Bioorganic Chemistry 138 (2023) 106675

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Chemoenzymatic approach towards the synthesis of the antitumor and antileishmanial marine metabolite (+)-Harzialactone A *via* the stereoselective, biocatalyzed reduction of a prochiral ketone

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ARTICLE INFO

Keywords:
Harzialactone A
Marine metabolite
Biocatalysis
Stereoselective reduction
Oxidoreductases
Whole cell bioreactor
Prochiral ketones

ABSTRACT

As a rich source of biological active compounds, marine natural products have been increasingly screened as candidates for developing new drugs. Among the several marine products and metabolites, (+)-Harzialactone A has drawn considerable attention for its antitumor and antileishmanial activity. In this work a chemoenzymatic approach has been implemented for the preparation of the marine metabolite (+)-Harzialactone A. The synthesis involved a stereoselective, biocatalyzed reduction of the prochiral ketone 4-oxo-5-phenylpentanoic acid or the corresponding esters, all generated by chemical reactions. A collection of different promiscuous oxidoreductases (both wild-type and engineered) and diverse microorganism strains were investigated to mediate the bioconversions. After co-solvent and co-substrate investigation in order to enhance the bioreduction performance, *T. molischiana* in presence of NADES (choline hydrochloride-glucose) and ADH442 were identified as the most promising biocatalysts, allowing the obtainment of the (*S*)-enantiomer with excellent *ee* (97% to > 99% respectively) and good to excellent conversion (88% to 80% respectively). The successful attempt in this study provides a new chemoenzymatic approach for the synthesis of (+)-Harzialactone A.

1. Introduction

Medicinal chemists have frequently been inspired by nature during the scaffold hopping stage albeit the structural complexity of the natural products compared to the synthetic drug-like molecules is often a limit. Indeed, either natural products or their metabolites offer unexplored frameworks for the development of innovative drugs, new leads or even are already pharmacologically active substances.

Fungi play an irreplaceable role within this context since they have exceptional abilities to produce a variety of metabolites characterized by unique structures and diverse biological activities. In particular, fungi coming from the marine environment have attracted a lot of interest because are very distinctive since they grow under pressure, high salinity and low temperature [1,2]. Among these fungi, *Trichoderma* species have gained a lot of attention since they are able to produce plentiful of secondary metabolites which possess attractive chemical structures and remarkable biological activities. More precisely *Trichoderma harzianum* probably contributed the most secondary metabolites (SMs) originating from *Trichoderma* species [3,4].

Among the SMs generated by *Trichoderma harzianum*, the metabolite (+)-Harzialactone A (Fig. 1) has drawn our attention since it possesses a recognized antitumor activity and a promising growth inhibitor activity against *Leishmania amazoniensis* [5–8]. Moreover, Harzialactone A is also structurally characterized by a γ-valerolactone (GVL) core, a valuable chiral building block of which we have already expertise [9–11]. From a synthetic point of view, the stereoisomers of (+)-Harzialactone A have been prepared according to different strategies comprising both chiral pool-based approaches or chemically catalyzed stereoselective reactions [12–16]. In this context, only one example dealing with the lactonase-mediated kinetic resolution of racemic Harzialactone A can be

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Abbreviations: NADES, Natural Deep Eutectic Solvent; ChCl, choline hydrochloride; Glu, glucose; Gly, glycerol; *i*-PrOH, isopropanol; EtOAc, ethyl acetate. PB, phosphate buffer; PTSA, *p*-Toluenesulfonic acid; YPD, Yeast Extract Peptone Dextrose.

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https://doi.org/10.1016/j.bioorg.2023.106675
Received 31 March 2023; Received in revised form 29 May 2023; Accepted 9 June 2023

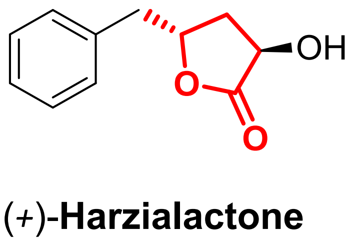


Fig. 1. The metabolite (+)-Harzialactone A; depicted in red the GVL moiety.

found as a biocatalyzed entry to this bioactive molecule [17]. To the best of our knowledge, in fact, no reports dealing with the preparation of 5-phenyl-γ-valerolactones, the core of the molecular skeleton of (+)-Harzialactone A, *via* the stereoselective biocatalyzed reduction of the corresponding γ-ketoesters or acids can be found in the literature.

For all these reasons, we became interested in the possibility of proposing a stereoselective, biocatalyzed synthesis for (+)-Harzialactone A *via* the enantioselective reduction of prochiral ketones.

The enzyme mediated preparation of chiral γ-lactones is a topic that has been already explored in the literature. Some reported examples are, again, based on the kinetic resolution of chemically prepared racemic lactone-precursors catalyzed by different lipases (Fig. 2a) [18,19].

More elegant preparations have been achieved *via* stereoselective biocatalyzed syntheses. Specifically, the enzymatic reduction and tandem cyclization of differently decorated γ-ketoesters, regarded as "bulky-bulky" substrates has been reported using both wild-type and engineered enzymes [20–25]. Among the γ-ketoesters investigated, the enantioselective reduction of methyl 4-oxo-4-phenylbutanoate (**8**, Fig. 2b), a compound structurally related to the focus of this work *i.e.* methyl 4-oxo-5-phenylpentanoate (**13**, Scheme 1), resulted in the preparation of enantiomerically enriched (*S*)-5-phenyl γ-valerolactone ((*S*)-**10**, Fig. 2b) with excellent isolated yield and enantiomeric excess (*ee*) using *Ralstonia* sp. or *Sphingobium yanoikuyae* alcohol dehydrogenase (Ras-ADH and Sy-ADH, respectively) as biocatalysts. More recently, a novel chemoenzymatic entry to 5-aryl or 5-alkylaryl valerolactone derivatives has been proposed by Özgen *et al.* who reported an integrated photo- and biocatalyzed synthesis of chiral γ-lactones using simple aldehydes, acrylates, or unsaturated acid as starting materials [26]. Furthermore, the microbial bioreduction of γ-ketoesters or acids was only accomplished for the preparation of 5-alkyl or 5-phenyl γ-lactones [27].

On the basis of these considerations, a novel chemoenzymatic synthesis of the two enantiomers of **16** (Scheme 1), direct precursors of enantiomerically enriched Harzialactone A isomers [15], has been here investigated and successfully validated. Seeking for good conversions and high *ee* in the bioreduction of the "bulky-bulky" substrates **12**–**15** to the corresponding γ-hydroxyester, different biocatalysts have been screened. Specifically: (1) a small family of ADHs of different nature and substrate scope [28–31]; (2) a collection of promiscuous hydroxysteroid dehydrogenases (HSDHs) [32–34], (3) a commercial kit of ADHs from Evoxx® and (4) different strains of *Torulopsis* sp. yeasts, selected among those species that have previously been exploited in the microbial reduction of structurally complex ketones [35].

2. Materials and Methods

2.1. General

All chemicals and solvents were purchased from Merck KGaA, Darmstadt, Germany, and TCI and used as commercially distributed. All purifications were performed by flash chromatography using prepacked Biotage Sfär columns or silica gel (particle size 40–63 µm, Merck) on an Isolera (Biotage, Uppsala, Sweden) apparatus. Thin-layer chromatography (TLC) analyses were performed on aluminum plates precoated with silica gel 60 matrix with a fluorescent indicator and visualized in a TLC UV cabinet followed by an appropriate staining reagent. The content of solvents in eluent mixtures is given as v/v percentage. R_f values are given for guidance. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian NMR System 300 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual solvent ($CHCl_3$, MeOH, or dimethyl sulfoxide (DMSO)) as an internal standard. Melting points were determined by a Buchi Melting Point B-540 apparatus.

The Alcohol Dehydrogenases Screening Kit (cat. n° evo-1.1.100) was purchased from Evoxx technologies GmbH (Monheim am Rhein, Germany) and applied in the screening according to its technical bulletin. Plasmid pMS470/pEamTA was kindly donated by Prof. W. Kroutil, University of Graz, Austria.

