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Stereoselective Reduction of Prochiral Cyclic 1,3-Diketones Using Different Biocatalysts

Martina Letizia Contente[1,3] · Federica Dall'Oglio[2] · Francesca Annunziata[2] · Francesco Molinari[3] · Marco Rabuffetti[3] · Diego Romano[3] · Lucia Tamborini[2] · Dörte Rother[4] · Andrea Pinto[3]

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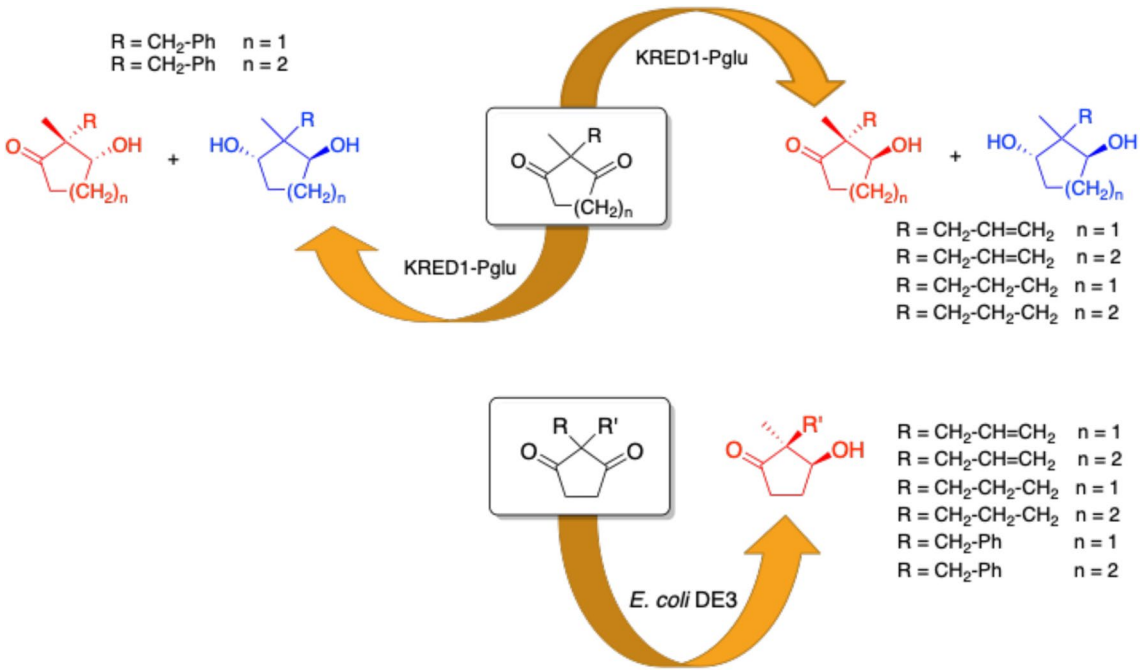
Abstract

We have developed biocatalytic methods for the stereoselective reduction of cyclic prochiral 1,3-diketones for the production of optically active *β*-hydroxyketones and/or 1,3-diols. The recombinant ketoreductase KRED1-Pglu (formulated as purified catalyst) and whole cells of wild type *Escherichia coli* DE3 Star were used as biocatalysts, displaying different and sometimes complementary stereoselectivity, thus allowing the preparation of stereochemically pure β-hydroxyketones (12–66% isolated yields, > 99% e.e.) and 1,3-diols (40–60% isolated yields, > 99% e.e.).

Graphic Abstract



Keywords Biocatalytic reduction · 1-3 diketones · β-hydroxyketones · 1,3-Diols · Enzymatic · Whole cells

Martina Letizia Contente and Federica Dall'Oglio have contributed equally to this work.

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Extended author information available on the last page of the article

1 Introduction

Biocatalytic reduction of prochiral diketones is an attractive method for the preparation of optically active hydroxyketones [1–3] and diols [4–6]. Chiral diols have a wide range

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of synthetic applications [7, 8] and can be obtained using diketoreductases (DKR), which are enzymes able to reduce both carbonyl groups of diketones [4]; these enzymes have been used for different biotransformations, including the preparation of (3*R*,5*S*)-ethyl-6-(benzyloxy)-3,5-dihydroxyhexanoate, the chiral side chain of antihypertensive rosuvastatin, using a DKR from *Acinetobacter baylyi* [4]. Alternatively, monoreduction of 1,2-diketones and 1,3-diketones affords α- and β-hydroxyketones, respectively, which are highly valuable chiral synthons for the synthesis of natural products and pharmaceuticals [9–11].

Regio- and stereoselective reductions of diketones affording different enantiopure hydroxy ketones or diols can be catalyzed by isolated alcohol dehydrogenases (ADHs) [12–16]. Linear aliphatic diketones are reduced by ADHs with different selectivity leading to ketols or diols depending on the substrate-binding modes in the active site of the enzyme [16]. Preparation of chiral 1,4-diaryl-1,4-diols from sterically hindered 1,4-diaryl-1,4-diketones has been accomplished using the ADH from *Ralstonia* sp. [17]; in this case, diols were the main products with no significant accumulation of the intermediate hydroxyketones. Enzymatic reduction of 2,2-disubstituted 1,3-alkanediones (with different substitution patterns on the tetrahedral carbon C2) is remarkably attractive from a synthetic point of view, since the biocatalyst may accomplish enantioselective hydrogen transfer with simultaneous stereo-discrimination between the two groups bond to the tetrahedral center C2, thus giving one stereoisomer as major final product. Different 2,2-disubstituted 1,3-cycloalkanediones were reduced using *Saccharomyces cerevisiae* giving good yields and sometimes excellent stereoselectivity, with formation, in all the cases, of the stereoisomers having (*S*)-configuration at the carbon bearing the hydroxy group [18, 19]. An example of industrial relevant application of this type of stereoselectivity is offered by the reduction of ethyl secodione, which can be enzymatically reduced to (13*R*,17*S*)-ethyl secol, applied for the synthesis of contraceptive hormones [20, 21].

