8** gives *cis*-**1**). Therefore, we found more convenient to assess the selectivity of each ADH on the lactone, after complete conversion of hydroxyesters into the latter (by treatment with TFA/Et_2O). Indeed, under the biotransformation conditions, even after prolonged reaction times (up to 24 h), complete conversions of **8** into the corresponding lactone could not be observed.



Scheme 5. ADH mediated reduction of optically pure saturated ketoesters **7a-b**.

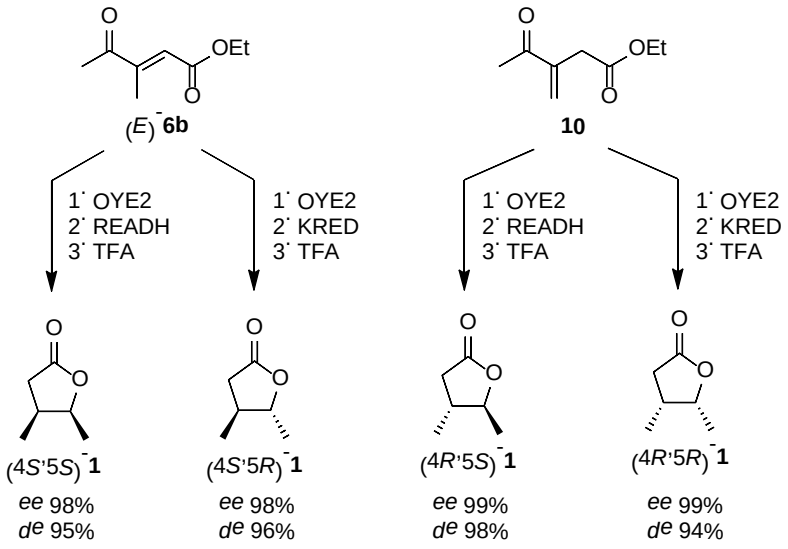
Table 2. ADH mediated reduction of optically pure saturated ketoesters (*S*)-**7b** and (*R*)-**7b**.

	Substrate	CPADH	READH	BYADH	HLADH	PLADH	DRADH	LKADH	KRED
Conv. (%)[a]	(*S*)-**7b**	16	96	<5	<5	>99	18	97	>99
	(*R*)-**7b**	<5	99	<5	<5	>99	21	>99	>99
de (%)[b]	(*S*)-**7b**	–96	–99	n.d.	n.d.	+92	–81	+94	+95
	(*R*)-**7b**	n.d.	+94	n.d.	n.d.	–51	+59	–98	–98
Stereochemistry of new center	(*S*)-**7b**	(*S*)	(*S*)	(*S*)	(*S*)	(*R*)	(*S*)	(*R*)	(*R*)
	(*R*)-**7b**	(*S*)	(*S*)	(*S*)	(*S*)	(*R*)	(*S*)	(*R*)	(*R*)

Expt. cond.: 2.0 g L^{-1} substrate, 100 µg mL^{-1} ADH, 4 U mL^{-1} GDH, 4 eq. glucose, 0.1 mM NAD^+, 0.1 mM $NADP^+$, 50 mM KP_i buffer pH 7.0, 30°C, 24 h; a) by GC; b) *de* of *trans*-**1**, by GC.

Among all screened enzymes, READH, LKADH and KRED gave the best results in terms of conversion (almost quantitative) and stereoselectivity (*de* >94%). In addition, we found that the READH exhibits a pro-*S* stereoselectivity (Prelog ADH),[22] while the other two enzymes were pro-*R* (anti-Prelog ADH).[23]

Finally, we performed the telescopic one-pot three steps sequence on both substrates (*E*)-**6b** and **10**, with OYE2 as the ER, and READH or KRED as ADHs, including the TFA-catalyzed ring closure (Scheme 6), yielding all four stereoisomers of *N. tabacum* lactone on a preparative scale, with an excellent *de* (>94%) and without any appreciable loss of optical purity (*ee* >98%). No tedious purification procedures were necessary, since the products were recovered with a very high purity.



Scheme 6: Sequential one-pot three-steps chemoenzymatic synthesis of all four stereoisomers of **1**.