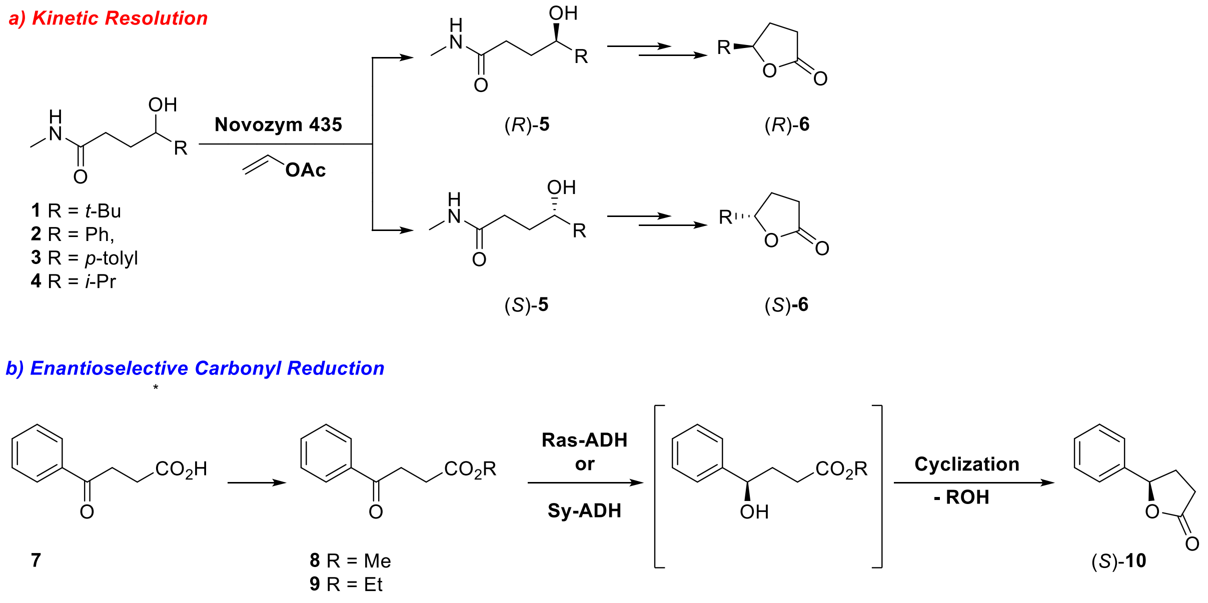
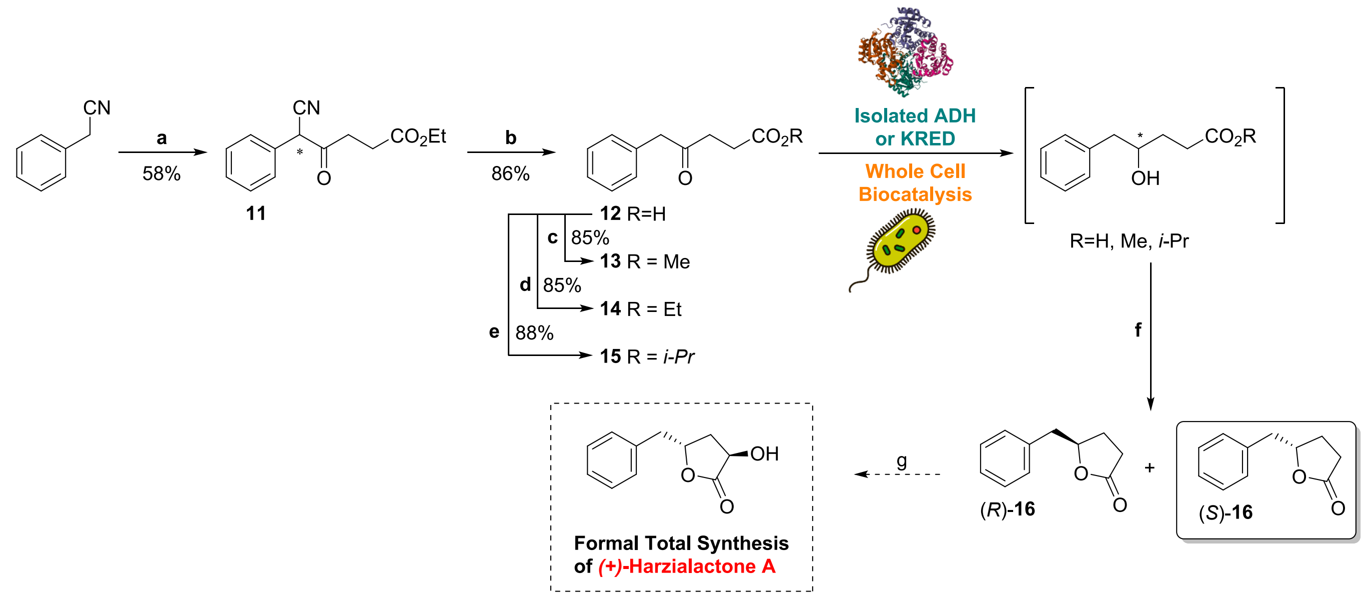


Fig. 2. Biocatalytic entries to chiral GVLs.



Scheme 1. Novel biocatalytic approach to enantiomerically enriched 5-benzyltetrahydrofuran-2-one (**16**). Reagents and conditions: (a) Na, diethyl succinate, EtOH, RT; (b) H_2O, Acetic Acid, Hydrochloric acid 37%, Reflux; (c) PTSA, MeOH, Trimethyl orthoformate, RT; (d) PTSA, EtOH, Triethyl orthoformate, RT; (e) $SOCl_2$, *i*-PrOH, reflux; (f) 10% aqueous solution of HCl; (g) 2-[(4-Methylphenyl)sulfonyl]-3-phenyloxaziridine, KHMDS, THF, −78 °C [15].

2.2. *Synthesis*

2.2.1. *Preparation of 4-oxo-4-phenylbutanoic acid (7)*

To a solution of succinic anhydride (500 mg, 4.99 mmol) in benzene (2.5 mL, 28.03 mmol) aluminum chloride was added (1.645 g, 12.34 mmol). The reaction was heated to reflux under stirring for 45 min, checking the progress via TLC. Afterward, the mixture was cooled down to room temperature and poured into ice. Then, the residue was diluted in dichloromethane (15 mL) and extracted with 10% aqueous solution of $NaHCO_3$ (7 mL). The basic extract was acidified with 10% aqueous solution of HCl, and the keto acid was extracted with dichloromethane (3 × 15 mL). The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording the pure product **7** as a white solid (396 mg, 2.22 mmol). Yield: 44.5% Mp: 109 °C. TLC (cyclohexane/ethyl acetate 7:3 + 1% formic acid) R_f: 0.22 ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.03–7.94 (m, 2H), 7.62–7.54 (m, 1H), 7.47 (t, $J = 7.5$ Hz, 2H), 3.32 (t, $J = 6.6$ Hz, 2H), 2.82 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.1, 179.1, 136.6, 133.5, 128.8, 128.2, 33.4, 28.3 NMR data matches reported literature data [36].

2.2.2. *Preparation of methyl 4-oxo-4-phenylbutanoate (8)*

Sulfuric acid (1.1 mL, 20.6 mmol) was added to a solution of **7** (656 mg, 3.68 mmol) in methanol (5 mL) and the mixture was heated at 50 °C for 12 h. The solution was evaporated under reduced pressure, and the residue was partitioned between water (5 mL) and dichloromethane (20 mL). The organic phase was washed with 10% aqueous solution of $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **8** (441 mg, 2.29 mmol) as a yellowish oil. Yield: 62.2%. TLC (cyclohexane/ethyl acetate 7:3) R_f: 0.44 ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.03–7.94 (m, 2H), 7.61–7.53 (m, 1H), 7.52–7.42 (m, 2H), 3.71 (s, 3H), 3.33 (t, $J = 6.6$ Hz, 2H), 2.77 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.2, 173.5, 136.6, 133.4, 128.7, 128.1, 51.9, 33.5, 28.1. NMR data match reported literature data [37].