In this work, we have studied the reduction of various 2,2-disubstituted 1,3-cyclopentanediones and 2,2-disubstituted 1,3-cyclohexanediones using a heterologously expressed ketoreductase (KRED1-Pglu) in a purified formulation [22, 23]. KRED1-Pglu has been previously used as recombinant protein over-expressed in a whole cell system [24, 25], free enzyme [26], and immobilized one onto agarose beads [27] for the stereoselective reduction of several aromatic and cyclic mono- and diketones. Whole cells of *E. coli* DE3 Star were also used as biocatalyst for the reduction of the same substrates, exploiting the ketoreductase(s) expressed in the cells (Scheme 1).

2 Materials and Methods

2.1 Chemicals

All reagents and solvents were purchased from Sigma–Aldrich. Flash column chromatography was performed on Merck Silica Gel (200 and 400 mesh).

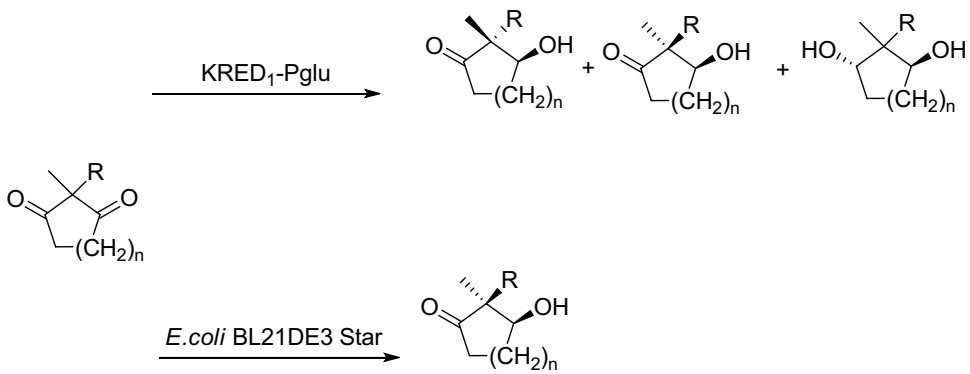
2.2 Analytical Methods

^{1}H NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are expressed in Hz.

Gas-chromatographic (GC) analysis were carried out using a Carlo Erba Fractovap GC (FID detector) equipped with a fused-silica capillary column MEGA-DEX DMP-Beta (dimethyl pentyl-β-cyclodextrin; 25 m × 0.25 mm i.d.), with the injector temperature at 200 °C. Different temperature gradients were applied depending on the biotransformation:

Bioreduction of **3**: at 90 °C for 10 min, then a temperature gradient of 4 °C/min to 180 °C;
bioreduction of **4**: at 80 °C for 10 min, then a temperature gradient of 2 °C/min to 180 °C;
bioreduction of **5**: at 90 °C for 10 min, then a temperature gradient of 3 °C/min to 180 °C;
bioreduction of **6**: at 90 °C for 10 min, then a temperature gradient of 2 °C/min to 180 °C;

Scheme 1 Aim of the work



bioreduction of **7**: at 90 °C for 10 min, then a temperature gradient of 3 °C/min to 180 °C;
bioreduction of **8**: at 90 °C for 10 min, then a temperature gradient of 2 °C/min to 180 °C.

Specific optical rotation measurements were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. Elemental analyses were carried out on a Carlo Erba Model 1106 (Elemental Analyzer for C, H, and N), and the obtained results are within 0.4% of theoretical values.

2.3 Synthesis and Characterization of Substrates

2-Allyl-2-methylcyclopentane-1,3-dione (**3**) [18]: to a solution of compound **1** (673 mg, 6.0 mmol) in 1 M NaOH (6.0 mL) allyl bromide was added (1.45 g, 12.0 mmol). The reaction was stirred for 24 h at room temperature, and monitored by TLC (9:1 DCM/MeOH). The mixture was extracted with dichloromethane (5 × 5 mL) and the organic phase was dried over Na_2SO_4, filtered and evaporated. The crude extract was then purified by column chromatography (9:1 cyclohexane/ethyl acetate) to obtain compound **3** as a pale-yellow oil (yield: 50%, 470 mg, 3.0 mmol). $R_f = 0.55$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.10 (3H, s), 2.35 (2 H, d, $J = 8$ Hz), 2.60-2.80 (4 H, m), 5.00–5.10 (2 H, m), 5.50–5.70 (1 H, m). Anal. Calcd for $C_9H_{12}O_2$: C, 71.03; H, 7.95. Found: C, 71.12; H, 7.83. Chiral GC retention time: 15.3 min.

2-Allyl-2-methylcyclohexane-1,3-dione (**4**) [18]: to a solution of compound **2** (757 mg, 6.0 mmol) in 1 M NaOH (6.0 mL) allyl bromide was added (1.45 g, 12.0 mmol). The reaction was stirred for 24 h at room temperature, and monitored by TLC (9:1 DCM/MeOH). The mixture was extracted with dichloromethane (3 × 5 mL) and the organic phase was dried over Na_2SO_4, filtered and evaporated. The crude extract was then purified by column chromatography (8:2 cyclohexane/EtOAc) to obtain compound **4** as a pale-yellow oil (yield: 50%, 500 mg, 3.0 mmol). $R_f = 0.55$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.25 (3 H, s), 1.80–1.95 (1H, m), 1.95–2.05 (1 H, m), 2.45-2.55 (2 H, d, $J = 8$ Hz), 2.60–2.68 (4H, m), 5.00–5.10 (2H, m), 5.50–5.63 (1H, m). Anal. Calcd for $C_{10}H_{14}O_2$: C, 72.26; H, 8.49. Found: C, 72.34; H, 8.29. Chiral GC retention time: 22.7 min.

2-Methyl-2-propylcyclopentane-1,3-dione (**5**) [18]: a solution of compound **3** (700 mg, 4.6 mmol) was prepared in MeOH (70 mL) and it was submitted to hydrogenation in a ThalesNano H-Cube Mini (T = 30 °C, P = 1 bar, CatCart Pd/C 5%, flow rate: 0.5 mL/min). The reaction was monitored by TLC (7:3 cyclohexane/EtOAc). The exiting flow stream was collected and the solvent was evaporated. Compound **5** was obtained in 95% yield (674 mg, 4.4 mmol). $R_f = 0.55$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.85 (3 H, t, $J = 7$ Hz), 1.12 (3 H, s), 1.15-1.30 (2 H, m), 1.55–1.65 (2H, m), 2.70–2.80 (4H, m). Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found C, 69.95; H, 9.25. Chiral GC retention time: 16.2 min.