Even though this, in principle, can be regarded as a sequential multienzymatic cascade system,[14b] the integration of both biocatalytic transformations in a proper

cascade system[24] (in which both the catalysts are introduced at the beginning) would be particularly appealing. However, such a goal would rely on a high chemoselectivity of the ADH towards the saturated ketoester, otherwise the reduction product (the allylic alcohol **9**) would accumulate in the reaction mixture because it is not an accepted substrate for ERs.[13]

To this aim, equimolar mixtures of the *α*,*β*-unsaturated ketoester and its corresponding saturated ketoester (*i.e*. (*E*)-**6b** + **7b** or **10** + **7b**), were submitted to the ADH mediated reduction using the previously optimized experimental conditions. A very complex distribution of products was obtained, not only due to the partial ring closure and to the formation of allylic alcohols, but mainly because some of the commercial ADH preparations also showed ER activity to some extent. Indeed, the ratio between the total amounts of saturated compounds and of unsaturated ones was different from the initial 1:1. This could be ascribed either to a promiscuous activity of some of the ADHs or, much more likely, to the presence of traces of ERs in the commercial enzymatic preparations. As demonstrated above, ERs are exceptionally active on substrates like **6a-b** and **10**, and even traces of these enzymes can afford high conversions. Anyway, even though this problem makes the assessment of the real chemoselectivity of the ADHs very difficult, none of the ADH preparations showed a satisfactory balance between chemoselectivity and conversion for all substrates.

Nevertheless, the examples in which the ADHs show an additional ER activity suggest that a combination of these two enzymes might lead to a high chemoselectivity even if the ADH is intrinsically not chemoselective. Indeed, if the ER is kinetically much faster than the ADH, the amount of *α*,*β*-unsaturated ketoester that could be reduced to give the side product allylic alcohol would become negligible. Indeed, the reduction of (*E*)-**6b** or **10** with OYE2 (40 μg mL^{-1}) in combination with

pro-*S* READH or pro-*R* KRED (100 μg mL^{-1}), followed by treatment with TFA, gave each stereoisomer of **1**, with *de*s very similar to those obtained in the sequential system (Table 3).

Table 3: Cascade approach to the chemoenzymatic synthesis of all four stereoisomers of **1**.

Substrate	Biocatalysts	Product	Yield (%)[a]	*ee* (%)[b]	*de* (%)[b]
(*E*)-**6b**	OYE2+READH	(4*S*,5*S*)-**1**	78	98	95
(*E*)-**6b**	OYE2+KRED	(4*S*,5*R*)-**1**	82	98	96
10	OYE2+READH	(4*R*,5*S*)-**1**	80	99	98
10	OYE2+KRED	(4*R*,5*R*)-**1**	83	99	94

a) isolated yield; b) by GC.

4. Conclusion

The improvement of efficiency and productivity of biocatalytic processes does not rely only on biocatalyst optimization, but also on process optimization. Multienzymatic cascade synthesis is certainly a suitable example of how the thoughtful selection of substrates, enzymes and conditions (*e.g.* temperature, concentrations, enzyme ratios) can drastically improve the yields and the practicality of a process.

As an addition to the recently published study on the biocatalytic synthesis of all four stereoisomers of **1**, we proved the feasibility of the same system with a different set of biocatalysts, and we introduced a new substrate-engineering strategy that enabled to obtain perfect enantioselectivity in spite of the fact that in this case the OYE

catalyzed reduction was not stereospecific, since the classical approach based on *E/Z* substrates failed. Thus, the “substrate engineering” strategy (as opposed to the more expensive and time-consuming protein engineering) can be a very valid alternative to compensate a low stereospecificity of wild-type biocatalysts.

Finally, we demonstrated that the chemoselectivity of ADHs is not always necessary in order to set-up a very efficient cascade system with an ER. Indeed, in this case the unusual relative high reaction rate of OYEs with respect to that of ADHs, allows their combination leading to the stereoselective cascade synthesis of **1** with an excellent chemoselectivity and diastereoselectivity.

Supporting Information

Experimental details and NMR spectra of the synthesized compounds.

Supporting Information File 1:

File Name: Text

File Format: Text

Title: Text

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