2.2.3. *Preparation of (±)-5-phenyltetrahydrofuran-2-one (10)*

Under nitrogen atmosphere, $NaBH_4$ (33 mg, 0.85 mmol) was added to a solution of **7** (126 mg, 0,704 mmol) in NaOH 5%. (1.4 mL). The reaction mixture was stirred at room temperature 18 h until TLC indicated the disappearance of the starting material. The reaction mixture was acidified with a 10% aqueous solution of HCl and extracted with dichloromethane (6 mL). The organic phase was washed with brine (2 mL) and dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording of the pure product **10** (82 mg; 0.45 mmol) as a colorless oil. Yield: 64.3%. TLC (cyclohexane/ethyl acetate 1:1 + 1% formic acid) R_f: 0.69 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45–7.27 (m, 5H), 5.52 (dd, $J = 7.9$, 6.3 Hz, 1H), 2.74–2.60 (m, 3H), 2.27–2.09 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.9, 138.9, 129.2, 128.5, 125.0, 80.9, 30.8, 28.9. NMR data match reported literature data [38].

2.2.4. *Preparation of Ethyl-5-phenyl-5-cianolevulinate (11)*

Under nitrogen atmosphere, benzyl cyanide (5 mL; 42.68 mmol) and diethyl succinate (11 mL, 65.73 mmol) were added to 21 mL of a freshly prepared sodium ethoxide solution. The resulting mixture was stirred at room temperature overnight, until TLC indicated the disappearance of the starting material. The solution was then diluted with water (50 mL) and washed three times with toluene (3 × 15 mL). The basic aqueous layer was acidified with concentrated HCl and extracted three times with ethyl ether (3 × 20 mL). The organic phase was washed with 10% aqueous solution of $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **11** (6.01 g, 24.54 mmol) as a yellow oil. Yield: 57.5%. TLC (cyclohexane/ethyl acetate 7:3 + 1% formic acid) R_f: 0.47 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.47–7.36 (m, 5H), 4.80 (s, $J = 2.0$ Hz, 1H), 4.09 (q, $J = 7.2$ Hz, 2H), 2.83 (t, $J = 6.4$ Hz, 3H), 2.61 – 2.53 (m, 2H), 1.21 (t, $J = 7.2$ Hz, 3H).

2.2.5. *Preparation of 5-phenyllevulinic acid (12)*

Compound **11** (6.01 g, 24.5 mmol) was dissolved in a mixture of water (0.85 mL), conc. HCl (22 mL), and glacial acetic acid (29 mL). The reaction was heated to reflux overnight under stirring the progress was checked via TLC. The mixture was diluted with water (30 mL) and extracted with dichloromethane (3 × 10 mL). Then the organic layer was extracted with 10% aqueous solution of $NaHCO_3$ (15 mL). The basic extract was acidified with aqueous HCl, and the keto acid was extracted with dichloromethane (3 × 15 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **12** (4.038 g, 21.0 mmol) as a yellow solid. Yield:

85.7%. Mp: 57 °C. TLC (cyclohexane/ethyl acetate 7:3 + 1% formic acid) R_f: 0.32 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.13 (m, 5H), 3.74 (s, 2H), 2.75 (t, $J = 6.6$ Hz, 2H), 2.60 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.5, 178.1, 134.0, 129.6, 128.9, 127.3, 50.2, 36.3, 27.9 NMR data match reported literature data [39].

2.2.6. Preparation of methyl 4-oxo-5-phenylpentanoate (13)

Under nitrogen atmosphere, PTSA (25 mg, 0.13 mmol) and trimethyl orthoformate (284 μL, 2.6 mmol) were added to a solution of **12** in methanol (15 mL). The reaction mixture was stirred at room temperature 18 h until TLC indicated the disappearance of the starting material. Afterward, the solvent was evaporated *in vacuo*, and the crude was dissolved in ethyl acetate (15 mL). The organic phase was washed with 10% aqueous solution of $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **13** (441 mg; 2.29 mmol) as a yellowish oil. Yield: 84.7%. TLC (cyclohexane/ethyl acetate 7:3) R_f: 0.43 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.16 (m, 5H), 3.74 (s, 2H), 3.65 (s, 3H), 2.76 (t, $J = 6.6$ Hz, 2H), 2.56 (t, $J = 6.6$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.40, 173.09, 134.06, 129.45, 128.70, 127.04, 51.72, 49.92, 36.42, 27.77. NMR data match reported literature data [40].

2.2.7. Preparation of ethyl 4-oxo-5-phenylpentanoate (14)

Under a nitrogen atmosphere, PTSA (25 mg, 0.13 mmol) and triethyl orthoformate (540 μL, 3.25 mmol) were added to a solution of **12** in ethanol (15 mL). The reaction mixture was stirred at room temperature 18 h until TLC indicated the disappearance of the starting material. Afterward, the solvent was evaporated *in vacuo*, and the crude was dissolved in ethyl acetate (15 mL). The organic phase was washed with 10% aqueous solution of $NaHCO_3$ (5 mL) and of brine (5 mL), dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **14** (490 mg, 2.22 mmol) as a yellow oil. Yield: 84.7%. TLC (cyclohexane/ethyl acetate 7:3) R_f: 0.49 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41–7.12 (m, 5H), 4.11 (q, $J = 7.1$ Hz, 2H), 3.74 (s, 2H), 2.75 (t, $J = 6.6$ Hz, 2H), 2.55 (t, $J = 6.6$ Hz, 2H), 1.23 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.3, 172.5, 134.2, 129.4, 128.6, 126.9, 53.2, 49.8, 36.4, 28.0, 14.1.

2.2.8. Preparation of isopropyl 4-oxo-5-phenylpentanoate (15)

Under a nitrogen atmosphere, $SOCl_2$ (283 μL, 3.9 mmol) was added to a solution of **12** (500 mg, 2.6 mmol) in isopropanol (15 mL). The reaction mixture was heated to reflux for 18 h until TLC indicated the disappearance of the starting material. Afterward, the solvent was evaporated *in vacuo*, and the crude was dissolved in ethyl acetate (15 mL). The organic phase was washed with 10% aqueous solution of $NaHCO_3$ (5 mL) and brine (5 mL), dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording compound **15** (536 mg, 2.28 mmol) as a yellow oil. Yield: 87.9%. TLC (cyclohexane/ethyl acetate 7:3) R_f: 0.56 ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.46–6.95 (m, 5H), 5.04–4.89 (m, 1H), 3.74 (s, 2H), 2.74 (t, $J = 6.6$ Hz, 2H), 2.52 (t, $J = 6.6$ Hz, 2H), 1.21 (d, $J = 0.8$ Hz, 3H), 1.19 (d, $J = 0.8$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.3, 172.0, 134.1, 129.4, 128.6, 127.0, 67.8, 60.1, 49.9, 36.5, 28.3, 21.7.

2.2.9. Preparation of (±)-5-benzyltetrahydrofuran-2-one (16)

Under nitrogen atmosphere, $NaBH_4$ (17 mg, 0.45 mmol) was added to a solution of **13** (76.7 mg, 0.37 mmol) in 5% aqueous solution of NaOH (0.8 mL). The reaction mixture was stirred at room temperature 18 h until TLC indicated the disappearance of the starting material. The reaction mixture was acidified with a 10% aqueous solution of HCl and extracted with dichloromethane (3 X 2 mL). The organic phase was washed with brine (2 mL) and dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, affording the pure product **16** (65 mg, 0.33 mmol) as a colorless oil. Yield: 91.5%. TLC (cyclohexane/ethyl acetate 7:3 + 1% formic acid) R_f: 0.50 ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.40–7.13 (m, 5H), 4.78–4.61 (m, 1H), 3.02–2.79 (m, 2H), 2.56–2.28 (m, 3H), 2.25–2.08 (m, 1H), 1.97–1.75 (m, 1H). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.22 (m, 5H), 4.76–4.70 (m, 1H), 3.09–2.90 (m, 2H), 2.51–2.33 (m, 2H), 2.29–2.21 (m, 1H), 2.00–1.90 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.9, 135.9, 129.4, 128.6, 126.9, 80.7, 41.3, 28.6, 27.1. $CDCl_3$ NMR data match reported literature data [16].