2-Methyl-2-propylcyclohexane-1,3-dione (**6**) [18]: a solution of compound **4** (700 mg, 4.2 mmol) was prepared in MeOH (70 mL) and it was submitted to hydrogenation in a ThalesNano H-Cube Mini (T = 30 °C, P = 1 bar, CatCart Pd/C 5%, flow rate: 0.5 mL/min). The reaction was monitored by TLC (7:3 cyclohexane/EtOAc). The exiting flow stream was collected and MeOH was evaporated. Compound **6** was obtained in 95% yield (671 mg, 3.9 mmol). $R_f = 0.55$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (3H, t, $J = 7$ Hz), 1.05–1.25 (2H, m), 1.28 (3H, s), 1.70–1.95 (3H, m), 1.95–2.10 (1H, m) 2.58–2.80 (4H, m). Anal. Calcd for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59. Found C, 71.42; H, 9.48. Chiral GC retention time: 25.8 min.

2-Methyl-2-benzylcyclopentane-1,3-dione (**7**) [28]: to a solution of compound **1** (650 mg, 5.8 mmol) in 1 M NaOH (5.8 mL) benzyl bromide was added (1.98 g, 11.6 mmol). The reaction was stirred for 36 h at room temperature and monitored by TLC (7:3 cyclohexane/EtOAc). The mixture was then extracted with ethyl acetate (3 × 5 mL) and the organic phase was dried over Na_2SO_4 and evaporated. The crude extract was purified by column chromatography (9:1 cyclohexane/EtOAc). Compound **7** was obtained as a white solid (yield: 50%, 587 mg, 2.9 mmol); mp 50 °C; $R_f = 0.58$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.20 (3H, s), 2.00–2.10 (2H, m), 2.50–2.60 (2H, m), 2.95 (2H, s), 7.00–7.05 (2H, m), 7.20–7.30 (3H, m). Anal. Calcd for $C_{13}H_{14}O_2$: C, 77.20; H, 6.98. Found C, 77.42; H, 7.06., Chiral GC retention time: 18.1 min.

2-Methyl-2-benzylcyclohexane-1,3-dione (**8**) [29]: to a solution of compound **2** (650 mg, 5.2 mmol) in 1 M NaOH (5.2 mL) benzyl bromide was added (1.78 g, 10.4 mmol). The reaction was stirred for 36 h at room temperature and monitored by TLC (7:3 cyclohexane/EtOAc). The mixture was then extracted with ethyl acetate (3 × 5 mL) and the organic phase was dried on Na_2SO_4 and evaporated. The crude extract was purified by column chromatography (9:1 cyclohexane/EtOAc). Compound **8** was obtained as a white solid (yield: 50%, 562 mg, 2.6 mmol); mp 43–45 °C; $R_f = 0.58$ (7:3 cyclohexane/ethyl acetate); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.28 (3H, s), 1.42–1.55 (1H, m), 1.67–1.80 (1H, m), 2.25–2.38 (2H, m), 2.50–2.60 (2H, m), 3.12 (2H, s), 7.00–7.08 (2H, m), 7.18–7.30 (3H, m). Anal. Calcd for $C_{14}H_{16}O_2$: C, 77.75; H, 7.46. Found C, 77.53; H, 7.28. Chiral GC retention time: 46.7 min.

2.4 Preparation of Recombinant *Escherichia coli* BL21 (DE3) Star Cells Expressing KRED1-Pglu

The gene sequence of KRED1-Pglu (GenBank: AKP95857) was previously identified from the genome of the non-conventional yeast *Pichia glucozyma* [26] (subsequently reclassified as *Ogataea glucozyma*). Cultures of *E. coli* BL21 (DE3) Star containing plasmid pKJE7 of Takara Chaperone plasmid set (for *dnaK-dnaJ-grpE* expression) transformed with the plasmid pET26 KRED1-Pglu [30] were pre-inoculated in 100 mL Erlenmeyer baffled flasks containing 20 mL of LB medium [yeast extract (5 g L^{-1}), tryptone (10 g L^{-1}) and NaCl (10 g L^{-1})] supplemented with 25 µg/mL kanamycin and 25 µg/mL chloramphenicol. After 16 h of growth (37 °C at 150 rpm) the starting culture was inoculated in the 200 mL of TB medium [yeast extract (24 g L^{-1}), tryptone (12 g L^{-1}), glycerol (4 g L^{-1}), 10% of 100 mM phosphate buffer pH 7.0] supplemented with 25 µg/mL kanamycin, 25 µg/mL chloramphenicol and 0.5 mg/mL L^{-1} arabinose to an initial OD_{600} of 0.1. The new culture was grown at 37 °C, 90 rpm until OD_{600} of 0.5. The induction was performed with isopropyl-β-D-thiogalactopyranoside (IPTG) final concentration of 0.5 mM. After 72 h at 10 °C and 90 rpm cells were harvested by centrifugation (8000 rpm, 4 °C, 45 min), washed once with 20 mM phosphate buffer pH 7.0 and stored at − 20 °C.

2.5 Preparation of Lyophilized Wild Type *E. coli* BL21 DE3 Star cells

Cultures of *E. coli* BL21 (DE3) Star containing plasmid pKJE7 of Takara Chaperone plasmid set (for *dnaK-dnaJ-grpE* expression) were pre-inoculated in 100 mL Erlenmeyer baffled flasks containing 20 mL of LB medium [yeast extract (5 g L^{-1}), tryptone (10 g L^{-1}) and NaCl (10 g L^{-1})] supplemented with 25 µg/mL chloramphenicol. After 16 h of growth (37 °C at 150 rpm) the starting culture was inoculated in the 200 mL of TB medium [yeast extract (24 g L^{-1}), tryptone (12 g L^{-1}), glycerol (4 g L^{-1}), 10% of 100 mM phosphate buffer pH 7.0] supplemented with 25 µg/mL chloramphenicol to an initial OD_{600} of 0.1. After 16 h at 37 °C, 90 rpm, microbial cells were harvested by centrifugation (8000 rpm, 4 °C, 45 min), washed once with 20 mM phosphate buffer pH 7.0, lyophilized and stored at − 20 °C.