2.3. Enzymatic and microbial bioreduction

2.3.1. Enzymes preparation.

Recombinant expression in *E. coli* and purification by affinity chromatography of the following enzymes was carried out as previously described: Ml-ADH [30], Lb-ADH [30] HSDHs [33], Is2-SDR [33], BmGDH [41] RasADH, ADH-A and SyADH were expressed in *E. coli* BL21 DE3 according to [30,42]. After cell pellet recovery by centrifugation, cells were resuspended in water and lyophilized.

2.3.2. Culture conditions.

The yeasts were routinely maintained on YPD medium slants (18 g/L agar, 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, pH = 5.6). The strain, grown on YPD medium slants for 72 h at 28 °C, was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of the same liquid medium (without agar) and incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C. In the case of solid–solid-liquid growth, the microorganism was grown in solid medium for 24 h and later it was inoculated in 100 mL liquid medium in a 500 mL Erlenmeyer flask. The microorganism was incubated on a reciprocal shaker (150 rpm) for 48 h at 28 °C before testing. Cell growth was evaluated by cells dry weight. 20 mL of broth culture were centrifuged (4500 rpm for 15 min), the pellet was washed twice with milliQ water and re-suspended in milliQ water. The cell suspension was placed in an oven for 12 h at 104 °C.

2.3.3. Enzymatic screening for 8 and 13 bioreduction.

Reactions were run in analytical scales working with a final volume of 0.5 mL. Substrates **8** and **13** were dissolved in PB (pH 7, 50 mM) at a final concentration of 10 mM starting from stock solutions prepared in DMSO (200 mM) reaching a co-solvent percentage of the 5% (v/v). The selected oxidoreductase was then added to the substrate solution based on its activity reaching a final concentration of 1 U/mL for Is2-SDR and all the HSDHs and of 0.5 U/mL in the case of Ml-ADH. Lb-ADH was used with a loading of 2 mg/mL. *E. coli*/SyADH, *E. coli*/ADH-A and *E. coli*/RasADH were instead added with concentrations of 15 mg of lyophilized cells per mL. Glucose (40 mM), glucose dehydrogenase BmGDH (0.5 U/mL) and NAD(P)$^+$ (0.4 mM) were added to the reaction mixtures which were then incubated for 24–48 h at 25 °C and 180 rpm. Stock solutions of cofactor (prepared in MilliQ-water at concentration of 20 mM) and glucose (prepared in PB buffer -pH 7, 50 mM- at a concentration of 100 mM) were used while BmGDH was added accordingly to its activity. In the case of *E. coli*/RasADH, following literature protocols [42], a final concentration of 1 mM of NAPD$^+$ and an operational temperature of 30 °C were used. Reactions were followed by TLC analysis *via* micro-extractions (*ca* 20 μL of reaction extracted with 40 μL of EtOAc). After 24 h, reaction mixtures were acidified *ca* to pH 2 with 2 N $HCl_{(aq)}$, extracted two times with 500 μL of EtOAc. The organic phases were combined and dried over Na_2SO_4 and were concentrated *in vacuo* to be analyzed by means of ^{1}H NMR (400 MHz, $CDCl_3$) and HPLC (Method B).

2.3.4. Microbial biotransformations: General procedure.

Cells obtained by centrifugation (4500 rpm for 15 min) of the culture broth were washed twice with tap water. The pellet was suspended in Tris/HCl buffer containing 50 g/L of glucose (pH 7, 0.1 M) to obtain a doubled cells concentration. Substrates (**8**, **12** or **13**) were dissolved in DMSO or isopropanol added to the biotransformations in a final concentration of 0.5, 1 or 2 g/L; keeping the co-solvent v/v ratio equal to the 5% (DMSO) and the 10% (isopropanol). The reactions were carried out at 28 °C under magnetic stirring. After 96 h, the biotransformations were acidified with an HCl solution ($[HCl]_{biotransformation} = 2$ M), left under magnetic stirring for 24 h and then extracted twice with ethyl acetate. The combined organic phases were dried over anhydrous Na_2SO_4 and were evaporated *in vacuo*. The samples were analyzed by chiral HPLC analyses according to Method A (see Supplementary Materials for details).

2.3.5. NADES preparation.

NADES were prepared by mixing choline hydrochloride (ChCl) and glucose (Glu) in a molar ratio of 1.5:1 or glycerol (Gly) and glucose (Glu) in a molar ratio of 2:1. The compounds were placed under magnetic stirring at a temperature of 75 °C until a colorless liquid mixture was obtained.

2.3.6. HPLC analyses.

Molar conversion and *ee* were determined by HPLC equipped with a chiral column according to one of the two different methods reported below.

Method A: Merck-Hitachi L-7100 instrument coupled with a UV6000LP detector and a Chiralcel OD-H chiral column (250 mm × 4.6 mm). HPLC conditions: flow rate = 0.8 mL/min, detection $\lambda = 210$ nm; temperature = 30 °C, mobile phase: 90% of hexane, 10% of *i*-PrOH and 0.1% of formic acid. Compound, R_t: **7**, 12.6 min; **8**, 10.9 min; (*S*)- **10**, 19 min; (*R*)-**10**, 18 min; **12**, 13.1 min; **13**, 12.45 min; **14**, 9.9 min; **15**, 8 min (*S*)-**16**, 18 min; (*R*)-**16**, 21 min.

Method B: Shimadzu LC-20AD high performance liquid chromatography system equipped with a Shimadzu SPD-20 A UV detector and a Phenomenex Lux 3u Cellulose-1 chiral column (250 mm × 4.6 mm). HPLC conditions: flow rate = 1 mL/min 1; detection $\lambda = 280$ nm; temperature = 30 °C, mobile phase: 70% of petroleum ether and 30% of *i*-PrOH. Compounds*:* R_t = **7**, 5.88 min; (*S*)-**10**, 6.9 min; (*R*)-**10**, 7.3 min; **13**, 6.27 min; (*S*)-**16**, 7.4 min; (*R)*-**16**, 8.3 min.

2.4. Semi-preparative scale preparation of (R)- and (S)-16

2.4.1. Preparation of (+)-5-benzyltetrahydrofuran-2-one (S)-16

The liquid culture inoculum of *T. molischiana* was prepared by making two passages in solid YPD medium by incubating the plates in both cases at 28 °C for 24 h. Cells from 50 mL of broth culture were centrifuged, washed and suspended in Tris/HCl buffer (25 mL, pH 7, 0.1 M) containing 10% v/v ChCl-Glc NADES and 25 mg of compound **12**. After 96 h at 30 °C under magnetic stirring, the biotransformation was acidified with HCl and extracted twice with ethyl acetate. The combined organic phases were dried over Na_2SO_4 and were concentrated *in vacuo* affording crude (*S*)-**16** which was purified by preparative TLC (Eluent: cyclohexane: ethyl acetate = 7:3). (*S*)-**16** was obtained as yellowish oil with a 65% isolated yield and an *ee* > 97%. $[\alpha]_D^{25} = +11.37$ (*c* 1.0, $CHCl_3$) [43], HRMS (ESI): *m/z* calculated for $[M + Na]^+$ $C_{11}H_{12}O_2$ 199.0735; found 199.0736.

2.4.2. Preparation of (-)-5-benzyltetrahydrofuran-2-one (R)-16

Glucose (40 mM), BmGDH (0.5 U/mL) and $NADP^+$ (0.4 mM) were added to a solution of **13** (30 mg, 10 mM) prepared in 10% v/v mixture of DMSO in PB buffer (pH 7.0, 50 mM). Reduction was started by adding *E. coli*/SyADH (10 mg/mL of lyophilized *E. coli* cells) and it was controlled by TLC analysis (ethyl acetate/petroleum ether = 7:3 as mobile phase, UV light and phosphomolybdic acid solution to stain) over the course of 48 h. After that, the mixture was extracted with EtOAc (3 X 500 μL), the organic layers were combined, dried over Na_2SO_4, and were concentrated *in vacuo* affording crude (*R*)-**16** which was purified by preparative TLC (Eluent: cyclohexane: ethyl acetate = 7:3). (*R*)-**16** was obtained as yellowish oil with a 70% isolated yield and an *ee* > 99%. (*R*)-**16** $[\alpha]_D^{25} = -10.98$ (*c* 1.0, $CHCl_3$) [44], HRMS (ESI): *m/z* calculated for $[M + Na]^+$ $C_{11}H_{12}O_2$ 199.0735; found 199.0735.

2.5. Computational Methods

Docking simulations were performed by using the resolved structure of the RasADH in complex with $NADP^+$ (PDB ID: 4I5D) [45] which was prepared by adding the hydrogen atoms according to the physiological pH equal to 7.4 by using the VEGA suite of programs [46]. The precise arrangement of the substrates was extrapolated by the resolved structure of F12 RasADH in complex with NADPH and A6O (PDB ID: 6IHH) [47]. In detail, the A60 was manually inserted into the catalytic cavity of the first structure by superimposing the common cofactor and the docking searches were focused within a 10 Å radius sphere around the so inserted A60 ligand. Docking simulations were carried out by PLANTS [48]; 10 poses per ligand were generated and ranked by the ChemPLP scoring function with speed equal to 1. The generated complexes were minimized by using Namd and rescored by Rescore$^+$ [49].

3. Results & discussion

3.1. Chemistry

3.1.1. Synthesis of the suitable substrates

The substrate **8** was synthesized starting from benzene that underwent a Friedel-Crafts acylation and a subsequent Fisher esterification as reported by Alazet *et al.* whose procedure was properly adapted [39]. The series of suitable prochiral ketones **12**–**15** were synthesized as shown in Scheme 1. The synthesis started with the commercially available benzyl cyanide that reacted with diethyl succinate to give compound **11** which was subsequently treated with a mixture of acetic acid/hydrochloric acid to afford the derivative **12**. Then, compound **12** underwent esterification with methanol, ethanol, and isopropanol to obtain compounds **13**, **14**, and **15** respectively. Furthermore, compounds *rac*-**10** and *rac*-**16** were synthesized as references for HPLC analyses.

Copies of the NMR spectra and HPLC chromatograms can be found in the Supplementary Materials.

3.2. Bioreduction of 13: Biocatalysts screening

3.2.1. Wild-type, recombinant oxidoreductases

The biocatalyzed stereoselective reduction of **13** and the one-pot tandem conversion of the obtained γ-hydroxyester into the corresponding γ-valerolactone **16** were investigated using a collection of NAD^+ or $NADP^+$ dependent oxidoreductases of different origin, both belonging to the alcohol dehydrogenase (ADHs) and hydroxysteroid

Table 1
Recombinant $NAD(P)^+$-dependent oxidoreductases screened for the stereoselective reduction of **8** and **13**.

Enzyme	Source, Reference	Cofactor
Alcohol Dehydrogenases		
Is2-SDR	*Metagenome sample,* [32]	$NADP^+$
Ml-ADH	*Micrococcus luteus,* [50]	NAD^+
E. coli/ADH-A[1]	*Rhodococcus ruber,* [51]	NAP^+
Lb-ADH	*Lactobacillus brevis,* [52]	$NADP^+$
E. coli/RasADH[1]	*Ralstonia* sp.*,* [42]	$NADP^+$
E. coli/SyADH[1]	*Sphingobium yanoikuyae,* [29,42]	$NADP^+$
Hydroxysteroids Dehydrogenases		
Ca7α-HSDH	*Clostridium absonum,* [33]	$NADP^+$
Dm7α-HSDH	*Deinococcus marmoris,* [32]	NAD^+
Hh7α-HSDH	*Halomonas halodenitrificans,* [32]	NAD^+
Ngi1_7α-HSDH	*Metagenome sample,* [32]	NAD^+
Bsp7β-HSDH	*Brucella* sp.*,* [32]	NAD^+
Ca7β -HSDH	*Clostridium absonum,* [53]	$NADP^+$
Rs7β-HSDH	*Rhodobacter sphaeroides,* [32]	NAD^+
Sc7β-HSDH	*Stanieria cyanosphaera,* [32]	NAD^+
Csp12α-HSDH	*Clostridium* sp.*,* [54]	$NADP^+$

[1] Lyophilized recombinant *E. coli* cell pellets were used in the biotransformations [29,42].

dehydrogenases (HSDHs) groups (Table 1). The glucose/glucose dehydrogenase from *Bacillus megaterium* (BmGDH) system was used for cofactor regeneration (Scheme 2), and enzymes were produced as recombinant proteins in *E. coli* according to established literature protocols (Table 1). Compound **8**, whose bioreduction was already reported in the literature, was used as model substrate to compare the performances of the selected biocatalysts on both the bulky-bulky γ-ketoester substrates. At variance to **8**, compound **13** is in fact characterized by a more flexible benzyl substituent as Cα decoration.

Table 1 summarizes the source and redox cofactor of the screened biocatalysts, all of them characterized by different synthetic features. Specifically, Is2-SDR, a short-chain dehydrogenase discovered by the authors by metagenome mining, and the alcohol dehydrogenase from *Micrococcus luteus* (Ml-ADH) are enzymes known for their broad substrate promiscuity and ability to reduce bulky or structurally complex substrates [32,50]. The above mentioned *E. coli*/RasADH and *E. coli*/SyADH showed good activity (60–70%) and excellent enantioselectivity in the reduction of **8**, while the alcohol dehydrogenase from *Rhodococcus ruber* (ADH-A) and *Lactobacillus brevis* (Lb-ADH) did not convert this model substrate [21,26,30]. Finally, a small collection of the different hydroxysteroid dehydrogenases (HSDHs) available to SCITEC-CNR laboratories was selected based on their high regio- and stereoselectivity towards steroid skeletons (*i.e.* 7α, 7β and 12α HSDHs) and substrate promiscuity [32,34].

All the described enzymes were thus used in analytical scale as biocatalysts for the stereoselective reduction of compounds **8** and **13** conducing their one-pot, tandem transformation into **10** and **16** respectively without isolating the corresponding γ-hydroxyester intermediate. Reactions were run for 24 h (for details of the screening protocol see the *Material and Methods* section); after extraction with ethyl acetate, conversion and *ee* for compounds **10** and **16** were determined *via* HPLC using chiral stationary phase methods already reported in the literature [16,20]. The successful biotransformations obtained during the screening process are summarized in Table 2.

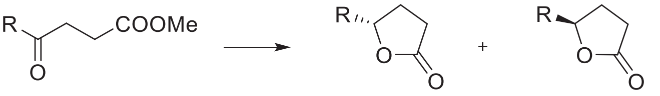
To our surprise, none of the promiscuous HSDHs, which successfully catalyzed the reduction of β-ketoesters [32,34], were able to convert compounds **8** or **13** to **10** or **16** respectively.

Interestingly, both Is2-SDR and Ml-ADH reduced **8** with modest conversion (< 40%) and complementary enantioselectivity. The former produced (*S*)-**10** (the Prelog product) with 78% *ee* while the latter catalyzed the formation of the (*R*)-**10** (the *anti*-Prelog product) with an excellent *ee* (> 99%). However, both these biocatalysts did not convert the bulkier substrate **13**.

In agreement with previous works [21,26,30], neither Lb-ADH nor ADH-A converted **8**, while both *E. coli*/RasADH and *E. coli*/SyADH reduced **8** with excellent *ee* (> 9%) in favor of the Prelog product (*S*)-**10**. In agreement with what was reported for **8,** compound **13** was not converted by neither Lb-ADH nor ADH-A while it was reduced by *E. coli*/RasADH and *E. coli*/SyADH, though with lower conversions. Moreover, both *E. coli*/RasADH and *E. coli*/SyADH, at variance to the reduction of **8**, formed the *anti*-Prelog product *(R)*-**16** from **13** with a modest to an excellent *ee* (66% and > 99%, respectively).

Surprised by the unexpected *anti*-Prelog stereoselectivity of the bioreduction of **13,** an *in silico* investigation was performed to compare the docking poses of the two prochiral γ-ketoesters in RasADH active site (PDB ID: 4I5D) [45].

Table 2
Screening results for the bioreduction of compounds **8** and **13**.