2.6 Purification of KRED1-Pglu Enzyme

For the expression of KRED1-Pglu enzyme, the hosting cells of *E. coli* BL21 DE3 Star-pKJE7 were prepared as described above. Cells were suspended in 50 mM phosphate buffer and 300 mM NaCl at pH 8.0, 20 mM imidazole. Proteins were extracted by sonication (10 cycles of 1 min on and 1 min off) and cell debris were harvested by centrifugation at 21000 rpm for 30 min at 4 °C. The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel using an ÄKTÄ system. Briefly, the column was equilibrated with 50 mM phosphate buffer, 300 mM NaCl, pH 8.0, 20 mM imidazole and the crude extract loaded. Subsequently, the column was washed with 50 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0. Finally, the adsorbed enzyme was eluted with 50 mM phosphate buffer, 300 mM NaCl, 250 mM imidazole, pH 8.0. Pellet, crude extract and collected fractions were analyzed by SDS-PAGE. The fractions showing the presence of a band of the expected size (27 kDa) were pooled, dialyzed against 50 mM phosphate buffer pH 8.0, and stored at − 20 °C. After 24 h, frozen samples were lyophilized. From 4 g of whole cells 600 mg of lyophilized enzyme were obtained.

2.7 KRED1-Pglu Activity Test

Activity measurements were performed following a protocol previously reported [26]. The test is based on a spectrophotometric assay at 340 nm by determining the consumption of NAD(P)H at 25 °C in a half-microcuvette (total volume 1 mL) for 5 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 µmol of NAD(P)H per minute under reference conditions, namely with 0.25 mM NAD(P)H and 0.47 mM benzyl as substrate (added as concentrated DMSO solution; final DMSO concentration in cuvette is 0.1% v/v), in 50 mM Tris–HCl buffer, pH 8.0.

2.8 General Procedure for Bioreductions with Lyophilized Cells

A solution of the selected substrate (**3**–**8**) (14 mM) was prepared in 20 mL of phosphate buffer 50 mM pH 8.0 with 1% of $CHCl_3$. In the same solution glucose (40 mM), $NADP^+$ (0.1 mM) and 1 U mL^{-1} of GDH from *Bacillus megaterium* for cofactor regeneration and lyophilized *E. coli* cells BL21 DE3 Star-pKJE7 (5 mg/mL); compound **5** was tested in the same reaction conditions with *E. coli* cells BL21 DE3 Star- pKJE7 expressing heterologous KRED1-Pglu. After 72 h, the reactions were acidified with HCl 2 N, extracted with ethyl acetate (3 × 10 mL) and the organic phase was dried over Na_2SO_4. After removal of the solvent, a chromatographic column was performed to separate the unreacted substrate from the products (8:2 cyclohexane/ethyl acetate).

2.9 General Procedure for Bioreductions with Purified KRED1-Pglu and Product Recovery

A solution of the selected substrate (**3**–**8**) (28 mM) was prepared in 20 mL of buffer TRIS HCl 50 mM pH 8.0 containing 5% of DMSO. In the same solution were added glucose

(40 mM), $NADP^+$ (0.1 mM) and 1 U mL^{-1} of GDH from *Bacillus megaterium*. 20 mU mL^{-1} of lyophilized KRED1-Pglu was used as biocatalyst. The reactions were monitored at different times, acidified with HCl 2 N, extracted with ethyl acetate (3 times) and the organic phases were dried over Na_2SO_4. After removal of the solvent, a chromatographic column was performed to separate the mono-alcohols and the diols (8:2 cyclohexane/ethyl acetate).

2.10 Product Characterization

(*2S,3S*)-2-Allyl-3-hydroxy-2-methylcyclopentanone (**9c**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +84.0$ (c 0.4, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 1.00 (3 H, s), 1.80 (1 H, bs), 1.92–2.02 (1 H, m), 2.16–2.52 (5 H, m), 4.15 (1 H, dd, $J = 4.0$, 3.0), 5.10–5.20 (2 H, m), 5.80–5.95 (1 H, m). Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.40; H, 9.43. Chiral GC retention time: 23.6 min (> 99% e.e.).

(*2S,3S*)-2-Allyl-3-hydroxy-2-methylcyclohexanone (**10c**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +27.5$ (*c* 0.42, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 1.19 (3H, s, CH_3), 1.60–1.80 (1H, m), 1.80–2.10 (4H, m), 2.35–2.45 (4H, m), 3.78–3.82 (1H, m), 5.05–5.18 (2H, m), 5.70–5.82 (1H, m). Anal. Calcd for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59. Found: C, 71.41; H, 9.45. Chiral retention time: 27.3 min (> 99% e.e.).

(*2S,3S*)-3-Hydroxy-2-methyl-2-propylcyclopentanone (**11c**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +61.0$ (c 1.94, $CHCl_3$,); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.94 (3 H, t, $J = 7.2$ Hz, CH,), 1.00 (3 H, s, CH,), 1.25–1.30 (1 H, m), 1.35–1.45 (1 H, m), 1.45-1.50 (2 H, m), 1.61 (1 H, br s, OH), 1.92–1.97 (1 H, m), 2.16-2.32 (2 H, m), 2.42–2.50 (1 H, m), 4.11 (1 H, t, $J = 4.5$ Hz). Anal. Calcd for $C_9H_{16}O_2$: C, 69.19; H, 10.32. Found: C, 68.97; H, 10.59. Chiral GC retention time: 24.0 min (> 99% e.e.).

(*2S,3S*)-3-Hydroxy-2-methyl-2-propylcyclohexanone (**12c**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +65.0$ (*c* 0.40, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.92 (3H, t, $J = 7.5$ Hz), 1.00–1.10 (1H, m), 1.18 (3H, s), 1.20–1.25 (1H, m), 1.50-1.65 (4H, m), 1.65–180 (1H, m), 1.95-2.05 (2H, m), 2.30–2.42 (2H, m), 3.65 (1H, dd, $J = 8.5$, 4 Hz). Anal. Calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 70.67; H, 10.56. Chiral GC retention time: 27.2 min (> 99% e.e.).

(*2S,3S*)-2-Benzyl-3-hydroxy-2-methylcyclopentanone (**13c**) [31]: $R_f = 0.42$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +74.9$ (*c* 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, s), 1.85–1.93 (2H, m); 2.15–2.22 (1H, m), 2.30–2.42 (1H, m), 2.50–2.60 (1H, m), 2.73 (1H, d, $J = 14.0$ Hz), 3.06 (1H, d, $J = 14.0$ Hz), 4.05 (1H, t, $J = 3.0$ Hz), 7.20–7.35 (5H, m). mp 88−90 °C; Anal. Calcd for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.59; H, 8.08. Chiral GC retention time: 34.4 min (> 99% e.e.).

(*2S,3S*)-2-Benzyl-3-hydroxy-2-methylcyclohexanone (**14c**) [32]: $R_f = 0.42$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +20.5$ (*c* 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 1.08 (3H, s), 1.70–1.90 (3H, m), 2.00–2.15 (2H, m), 2.55 (2H, t, $J = 6.9$ Hz), 2.96 (1H, d, $J = 13.7$ Hz), 3.10 (1H, d, $J = 13.7$ Hz), 3.75–3.80 (1H, m), 7.15–7.30 (5H, m). Anal. Calcd for $C_{14}H_{18}O_2$: C, 77.03; H, 8.31. Found: C, 76.80; H, 8.12. Chiral GC retention time: 52.7 min (> 99% e.e.).

(*2R,3S*)-2-Allyl-3-hydroxy-2-methylcyclopentanone (**9a**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = -73.0$ (c 0.26, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 1.02 (3H, s), 1.69 (1H, br s), 1.80-1.92 (1H, m), 2.10–2.30 (4H, m), 2.40–2.52 (1H, m), 4.23 (1H, t, $J = 6.4$ Hz), 5.05–5.15 (2H, m), 5.68–5.72 (1H, m). Anal. Calcd for $C_9H_{14}O_2$: C, 70.10; H, 9.15. Found: C, 70.06; H, 9.40. Chiral GC retention time: 23.0 min (> 99% e.e.).

(*2R,3S*)-2-Allyl-3-hydroxy-2-methylcyclohexan-1-one (**10a**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = -9.4$ (c 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 1.15 (3H, s), 1.60–1.75 (2H, m), 1.80-1.92 (1H, m), 1.95–2.10 (2H, m), 2.30–2.50 (4H, m), 3.85–3.95 (1H, m), 5.02–5.18 (2H, m), 5.70–5.82 (1H, m). Anal. Calcd for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59. Found: C, 71.43; H, 9.48. Chiral GC retention time: 26.5 min (> 99% e.e.).

(*2R,3S*)-3-Hydroxy-2-methyl-2-propylcyclopentanone (**11a**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = -110$ (c 1.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3 H, t, $J = 7.2$ Hz), 1.02 (3 H, s), 1.20–1.40 (4H, m), 1.65 (1 H, br s, OH), 1.85–1.90 (1H, m), 2.16–2.30 (2H, m), 2.41–2.47 (1H, m), 4.22 (1H, t, $J = 6.0$ Hz). Anal. Calcd for $C_9H_{16}O_2$: C, 69.19; H, 10.32. Found: C, 69.39; H, 10.45. Chiral GC retention time: 23.6 min (> 99% e.e.).

(*2R,3S*)-3-Hydroxy-2-methyl-2-propylcyclohexanone (**12a**) [18]: $R_f = 0.40$ (7:3 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = -40.8$ (*c* 0.54, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.85 (3H, t, $J = 7.5$ Hz), 1.00–1.10 (1H, m), 1.12 (3H, s), 1.20–1.35 (1H, m), 1.42–1.55 (1H, m), 1.60–1.70 (2H, m), 1.75–1.85 (2H, m), 2.00–2.20 (2H, m), 2.30–2.45 (3H, m), 3.90 (1H, dd, $J = 5.5$, 2.5 Hz). Anal. Calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 70.48; H, 10.71. Chiral GC retention time: 27.3 min (> 99% e.e.).

2-Allyl-2-methylcyclopentane-1,3-diol (**15**): $R_f = 0.32$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +69.7$ (*c* 2.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (3H, s), 1.42-1.60 (2H, m), 2.09–2.22 (2H, m), 2.22–2.35 (2H, m), 3.90 (1H, dd, $J = 4.3$ Hz), 4.11 (1H, t, $J = 6.4$ Hz), 5.05–5.20 (2H, m), 5.90–6.05 (1H, m).[6] Anal. Calcd for $C_9H_{16}O_2$. C, 69.19; H, 10.32. Found: C, 69.00; H, 10.09. Chiral GC retention time: 26.9 min (> 99% e.e.).

2-Allyl-2-methylcyclohexane-1,3-diol (**16**): $R_f = 0.32$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +42.0$ (*c* 2.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.94 (3H, s), 1.44–1.80 (6H, m), 2.32 (2H, d, $J = 4.8$), 3.70–3.75 (1H, m), 3.78–3.83 (1H,

m), 5.08–5.20 (2H, m), 5.90–6.05 (1H, m).[7] Anal. Calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 70.68; H, 10.80. Chiral GC retention time: 29.3 min (> 99% e.e.).

2-Methyl-2-propylcyclopentane-1,3-diol (**17**): $R_f = 0.32$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +44.6$ (*c* 2.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.92 (3H, s), 0.96 (3H, t, *J* = 7.2), 1.22–1.60 (7H, m), 2.05–2.22 (2H, m), 3.90 (1H, t, *J* = 4.5 Hz), 4.03 (1H, t, *J* = 6.0). Anal. Calcd for $C_9H_{18}O_2$: C, 68.31; H, 11.47. Found: C, 68.45; H, 11.58. Chiral GC retention time: 27.1 min (> 99% e.e.).

2-Methyl-2-propylcyclohexane-1,3-diol (**18**): $R_f = 0.32$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +40.6$ (*c* 0.95, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.92 (3H, s), 0.95 (3H, t, *J* = 7.5 Hz), 1.20–1.35 (2H, m), 1.35–1.45 (2H, m), 1.35–1.76 (6H, m), 3.71 (1H, dd, *J* = 8.5, 4.0 Hz), 3.77 (1H, dd, *J* = 5.5, 2.5 Hz). Anal. Calcd for $C_{10}H_{20}O_2$: C, 69.72; H, 11.70. Found: C, 69.90; H, 11.79. Chiral GC retention time: 29.7 min (> 99% e.e.).