8 R = Ph
13 R = Bn

(*R*)-**10** R = Ph
(*R*)-**16** R = Bn

(*S*)-**10** R = Ph
(*S*)-**16** R = Bn

Entry	Oxidoreductases	Substrate 8 Product 10[1] *c* (%)[3]	*ee* (%)[3]	13 16[2] *c* (%)[3]	*ee* (%)[3]
1	**Is2-SDR**	31	78 (*S*)	N.C.	
2	**Ml-ADH**	38	> 99 (*R*)	N.C.	
3	***E. coli*/RasADH**	60	> 99 (*S*)	50	66 (*R*)
4	***E. coli*/SyADH**	70	> 99 (*S*)	45	> 99 (*R*)

N.C. = no conversion.

[1] Method: Lux Cellulose-1, λ 210 nm and 254 nm, mobile phase petroleum ether/isopropanol = 7:3, flow = 1 mL/min. R_t = (*S*)-**10**, 6.9 min; (*R*)-**10**, 7.3 min.

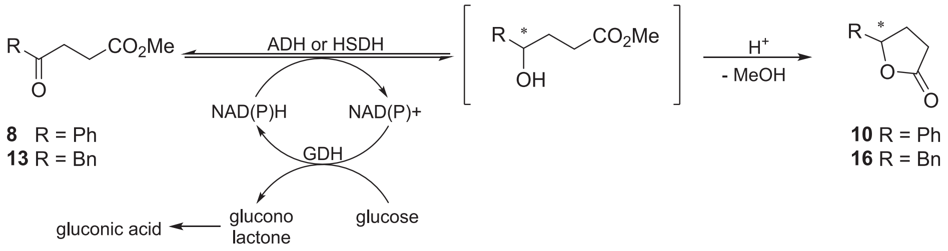
[2] Method: Lux Cellulose-1, λ 210 nm and 254 nm, mobile phase petroleum ether/isopropanol = 7:3, flow = 1 mL/min. R_t = (*S*)-**16**, 7.4 min; (*R*)-**16**, 8.3 min.

[3] Conversion (*c* (%)) and enantiomeric excesses (*ee* (%)) were determined by HPLC analysis on chiral column (see Materials and Methods section).

The docking results revealed that both the substrates, namely compounds **8** and **13,** can be suitably accommodated within the RasADH catalytic pocket where they assume two specular binding modes thus providing different enantiotopic faces as shown in Fig. 3. Both esters arrange the keto group in a pose conducive to the catalysis since the oxygen atom interacts with Ser137, His147 and Tyr150, while the nicotinamide ring of the cofactor points to the carbonyl carbon atom of the substrate. The differences between the two simulated substrates involve the relative arrangement of the phenyl ring and the ester moiety. Indeed, Fig. 3A reveals that the small substrate (**8**) is able to arrange the phenyl ring towards the cofactor with which it can stabilize π/π interactions further reinforced by similar stacking contacts with Phe205 plus hydrophobic interactions with surrounding alkyl-chain residues (e.g. Ile91, Ala162, Ile187 and Ile188). The ester group of compound **8** is facing the exit of the binding pocket where it can only stabilize hydrophobic interactions with Val138, Leu144 and Leu246. In contrast, Fig. 3B shows that compound **13** prefers to assume an overturned pose in which the ester group approaches the cofactor, and the phenyl assumes a more superficial arrangement. In detail, the ester moiety is engaged by a H-bond with Gln191 plus hydrophobic contacts with Ile91 and Ile187, while the phenyl ring elicits a clear π/π stacking with Phe205 plus hydrophobic contacts with Val138, Leu144 and Leu246.

3.2.2. *The Evoxx® alcohol dehydrogenase kit*

As discussed, compound **13** was reduced by *E. coli*/RasADH even though with low conversion and *ee*, and by *E. coli*/SyADH, with an



Scheme 2. Enzymatic reduction of compounds **8** and **13** and their tandem conversion into the corresponding GVLs (**10** and **16**).

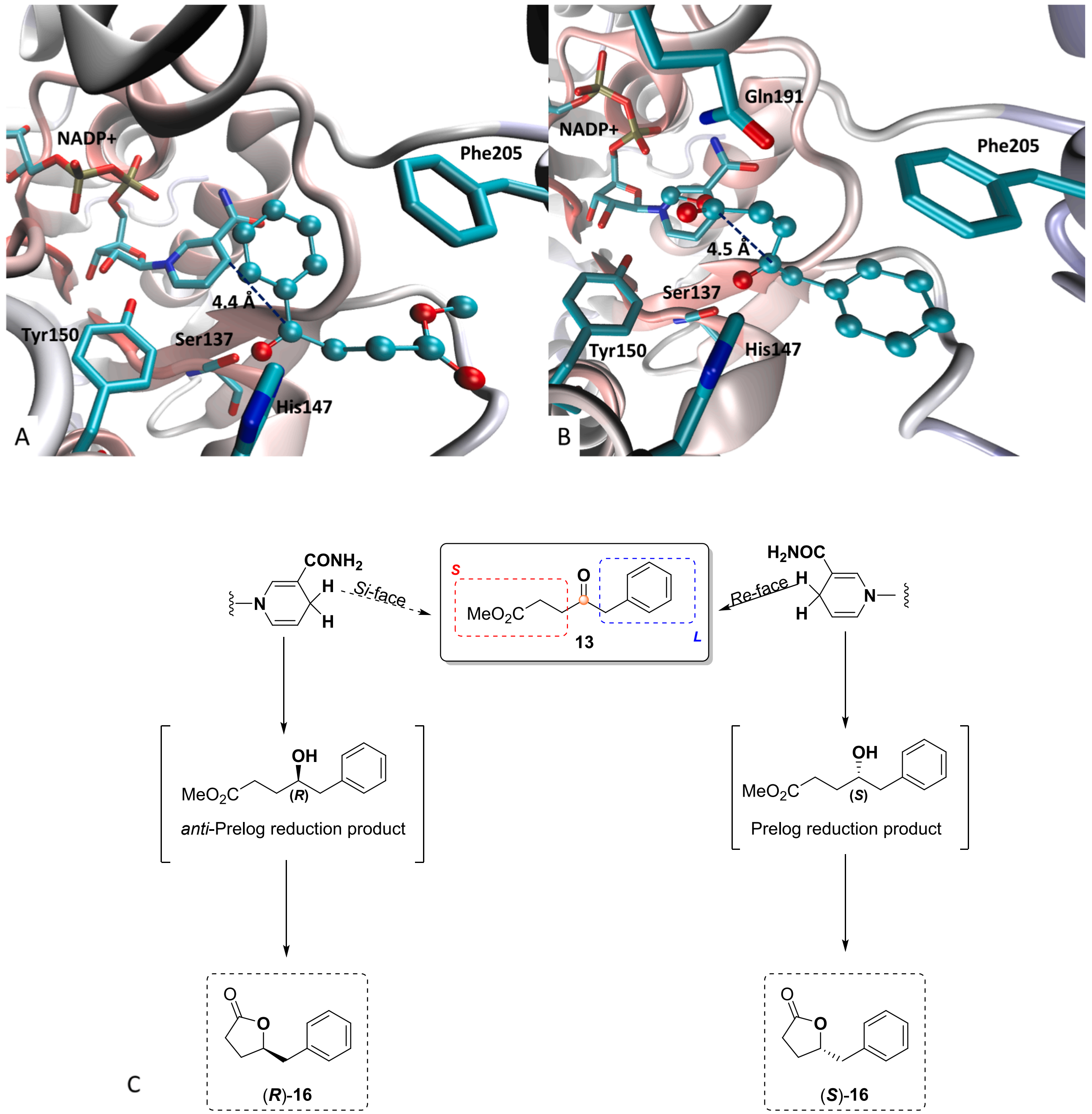


Fig. 3. Main interactions stabilizing the putative complexes for compounds **8** (A) and **13** (B) within the catalytic pocket of RasADH (PDB ID: 4I5D) [45]. (C) Schematic representation of Prelog and anti-Prelog reduction.

excellent *ee* and a lower conversion when compared to the reduction of compound **8**. Moreover, both *E. coli*/RasADH and *E. coli*/SyADH produced the (*R*)-enantiomer of **16**, which presents the opposite absolute stereochemistry of (+)-Harzialactone A (Fig. 1). Thus, seeking for an enantiocomplementary enzyme, a kit of commercially available engineered ADH (EvoXX® Technologies GmbH) whose good performances on bulky and structurally complex natural compounds have been already reported by us [30] was screened for the reduction of substrates **8** and **13**. Among the screened enzymes ADH442 was found to efficiently catalyzed the formation of (*R*)-**16** with excellent conversion and *ee*. Obtained data are reported in detail in the Supplementary Materials (Table S1).

3.2.3. *Microbial bioreduction of 13*

After screening isolated enzymes, looking for a low-cost biocatalyst to produce (*S*)-**16**, the core of (+)-Harzialactone A, we focused our attention on the microbial reduction of **13** (Scheme 1) because of no reports can be found in the literature dealing with the biotransformation of this substrate using whole cells as biocatalyst. Twenty different yeast strains selected from our in-house collection and belonging to different species and *genera* were exploited in an analytical scale screening. As planned for isolated enzymes, compound **13** was used as a model substrate (Fig. 2b) since, as demonstrated by Forzato *et al.*, its acid and the ethyl ester derivatives were stereoselectively reduced to the corresponding (*S*)-butyrolactones either by *P. etchelsii*, *P. glucozyma*, or *S. cerevisiae* [27].

Screening results (Table 3) are reported as conversion and *ee* of the

Table 3
Microorganisms screened for the bioreduction of compounds **8** and **13**.

Entry	Microorganism	Substrate					
		8			**13**		
		Product			**Product**		
		7	**10**		**12**	**16**	
		c (%)[1]	*c* (%)[1]	*ee* (%)[1]	*c* (%)[1]	*c* (%)[1]	*ee* (%)[1]
1	*Kluyveromyces marxianus* CBS 397	71	17	72 (*S*)	59	41	85 (*S*)
2	*K. marxianus* var. lactis CL69	53	41	75 (*S*)	78	10	37 (*S*)
3	*Lindnera fabianii* CBS 5640	5	95	95 (*S*)	65	35	66 (*S*)
4	*Pachysolen tannophylus* CBS 4044	5	75	22 (*S*)	47	45	33 (*R*)
5	*Pichia capsulata* CBS 1993	70	13	60 (*S*)	73	traces	
6	*Pichia etchelsii* MIM	24	51	47 (*S*)	48	traces	
7	*Pichia glucozyma* CBS 5766	34	30	45 (*S*)	13	traces	
8	*Pichia jadinii* CBS 4885	57	25	65 (*S*)	89	16	64 (*R*)
9	*Pichia pastoris* CBS 704	71	traces		80	traces	
10	*Rhodotorula rubra* MIM 146	79	traces		39	59	87 (*R*)
11	*Rhodotorula rubra* MIM 147	63	traces		35	12	55 (*R*)
12	*Saccharomyces cerevisiae* ZEUS	47	41	98 (*S*)	73	traces	
13	*Sporidiobolus pararoseus* SD2	96	traces		92	11	77 (*R*)
14	*Sporobolomyces holsaticus* NCYC 420	76	traces		75	traces	
15	*Sporobolomyces salmonicolor* MIM	0	0		46	traces	
16	*Slooffia tsugae* CBS 5038	51	38	98 (*S*)	73	18	81 (*S*)
17	*Torulopsis magnolie* IMAP 4425	8	82	75 (*S*)	51	32	71 (*S*)
18	*Torulopsis molischiana* CBS 837	14	71	92 (*S*)	68	16	58 (*S*)
19	*Torulopsis pinus* IMAP 4573	73	traces		68	13	4 (*S*)
20	*Torulopsis castelli* CBS 4332	11	11	50 (*S*)	67	15	91 (*S*)

Screening conditions: substrate 2 mg/mL, DMSO 5% v/v, glucose 50 mg/mL, biocatalyst double concentrated compared to the liquid culture, 96 h and 30 °C.

[1] Conversion (*c* (%)) and enantiomeric excesses (*ee* (%)) were determined by HPLC analysis on chiral column (see Materials and Methods section).

corresponding lactones determined *via* HPLC (See Materials and Methods section and Supplementary Materials). At variance to the use of isolated enzymes, both the substrates (**8** and **13**) underwent competitive hydrolytic processes in the presence of whole cells, thus they were also converted to the corresponding γ-ketoacids (**7** and **12**).

As expected, compound **8** was successfully reduced by *P. etchelsii*, *P. glucozyma*, and *S. cerevisiae* with good conversion and moderate to excellent *ee*; among them *S. cerevisiae* and *L. fabianii* were the best ones in term of enantioselectivity (*ee* ≥ 95%). In general, the yeast strains, that were found active, reduced compound **8** only to (*S*)-**10** with different degrees of enantioselectivity.

As far as the bioreduction of compound **13** concerns (Table 3), this bulkier substrate was generally transformed to the corresponding lactone with lower conversion with respect to compound **8**. Interestingly, the screened yeast strains showed different stereoselectivity in reducing **13** leading to the formation of both the enantiomers of **16** with different degrees of enantioselectivity. In details, five yeast strains (Entries 4, 8, 10, 11, 13) catalyzed the formation of the *anti*-Prelog lactone (*R*)-**16** with up to 87% of *ee*, while all the other microorganisms produce the Prelog product with poor to good *ee*.

This inversion of enantioselectivity in the reduction of **13**, with the respect to **8**, may be caused by different factors. As a matter of fact, several enzymes endowed by different affinity and selectivity towards the two substrates **8** and **13** could be present in the same yeast. In addition, the bulkier and more flexible nature of compound **13**, as previously demonstrated *in silico* in the case of *E. coli*/RasADH, could induce a different binding pose within the catalytic site of the same reductase thus offering a distinctive prochiral face to the cofactor.

Furthermore, as previously mentioned, in all the biotransformations modest to considerable amounts of **12** were formed attesting the high hydrolytic activity of these microorganisms toward compound **13**.

This preliminary screening (Table 3) allowed us to identify *T. castelli* CBS 4332 as the most promising biocatalyst for the reduction of **13** to the Prelog enantiomer (*S*)-**16** with a good *ee* (> 90%). Interestingly, *Torulopsis* sp. yeasts were already applied for the reduction of bulky substrate [35] and for these reasons *Torulopsis* strains were selected for upcoming experiments for the optimization of the biotransformations. Specifically, the undesired hydrolytic activity shown toward **13** has been studied to address if (1) it could be lowered avoiding competition with the desired reducing activity or if (2) it could be directly exploited to produce the target lactone **16** from **12** (Scheme 2). Also, the reasons at the basis of the modest conversions of **13** into **16** have been investigated in terms of substrate loading, substrate/product inhibitory activity and in-cell availability of redox cofactors via *ad hoc* designed experiments.

At first, we tried to suppress/lower the hydrolytic activity using bulkier esters as substrates, *i.e.* ethyl (**14**) and isopropyl (**15**) derivatives, in the presence of *T. magnolie*, *T. molischiana*, and *T. castelli*. Also, a hexane-buffer biphasic system was tested using **13** as substrate. As shown in Table 4 (Entries 1–3), the use of bulkier esters or a biphasic system did not produce a significant increase in the amount of **16** with the respect to **12**.

Thus, according to the potential inhibitory effects and/or different enzymatic activities described above , we focused our attention on the effects of using different concentrations of **13** on the **16**/**12** ratio (Table 4, Entries 4, 5). We discovered that increasing the concentration of compound **13** always lowered the **16**/**12** ratio, *de facto* facilitating the undesired hydrolytic processes.

In addition, based on these results we again hypothesized substrate/product inhibitory effects and/or a competition between hydrolytic and redox enzymes characterized by different kinetics.

Afterwards, a set of glucose-feeding experiments (feed every 50 g/L every 24 h) was conducted to investigate if the low conversion of compound **13** into **16** could be ascribed to poor redox cofactor availability in the cells. Percentages of compounds **16** and **12** seemed to be unmodified by this approach as the **16**/**12** ratio basically did not change in all the experiments (Data not shown).

Finally, considering the high hydrolytic activities of the *Torulopsis* yeasts and the possible alternative reaction pathways reported in Scheme 3 we checked whether the microorganisms could have directly reduced compound **12** with better conversion and enantioselectivity (Table 5).

In the meantime, we could also determine if the reduction of **12** would be enantioconvergent with the bioreduction of **13** using the same biocatalyst.

Surprisingly, the three microorganisms produced (*S*)-**16** with excellent *ees* (97–98%). These results corroborated the hypothesis of the presence of no-stereoconvergent ADHs operating on **13** and/or **12** that lowered the *ees* of the previously discussed biotransformations. This is of relevance in the case of bioreduction of **12** catalyzed by *T. magnolie* and *T. molischiana* whose *ees* were significantly higher when compared to the results of Table 3.

Once we discovered that compound **12** represented a more convenient substrate, we decided to optimize its biotransformation *via* an

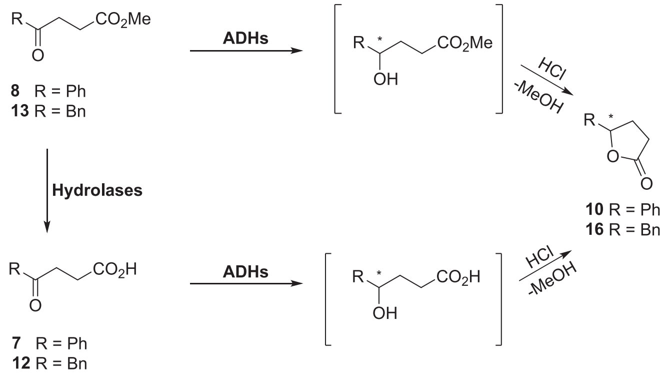
Table 4
Studies on the bioreduction of the different prochiral ketones.

Entry	Substrate	*T. magnolie*			*T. molischiana*			*T. castelli*		
		16 (%)	**12** (%)	**16/12**[1]	**16** (%)	**12** (%)	**16/12**[1]	**16** (%)	**12** (%)	**16/12**[1]
1	**14**	30	57	0.5	28	51	0.5	10	63	0.1
2	**15**	16	36	0.4	20	47	0.4	5	41	0.1
3	**13-Biphasic**[2]	0	37	N.C.	0	34	N.C.	0	43	N.C.
4	**13, 1 mg/mL**	29	67	0.4	51	49	1.0	25	60	0.4
5	**13, 2 mg/mL**	15	63	0.2	16	67	0.2	15	67	0.2

Cell grown for 72 h in solid medium and 48 h in liquid medium (S-L), 96 h of biotransformation, [substrates] = 2 mg/mL, 5% v/v DMSO, glucose 50 g/L.
N.C. = not calculated.

[1] Ratio between the HPLC abundance (%) of compounds **16** and **12**.

[2] Hexane: Tris/HCl buffer (0.1 M, pH 7) = 1:1.



Scheme 3. Different biotransformation pathways of compound **13** in cells.

Table 5
Studies on bioreduction of compound **12**.

Entry	Microorganism	*c* (%)[1]	*ee* (%)[1]
1	*T. magnolie*	44	98 (*S*)
2	*T. molischiana*	35	98 (*S*)
3	*T. castelli*	17	97 (*S*)

Cell grown for 72 h in solid medium and 48 h in liquid medium (S-L), 96 h of biotransformation, [12] = 1 mg/mL. *T. molischiana* [cells] = 21.2 mg/mL; *T. magnolie* [cells] = 20.4 mg/mL; *T. castelli* [cells] = mg/mL 15.7 mg/mL.

[1] Conversion (*c* (%)) and enantiomeric excesses (*ee* (%)) were determined by HPLC analysis on chiral column (see Materials and Methods section).

approach of reaction media engineering. Specifically, we tested: (1) possible inhibitory effects of compound **16**; (2) the concentration of compound **12** and, most importantly; (3) the effects of different co-solvents and co-substrates.

The presence of 0.5 mg/mL of **16** did not produce any inhibitory effects on bioreduction of compound **12**, while high substrate's concentrations sensibly lowered its conversion into **16** (from 22% in the presence of 2.0 mg/mL to the 68% in the presence of 0.5 mg/mL).

The effects of different co-substrates and co-solvents on the bioconversion of compound **12** into (*S*)-**16** are reported in Table 6. The use of increasing concentrations of glucose (Entries 2–3) or glycerol did not produce any appreciable effects on the conversion of compound **12** (Entry 5), while xylose slightly lowered it. At variance to what we discovered before for the bioreduction of bulky ketones mediated by *Torulopsis* sp. [35], the 10% v/v of isopropanol lowered the conversion of compound **12** (Entry 1), highlighting that different ADHs could be involved in the reduction of this specific substrate. Based on these results we decided to use a glucose-based NADES [55] (natural deep eutectic solvents, Entries 6 and 7) since they merged the characteristics of green and biocompatible co-solvent with a convenient co-substrate. This strategy allowed us to strongly increase the conversion of **12** into the target (*S*)-**16**. Additionally, a double growth passage on solid medium and then in liquid medium was found to increase the conversion of **12**.

Table 6
Co-substrates and co-solvents effects on bioreduction of **12**.

Entry	Co-substrate	*T. molischiana*		*T. magnolie*	
		c **(%)**[1]	***ee*** **(%)**[1]	***c*** **(%)**[1]	***ee*** **(%)**[1]
1	Isopropanol 10% v/v	10	98 (*S*)	N.C.	
2	Glucose 50 g/L	36	97 (*S*)	34	96 (*S*)
3	Glucose 100 g/L	43	93 (*S*)	33	93 (*S*)
4	Xylose 50 g/L	41	92 (*S*)	30	94 (*S*)
5	Glycerol 50 g/L	25	97 (*S*)	22	96 (*S*)
6	NADES (glu-gly)[2]	80	93 (*S*)	79	97 (*S*)
7	NADES (ChCl-glu)[3]	82	97 (*S*)	60	96 (*S*)

Cell grown for 48 h (S-L), 96 h of biotransformation, **12** 1 mg/mL. *T. molischiana* [cells] = 21.2 mg/mL; *T. magnolie* [cells] = 20.4 mg/mL.
N.C. = no conversion.

[1] Conversion (*c* (%)) and enantiomeric excesses (*ee* (%)) were determined by HPLC analysis on chiral column (see Materials and Methods section).

[2] NADES (glu-gly): Glucose-glycerol.

[3] NADES (CHCL-glu): Choline chloride-glucose.

3.2.4. *Semi-preparative scale preparation of (R)- and (S)-16*

(*R*)- and (*S*)-**16** were prepared using two different biocatalytic systems.

The *anti*-Prelog enantiomer (*R*)-**16** was prepared using *E. coli*/SyADH as biocatalyst (10 mg/mL of lyophilized *E. coli* cells) using a 10 mM solution of **13** prepared in the presence of 10% v/v DMSO in PB buffer (pH 7.0, 50 mM), glucose (40 mM), BmGDH (0.5 U/mL) and NADP$^+$ (0.4 mM). Product was obtained with an *ee* > 99% (80% molar conversion) and a 70% isolated yield (see the Material and Methods section).

The Prelog product (*S*)-**16**, the direct precursor of (+)-Harzialactone A [15], was obtained by biotransformation with whole cells of *T. molischiana* (18.9 mg/mL) grown in solid medium 24 h (double step) and liquid medium 48 h. The cells were suspended in Tris/HCl buffer (0.1 M, pH 7) containing 10% NADES (ChCl-glu) and 1 g/L of **12**. After 96 h (*S*)-**16** was obtained with a molar conversion of 88%, *ee* > 97% and 65% isolated yield (see the Material and Methods section).

4. Conclusions

In this work, after screening libraries of more than 15 wild type, promiscuous oxidoreductases, a commercial kit of ADHs and 20 different yeasts strains as whole-cell biocatalysts, we have successfully designed and realized a chemo-enzymatic entry to both the enantiomers of **16**. To the best of our knowledge this is the first time that a biocatalytic approach is applied for the preparation of (*S*)-**16**, a valuable synthon of the bioactive marine drug (+)-Harzialactone A.

This convenient, facile, and biocatalytic approach to entantiomerically enriched γ-lactones is now being exploited by us for the synthesis of the two enantiomers of the metabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, that we found to be active against *Candida albicans* when tested as a racemate [10].

Funding

This work was supported by Università degli Studi di Milano.

Supplementary Materials

The supporting information file for the manuscript was uploaded.

Author Contributions

Angelica Artasensi and Ivan Bassanini share the First author.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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