2-Benzyl-2-methylcyclopentane-1,3-diol (**19**): $R_f = 0.37$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +91.0$ (*c* 1.5, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, s), 1.45–1.62 (3H, m); 2.10–2.36 (2H, m), 2.67 (1H, d, *J* = 14.0), 2.91 (1H, d, *J* = 14.0), 3.89 (1H, dd, *J* = 1.5 Hz), 4.26 (1H, t, *J* = 3.0 Hz), 7.20–7.35 (5H, m). Anal. Calcd for $C_{13}H_{18}O_2$: C, 75.69; H, 8.80. Found: C, 75.88; H, 8.99.

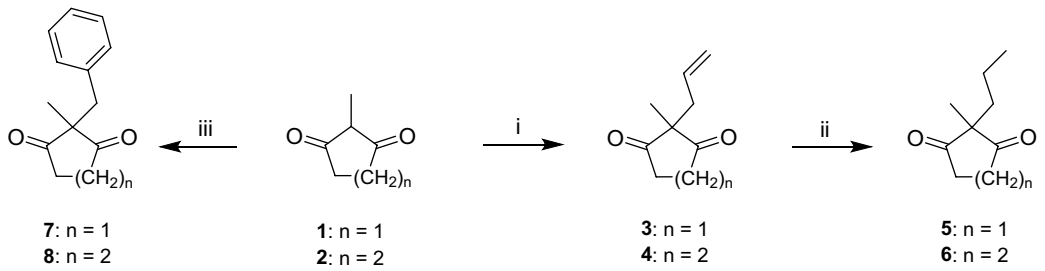
2-Benzyl-2-methylcyclohexane-1,3-diol (**20**): $R_f = 0.37$ (1:1 cyclohexane/ethyl acetate); $[\alpha]_D^{20} = +36.0$ (*c* 3.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 0.86 (3H, s), 1.46–1.91 (6H, m), 2.84 (1H, d, *J* = 13.7 Hz), 2.99 (1H, d, *J* = 13.7 Hz), 3.58–3.62 (1H, m), 3.88 (1H, dd, *J* = 3.0 Hz), 7.18–7.32 (5H, m). Anal. Calcd for $C_{14}H_{20}O_2$: C, 76.33; H, 9.15. Found: C, 76.56; H, 9.00.

3 Results and Discussion

3.1 Synthesis of 2,2-Disubstituted 1,3-Cyclopentanediones and Cyclohexanediones

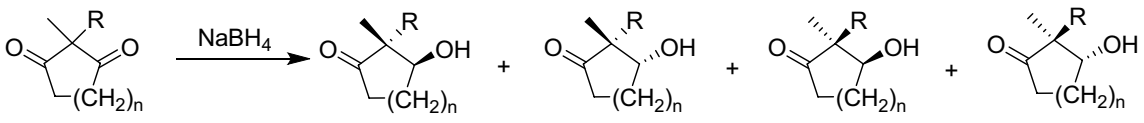
Substrates (**3**–**8**) were synthesized, as previously reported, starting from commercially available 2-methyl-1,3-cyclopentanedione (**1**) and 2-methyl-1,3-cyclohexanedione (**2**) (Scheme 2) [18, 28, 29].

Compounds **3**–**8** were monoreduced with $NaBH_4$ to obtain the four possible stereoisomers of the chiral ketols as



i: Allyl bromide (2eq.), 1N NaOH (1eq.), 50% yield; ii: MeOH, H-Cube Mini, CatCart Pd/C 5% T = 30 °C, P = 1 bar, flow rate: 0.5 mL/min, 95% yield ; iii: Benzyl bromide (2 eq.), 1N NaOH (1 eq.), 50% yield

Scheme 2 Preparation of 2,2-disubstituted 1,3-cycloalkanediones **3**-**8**



3, 9: n = 1; R = $-CH_2-CH=CH_2$
4, 10: n = 2; R = $-CH_2-CH=CH_2$
5, 11: n = 1; R = $-CH_2-CH_2-CH_3$
6, 12: n = 2; R = $-CH_2-CH_2-CH_3$
7, 13: n = 1; R = $-CH_2-Ph$
8, 14: n = 2; R = $-CH_2-Ph$

Scheme 3 Products obtained by monoreduction of **3**-**8**

analytical standards (Scheme 3). Absolute configuration of the four stereoisomers of **9**–**14** was assigned on the basis of chiral GC and ^{1}H NMR spectra in comparison with literature data [18, 31, 32].

3.2 Reduction with Isolated KRED1-Pglu

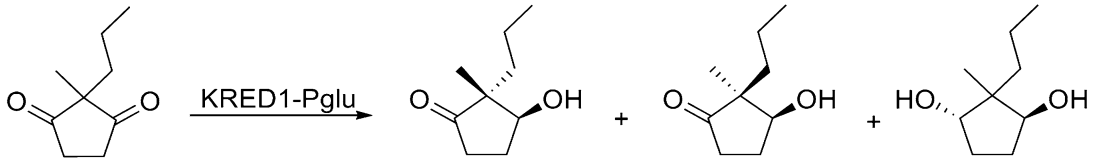
2-Methyl-2-propylcyclopentane-1,3-dione (**5**) was initially chosen as current substrate for the enzymatic reduction catalyzed by the purified ketoreductase (KRED1-Pglu) from *Ogataea glucozyma*. KRED1-Pglu was used as free enzyme in the presence of a catalytic amount of $NADP^+$ and a system for the regeneration of the cofactor, composed of glucose and glucose dehydrogenase (GDH) from *Bacillus megaterium*. The biotransformation was firstly carried out using different substrate concentrations (Table 1).

With higher substrate concentrations (> 28 mM, entries 7 and 8), monoreduction was the only transformation observed during a 24 h reaction time, with complete enantioselectivity of the carbonyl reduction (formation of *S*-stereocenter at position 3) and low diastereoselectivity, hence furnishing **11a/11c** with a 42/58 ratio. Notably, under these conditions no full conversion could be achieved and no traces of diol were observed, indicating a possible inhibitory effect on KRED1-Pglu exerted by an excess of substrate. When the reaction was carried out in conditions of lower substrate concentrations, carbonyl reduction occurred also on **11c**, affording (1*S*,3*S*)-2-methyl-2-propylcyclopentane-1,3-diol (**17**), while **11a** remained unreacted in the reaction mixture and could be recovered as pure stereoisomer at the end of the reaction (entries 3 and 6, Table 1). Absolute configuration and optical purity of diol **17** were assigned based on the fact that the recovered diol is chiral and that the (1*S*,3*S*)-configuration is formed independently of which of the two ketols (**11a** and **11c**) is further reduced to diol. In other words, KRED1-Pglu, when used at low substrate/enzyme ratio, was able to perform the second reduction with high enantioselectivity, but also with high stereo-recognition of the stereogenic center in position 2, thus carrying out the resolution of the diastereoisomeric mixture composed of **11a/11c**. It should be emphasized that this behavior was only observed when the initial substrate concentration was up to 28 mM, and most likely no substrate inhibition was significantly relevant on the enzymatic activity.

The results obtained using compound **5** as substrate led us to perform the biotransformations of the other 2,2-disubstituted 1,3-cycloalkanediones using an initial substrate concentration of 28 mM (Table 2).

All the tested substrates showed similar behavior in terms of general reactivity (Scheme 4): firstly, monoreduction occurred with high enantioselectivity and low diastereoselectivity, giving a (2*S*,3*S*)/(2*R*,3*S*) stereoisomeric mixture of

Table 1 Reduction of 2-methyl-2-propylcyclopentane-1,3-dione **5** with KRED1-Pglu using different initial substrate/enzyme ratios. Stereoisomers **11b** and **11d** were not detected



5 (2*R*,3*S*)-**11a** (2*S*,3*S*)-**11c** (1*S*,3*S*)-**17**

Entry	Substrate **5** (μM)	Time (min)	Remaining substrate[a]	(2*R*,3*S*)-**11a**[a,b]	(2*S*,3*S*)-**11c**[a,b]	(1*S*,3*S*)-**17**[a]
1	14	5	15	35	30	20
2	14	60	0	42	38	20
3	14	240	0	42	0	58
4	28	5	36	27	32	5
5	28	60	0	42	40	18
6	28	480	0	40	0	60
7	56	30	40	25	35	0
8	56	1440	36	26	38	0

Reaction conditions: 14–56 mM substrate, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0)

[a]As determined by chiral GC

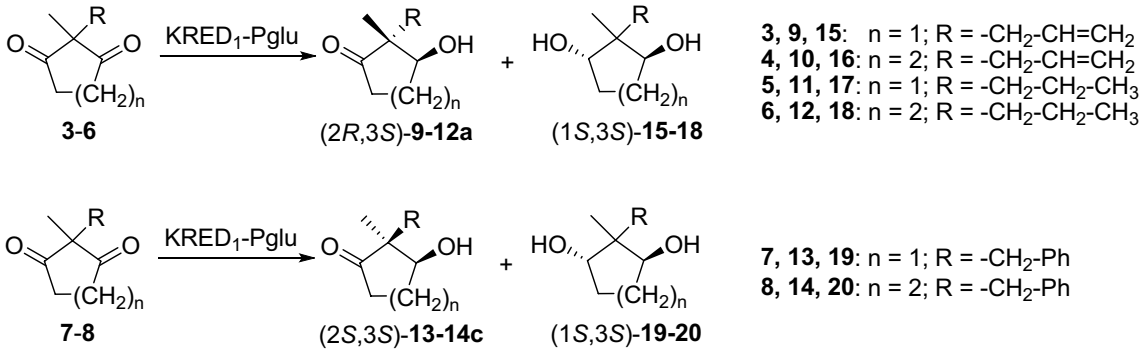
[b]Absolute configurations were assigned by comparison with ^{1}H NMR of the Mosher's derivatives.[18] Results are the mean of three independent assays performed in triplicate

Table 2 Reduction of 2,2-disubstituted 1,3-cycloalkanediones **3**–**8** using KRED1-Pglu

Entry	Substrate	Conv. (%)[a]	(2*R*, 3*S*)-ketol[a]	(2*S*, 3*S*)-ketol[a]	(1*S*, 3*S*)-diol	Time (h)
1	**3**	> 99	50 (**9a**)	0 (**9c**)	50 (**15**)	24
2	**4**	> 99	52 (**10a**)	0 (**10c**)	48 (**16**)	36
3	**6**	> 99	42 (**12a**)	0 (**12c**)	58 (**18**)	4
4	**7**	> 99	0 (**13a**)	40 (**13c**)	60 (**19**)	1
5	**8**	> 99	0 (**14a**)	60 (**14c**)	40 (**20**)	24

Reaction conditions: 28 mM substrate, 0.1 mM $NADP^+$, KRED1-Pglu (20 mU/mL), GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0)

[a]As determined by chiral GC. Results are the mean of three independent assays performed in triplicate



Scheme 4 Reduction of 2,2-disubstituted-1,3-cyclopentanediones and 1,3-cyclohexanediones with KRED1-Pglu

the corresponding β-hydroxyketones; the second reduction furnished the diol, leaving one stereoisomer unreacted.

Although similar reactivity was observed, reduction of 2,2-disubstituted cyclohexanediones was generally faster than cyclopentadiones. Reduction of 2-propyl and 2-allyl derivatives took place with the same stereobias in all the cases, allowing for the recovery of the (2*R*,3*S*)-stereoisomer of the ketol. On the other hand, the presence of the bulkier benzyl substituent in position 2 (Table 2, entries 4 and 5) had a striking effect on the stereorecognition of the second reduction, with preference for the consumption of the (2*R*,3*S*)-stereoisomers and thus final accumulation of (2*S*, 3*S*)-**13c** and (2*S*, 3*S*)-**14c**.

Notably, in all the cases, at the end of the reaction, the recovered diol was obtained as single, optically active stereoisomer, as determined by optical rotation and ^{1}H NMR. It should be noted that during the second reduction, the stereogenic center at C2 is lost and all the diols produced had (1*S*,3*S*)-configuration.

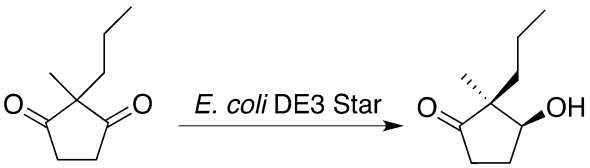
3.3 Reduction with Whole Cells of *Escherichia coli* DE3 Star

With the aim of preparing an easier to use biocatalyst, containing both recombinant ketoreductase and cofactors, lyophilized whole cells of *E. coli* DE3 Star expressing heterologous KRED1-Pglu were evaluated for the reduction of compound **5** [30]; indeed, the use of whole cells guarantees the supply of cofactors and systems for their recycling. Surprisingly, enantioselectivity was generally different from what observed with the purified KRED1-Pglu and strongly variable depending on the reaction conditions. These observations led us to the hypothesis that whole cells of *E. coli* DE3 Star contained endogenous ketoreductase(s) with activity towards **5**, but different enantioselectivity than what displayed by isolated KRED1-Pglu. Accordingly, we utilized wild-type whole cells of *E. coli* DE3 Star that did not express the recombinant ketoreductase for evaluating their intrinsic activity.

Lyophilized cells *E. coli* DE3 Star (5 mg/mL) lacking the plasmid coding for KRED1-Pglu (hereinafter simply called *E. coli* DE3 Star) were therefore employed for the reduction of **5**, showing the formation of **11c** as the only product of the reaction, although with low yields (24% after 72 h) and longer reaction time (Table 3, entry 1). The use of higher amounts of biocatalysts did not give a significant improve of the yields (28% conversion after 72 h using 25 mg/mL of lyophilized cells).

A screening of different organic co-solvents was performed. One reason was to potentially increase cell permeability and thus to enhance the yield of the biotransformation (Table 3) [33]. Further reasons were to determine the effect of solvent addition to both, conversion and selectivity, as it was seen in first quick and preliminary trials

Table 3 Reduction of 2-methyl-2-propylcyclopentane-1,3-dione **5** using lyophilized cells of *E. coli* DE3 Star



Entry	Co-solvent (% vol/vol)	Remaining substrate **5**[a] (%)	**11c**[a] (%)
1	No co-solvent	76	24
2	DMSO (5%)	72	28
3	DMSO (10%)	88	12
4	CH_2Cl_2 (1%)	84	16
5	CH_2Cl_2 (2%)	98	< 5
6	Acetone (2%)	80	20
7	Acetone (5%)	85	15
8	$CHCl_3$ (1%)	50	50
9	$CHCl_3$ (2%)	95	5
10	MTBE (2%)	48	42
11	MTBE (5%)	75	25
12	MTBE (10%)	97	< 5
13	MIBK[b] (2%)	75	25
14	MIBK[b] (5%)	80	20

Reaction conditions: 14 mM substrate, 5 mg of lyophilized *E. coli* DE3 Star, 0.1 mM $NADP^+$, GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0) in the presence of different amounts of co-solvent

[a]As determined by chiral GC. Results are the mean of three independent assays performed in triplicate

[b]Methyl Isobutyl Ketone

that reaction conditions strongly influenced the catalyst performance.

The addition of different co-solvents led to variable molar conversion (< 5–50%) of **11c** while no effect on the stereoselectivity was observed. The best results were obtained with 1% $CHCl_3$ (entry 8, Table 3); under this condition, the mono-alcohol **11c** was obtained as unique product with 50% of molar conversion, whereas higher concentration of $CHCl_3$ inhibited the enzymatic activity.

As proof of concept, we checked the reactivity of the other 2,2-disubstituted 1,3-cycloalkanediones with lyophilized cells of *E. coli* DE3 Star in the presence of $CHCl_3$ (1% vol/vol) (Table 4).

All substrates were converted into the corresponding (2*S*,3*S*)-β-hydroxyketones as the only product (Table 4), although with different yields. The ketoreductase(s) in the cells of *E. coli* DE3 Star is therefore able to catalyze the mono reduction of 2,2-disubstituted 1,3-cycloalkanediones with high enantioselectivity and diastereoselectivity.

Table 4 Reduction of 2,2-disubstituted 1,3-cycloalkanediones using whole cells of *E. coli* DE3 Star

Entry	Substrate	Conversion (%)[a]	Isolated (2*S*,3*S*)-ketol
1	**3**	41	35
2	**4**	34	30
3	**5**	70	66
4	**6**	30	25
5	**7**	74	70
6	**8**	16	12

Reaction conditions: 14 mM substrate, 5 mg of lyophilized *E. coli* DE3 Star, 0.1 mM $NADP^+$, GDH (1 U/mL), glucose 40 mM in Tris/HCl buffer pH 8.0 (0.05 M, pH 8.0) in the presence of different amounts of co-solvent

[a]As determined by GC. Results are the mean of three independent assays performed in triplicate

Notice that, in general, cyclopentanediones produced the corresponding ketol in higher yields compared to cyclohexanediones.

4 Concluding Remarks

In conclusion, we have reported on the stereoselective reduction of prochiral cyclic 1,3-diketones using different biocatalytic systems. Under selected conditions, whole cells of *E. coli* DE3 Star furnished only one stereoisomer, in most of the cases different than the one recovered at the end of the reaction with purified KRED1-Pglu. KRED1-Pglu, employed under conditions of low substrate/enzyme ratio, was also able to give (1*S*, 3*S*)-diols with excellent enantioselectivity (> 99% e.e). It is important to note that the strain *E. coli* DE3 Star, commonly used to produce recombinant proteins, showed relevant ketoreductase activity towards these particular substrates.

Purified KRED1-Pglu and whole cells of *E. coli* DE3 Star can be used as alternative biocatalysts to Baker's yeast [18] for the reduction of prochiral 2,2-disubstituted-1,3-cyclopentadiones and 1,3-cyclohexanediones, since different products and different stereoselectivity were obtained.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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Affiliations

Martina Letizia Contente[1,3] · Federica Dall'Oglio[2] · Francesca Annunziata[2] · Francesco Molinari[3] · Marco Rabuffetti[3] · Diego Romano[3] · Lucia Tamborini[2] · Dörte Rother[4] · Andrea Pinto[3]

✉ Andrea Pinto
andrea.pinto@unimi.it

[1] School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK

[2] Department of Pharmaceutical Sciences (DISFARM), University of Milan, via Mangiagalli 25, 20133 Milan, Italy

[3] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, via Mangiagalli 25, 20133 Milan, Italy

[4] Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany