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RESEARCH ARTICLE

A study on the enzyme catalysed enantioselective hydrolysis of methyl 2-methyl-4-oxopentanoate, a precursor of chiral γ-butyrolactones

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ABSTRACT

Porcine pancreas lipase (PPL) resolution of the α-methyl group of racemic methyl 2-methyl-4-oxopentanoate, a valuable synthetic precursor of fragrances and marine natural products, was enhanced by salt modulation of the enzymatic hydrolysis. For the enantioselective hydrolysis of the title ester, PPL was selected from a series of esterases and lipases, and its enantioselectivity was evaluated by changing the reaction medium parameters. The use of 1.6 mol L^{-1} sodium sulfate in phosphate buffer (pH 7.2) improved the enantioselectivity allowing the formation of methyl (2*R*)-(+)-2-methyl-4-oxopentanoate and (2*S*)-(–)-2-methyl-4-oxopentanoic acid with an enantiomeric excess of >99% and 71%, respectively. The study showed that a modulation of PPL enantioselectivity could be achieved by using kosmotropic salts in the reaction media. The present method consists of a practical and low-cost option to improve enzymatic kinetic resolution reactions.

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KEYWORDS
Enzymatic kinetic resolution; Hofmeister effect; salting-out; medium engineering; interfacial enzymes

Introduction

Enzymes have an important catalytic role in contemporary organic synthesis and allow access to a plethora of enantiomerically enriched compounds (Zheng and Xu 2011; Clouthier and Pelletier 2012; Reetz 2013; Nestl et al. 2014). In spite of this, biocatalysts are useful tools for synthesis even for industrial processes where problems related to scale-up of the reactions can be circumvented (Wells et al. 2012). The utility of biocatalysts for synthetic purposes resides in their ability to display promiscuity when subjected to react with different substrates of their natural substrate in some cases (Hult and Berglund 2007). Such reactions have been optimized for efficiency improvement. This goal is reached by managing the reaction media conditions or by modification of the biocatalyst itself by molecular biology techniques in the directed evolution of enzymes (Bornscheuer et al. 2012). The former strategy is straightforward because the optimization involves the variation of reaction conditions such as substrate concentration, pH, temperature and the use of co-solvents (Faber 2011).

A medium variable that modulate enzyme activity is the use of salt additives. Salt or ion effects are ubiquitous in biological and chemical sciences as recognized by Franz Hofmeister at the end of 19th century (Kunz, Henle, et al. 2004). Despite being a subject of continuous debate (Kunz, Lo Nostro, et al. 2004; Lo Nostro and Ninham 2012; Salis and Ninham 2014; Jungwirth and Cremer 2014), the enzyme activity modulation by salts as result of the direct interaction among ions and the protein surface has been shown for *Aspergillus niger* (Pinna et al. 2005) and *Candida rugosa* lipase (Salis et al. 2007), alkaline phosphatase (Yang et al. 2010), human kallikrein-related peptidase 3 (KLK3 or PSA) (Andrade et al. 2010; Huang et al. 2001) and HIV protease (Heyda et al. 2009) in aqueous media.

Hofmeister effects are related to the interactions of kosmotropic and chaotropic ions with enzymes or solvent that changes their structure, stability and catalytic properties (Baldwin 1996; Zhang and Cremer 2006; Peng et al. 2016; Endo et al. 2016, 2018). The intent of this work consists in the optimization of the enantioselective hydrolysis of methyl 2-methyl-4-oxopentanoate (methyl 2-methyllevulinate), a known precursor of chiral 2,4-disubstituted γ-butyrolactones of synthetic utility as building blocks for the synthesis of

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Supplemental data for this article can be accessed here.

jasplakinolide (Xu et al. 2013), geodiamolides A and B (Hirai et al. 1990), milbemycin β_3 (Barrett et al. 1986) and amphinolide B1 (Fürstner et al. 2009). The access to chiral 2-methyl levulinate derivatives was demonstrated by a stereoselective hydrogenation transfer from a Hantzsch ester catalyzed by a chiral binaphtol phosphate (Martin and List 2006) or by the stereospecific ene reductase activity of Old Yellow Enzyme (Korpak and Pietruszka 2011) on the same substrate, 2-methyl-4-oxopent-2-enoate (Figure 1).

In such scenario, a biocatalytic access of both enantiomers of the title γ-ketoester would be valuable for synthetic purposes.

Here, we report the optimization of the enantioselective kinetic resolution of lipases and esterases from different sources on methyl 2-methyl-4-oxopentanoate hydrolysis by screening medium conditions. Kosmotropic salt effects improved the biocatalyst performance and afforded (2*R*)-(+)-methyl 2-methyl-4-oxopentanoate and (2*S*)-(–)-methyl 2-methyl-4-oxopentanoic acid.

Materials and methods

General

All commercially available chemicals were purchased from Sigma-Aldrich (USA) and used without further purification. Solvents were purchased from Synth (Brazil) and used without further purification. CAL-B (*Candida antactica* lipase ≥10,000 U g^{-1}), *Pseudomonas cepacia* lipase, porcine pancreas lipase 100–400 unit mg^{-1}, Amano Lipase sp. > 500 U g^{-1}, *Candida rugosa* lipase >100 U g^{-1} and *Rhizopus niveus* lipase 3.6 U g^{-1} were purchased from Sigma-Aldrich Co. The esterase from *Sacharomyces cerevisiae* 2.2 U mg^{-1}, esterase from horse liver 0.52 U mg^{-1}, esterase from *Candida lipolytica* 0.10 U mg^{-1}, esterase from *Rhyzopus oryzae* 151 U g^{-1} and esterase from *Mucor miehei* 1.1 U mg^{-1} were purchased from Fluka Analytical.

Analytical thin-layer chromatography (TLC) was performed with aluminum-backed silica plates coated with a 0.25 mm thickness of silica gel 60 F_{254} (Merck), exposure to vanillin solution and heating. Standard chromatographic purification methods were followed using 35–70 mm (240–400 mesh) silica gel purchased from Sigma-Aldrich.

Nuclear magnetic resonance spectra were recorded on a Bruker Ultrashield 300 spectrometer at 300 MHz for ^{1}H- and 75 MHz for ^{13}C-NMR. The ^{1}H-NMR chemical shifts (δ) are reported in ppm relative to the TMS peak and ^{13}C-NMR chemical shifts (δ) are reported in ppm relative to $CDCl_3$. The coupling constants (*J*) are given in hertz (Hz). Optical rotations were determined on a JASCO DIP-378 polarimeter (Sodium D line at 589 nm). The enzymatic kinetic resolutions were conducted at Thermomixer Comfort from Eppendorf except during scale-up reactions that were conducted in an orbital shaker (Solab). Reactions under microwave irradiation were performed in a CEM Discover at 50 W and a maximum temperature of 75 °C.

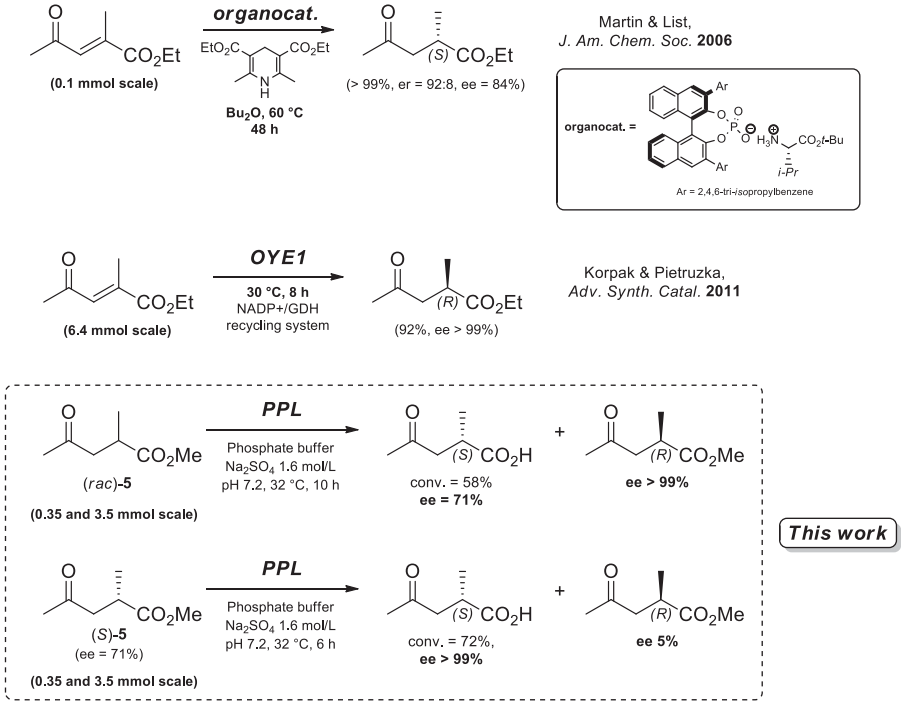

Figure 1. Approaches for the preparation of chiral 2-methyl-4-oxopentanoic acid derivatives.

The ratio of nitro esters was determined in a Varian type 450-GC flame ionization detector (FID) equipped with a Varian CP-Sil 5 CB capillary column (30 m × 0.25 mm ×0.25 um) using nitrogen as the carrier gas. The conditions used for separation are: injector 270 °C, detector 270 °C, hydrogen 300 mL min^{-1}, air 30 mL min^{-1}. The column flow was 1.05 mL min^{-1} with a maximum temperature of 250 °C. The temperature program was 50 °C for 5 min increasing 10.0 °C min^{-1} to 250 °C.

The enantiomeric excess of compounds was determined in a Shimadzu type GC-2010 flame ionization detector (FID) chromatograph fitted with a Chirasil-DEX CB β-cyclodextrin (25 m × 0.25 mm, 25 μm) fused silica capillary column using nitrogen as the carrier gas. The conditions used for the chiral separation are: injector 250 °C, detector 250 °C, split 29.9, hydrogen 400 mL min^{-1} and air 30 mL min^{-1}. The column flow was 1.05 mL min^{-1} at a maximum temperature of 170 °C. The temperature program was 50 °C for 14 min increasing to 0.5 °C min^{-1} to 67 °C and then 10 °C min^{-1} to 160 °C.

Purification of racemic keto ester was performed in a Kugelrohr Büchi B-580. The enantiomeric excess of keto ester and keto acid were calculated according to equation: $((E1 - E2)/(E1 + E2)) \times 100$, where $E1$ and $E2$ are the ratio of enantiomers of each compound (**5** and **6**) as determined by chiral GC analyses. The conversions were calculated according to equation: Conv. $= ee_e/(ee_a + ee_e) \times 100$. The enantiomeric ratios (E) were calculated according to equation: $E = \{\ln[eeP(1 - eeS)]/(eeP + eeS)\}/\{\ln[eeP(1 + eeS)]/(eeP + eeS)\}$(Faber 2011).

Screening of enzymes for kinetic resolution of racemic methyl 2-methyl-4-oxopentanoate (5)

The compound **5** was synthesized according to a previously reported procedure with some modifications (see supporting information).

Racemic methyl 2-methyl-4-oxopentanoate (**5**) (50 mg) and 25 mg of selected enzyme were added to 5 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution (pH 7.2) in a 15 mL Falcon tube. These mixtures were submitted to orbital stirring (500 rpm) at 25 °C for 2 h. After this period, each reaction mixture was filtered through a cotton plug, and the pH was adjusted to 1.0 and extracted with AcOEt (3 × 2 mL). The organic phases were combined, dried over $MgSO_4$, filtered and aliquots were analyzed by a chiral gas chromatography to determine the enantiomeric excess of products and unreacted starting materials (see supporting information S21).

Effect of addition of salts to reaction medium and temperature

Racemic methyl 2-methyl-4-oxopentanoate (**5**) (50 mg) and porcine pancreas lipase (PPL) (25 mg) were added to 5 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution (pH 7.2) in a 15 mL Falcon tube containing different salts as depicted in Table 2. These mixtures were submitted to orbital stirring (500 rpm) at 25 °C for 2 and 4 h. After this period, each reaction mixture was filtered through a cotton plug, and the pH was adjusted to 1.0 and extracted with AcOEt (3 × 2 mL). The organic phases were combined, dried over $MgSO_4$, filtered and aliquots were analyzed by a chiral gas chromatography to determine the enantiomeric excess of products and unreacted starting materials (Table 1).

The procedure described above was also applied in different temperatures (32 °C and 40 °C) (Table 2).

Enzymatic kinetic resolution of racemic methyl 2-methyl-4-oxopentanoate (5) by PPL

Racemic methyl 2-methyl-4-oxopentanoate (**5**) (50 mg) and PPL (25 mg) were added to 5 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution containing 1.6 mol L^{-1} of sodium sulfate at pH 7.2 in a 15 mL Falcon tube. This mixture was submitted to orbital stirring (500 rpm) at 32 °C for 10 h. Next, the solution was filtered in a cotton plug, and the pH was adjusted to 10–11 using a 2 mol L^{-1} solution of NaOH and extracted with AcOEt (4 × 2 mL). The organic phases were combined, dried over $MgSO_4$ and the solvent removed under reduced pressure to afford methyl (2*R*)-(+)-2-methyl-4-oxopentanoate (*R*)-**5** in >99% ee as a colorless oil $[\alpha]_D^{25} = +12.5$ (c 0.8, $CHCl_3$). The carboxylic acid (*S*)-**6** was recovered from the aqueous solution by saturating it with NaCl, and the pH was adjusted to 1.0 using 5 mol L^{-1} HCl solution and washing with AcOEt (5 × 2 mL). The organic phases were combined, dried over $MgSO_4$ and solvent removed under reduced pressure to afford the (2*S*)-(–)-2-methyl-4-oxopentanoic acid with 71% ee (71%).

This reaction was also performed using 500 mg of (*rac*)-**5** and PPL (250 mg) in 50 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution containing 1.6 mol L^{-1} of sodium sulfate at pH 7.2 at 32 °C. In this condition, the reaction was performed in Erlenmeyer flask using orbital stirring at 250 rpm affording methyl (2*R*)-(+)-2-methyl 4-oxopentanoate (*R*)-**5** 0.165 g (33% yield, >99% ee) and (2*S*)-(–)-2-methyl-4-oxopentanoic acid (*S*)-**6** 0.271 g (60% yield, 71% ee), CG t_R [(*R*)-**5**] = 44.21 min, [(*S*)-**5**] = 44.92 min, [(*R*)-**6**] = 44.21 min and [(*S*)-**6**] = 44.92 min, Chirasil-DEX CB β-cyclodextrin (25 m × 0.25 mm, 25

Table 1. Effect of salts in PPL-catalyzed hydrolysis of (*rac*)-**5**.

		2 hours				4 hours			
Salt additive	Medium Ionic Strength (mol L^{-1})	ee_e (%)	ee_a (%)	conv. (%)	*E*	ee_e (%)	ee_a (%)	conv. (%)	*E*
–	0.246	9.0	59.5	10.0	4.3	13.8	62.3	14.8	4.9
NaCl (1.6 mol L^{-1})	1.835	9.4	59.7	10.4	4.3	13.7	67.1	13.7	5.8
Na_2SO_4 (0.6 mol L^{-1})	2.030	13.2	68.7	14.2	6.1	24.4	71.7	25.3	7.7
Na_2SO_4 (1.1 mol L^{-1})	3.500	12.5	67.7	13.5	5.9	21.2	74.1	22.2	8.3
Na_2SO_4 (1.6 mol L^{-1})	4.950	21.5	80.4	22.5	11.3	46.5	84.4	47.5	18.7
KF (1.6 mol L^{-1})	1.835	1.8	3.1	2.8	1.1	0.8	5.6	1.8	1.1
K_2SO_4 (0.6 mol L^{-1})	1.998	19.4	90.4	17.7	24.0	20.7	87.8	19.1	18.8
K_2SO_4 (1.0 mol L^{-1})	NS	–	–	–	–	–	–	–	–
Sodium citrate (0.6 mol L^{-1})	3.554	26.4	87.3	23.2	19.1	35.6	86.0	29.3	18.8
Sodium citrate (1.0 mol L^{-1})	5.778	23.3	92.1	20.2	30.3	50.2	92.6	35.2	43.2
Sodium citrate (1.6 mol L^{-1})	NS	–	–	–	–	–	–	–	–

Conditions: Phosphate buffer 0.1 M (5.0 mL/pH 7.2), temperature = 25 °C, substrate = 50 mg, enzyme = 25 mg. ee_a = enantiomeric excess of carboxylic acid. ee_e = enantiomeric excess of keto ester. NS = Not soluble in phosphate buffer 0.1 M.

Table 2. Temperature effect on PPL-catalyzed hydrolysis of (*rac*)-**5**.

	2 hours				4 hours			
Temp. (°C)	ee_e (%)	ee_a (%)	Conv. (%)	*E*	ee_e (%)	ee_a (%)	Conv. (%)	*E*
25	21.5	80.4	22.5	11.3	46.5	84.4	47.5	18.7
32	32.8	81.5	28.7	13.4	64.9	83.1	43.8	21.1
40	43.5	79.3	35.4	13.3	64.5	80.4	44.5	17.8

Conditions: Phosphate buffer 0.1 M (5.0 mL/pH 7.2), temperature = 25 °C, substrate = 50 mg, enzyme = 25 mg. ee_a = enantiomeric excess of carboxylic acid. ee_e = enantiomeric excess of keto ester.

μm), temperature gradient: 50 °C (14 min iso), 0.5 °C min^{-1} to 67 °C, 10 °C min^{-1} to 160 °C.

Recycling of (2S)-(–)-2-methyl-4-oxopentanoic acid (S)-6 (71% ee)

To a 100 mL one neck round-bottomed flask a DMF (55 mL) solution of enantioenriched (2*S*)-(–)-2-methyl-4-oxopentanoic acid (71% ee) (2.60 g, 20 mmol) was cooled to 0 °C. Anhydrous potassium carbonate (5.53 g, 40 mmol) was slowly added to this solution and allowed to react for 15 min before addition of methyl iodide (60 mmol, 3.12 mL) in one portion. The reaction mixture was stirred at 0 °C for 30 min and then for another 1.5 h at room temperature. Next, water was added until complete dissolution of K_2CO_3. This solution was extracted with ethyl acetate as needed to extract all γ-keto esters. (The extraction was monitored by TLC using 50% ethyl acetate as the eluent). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford methyl (2*S*)-(–)-2-methyl-4-oxopentanoate (71% ee) as colorless oil in 85% yield (2.45g). The enantiomeric excess was unchanged as determined by chiral gas chromatography.

Enzymatic kinetic resolution of methyl (2S)-(–)-2-methyl-4-oxopentanoate (S)-5 (71% ee) by PPL

The enantioenriched (2*S*)-(–)-methyl 2-methyl-4-oxopentanoate (*S*)-**5** (50 mg) and 25 mg of PPL were added to 5 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution of phosphate containing 1.6 mol L^{-1} Na_2SO_4 at pH 7.2 and 32 °C. This mixture was submitted to orbital stirring (500 rpm) at 32 °C for 9 h.

Next, the mixture was filtered with a cotton plug, and the pH adjusted to 11 using a 2.0 mol L^{-1} solution of NaOH and extracted with AcOEt (4 × 2 mL) to remove the unreacted ester. The carboxylic acid was then recovered from the aqueous solution by saturating it with NaCl, and the pH was adjusted to 1–2 using 5 mol L^{-1} HCl and washed with AcOEt (5 × 2 mL). The organic phases were combined, dried over $MgSO_4$ and solvent removed under reduced pressure to afford the (2*S*)-(–)-2-methyl-4-oxopentanoic acid (*S*)-**6** with excellent enantiomeric excess (>99%), $[\alpha]_D^{25}$ –18.1 (c 0.8, $CHCl_3$), literature $[\alpha]_D^{25}$ –21.1 (c 0.8, $CHCl_3$) (Hoffman and Kim 1995).

This reaction was also performed in 500 mg scale of enantioenriched methyl (2*S*)-(–)-methyl 2-methyl-4-oxopentanoate (*S*)-**5** and PPL (250 mg) in 50 mL of a 0.1 mol L^{-1} sodium phosphate buffer solution containing 1.6 mol L^{-1} of sodium sulfate at pH 7.2 at 32 °C for 9 h.

Here, the reactions were made in Erlenmeyer flasks using orbital stirring at 250 rpm to afford unreacted ester **(**0.113 g) and (2*S*)-(–)-2-methyl-4-oxopentanoic acid (*S*)-**6** (0.267 g; 61% yield, > 99% ee).

^{1}H-NMR: (300 MHz, $CDCl_3$) δ 3.03–2.87 (m, 1H), 2.55–2.48 (m, 1H), 2.17 (s, 3H), 1.22 (d, *J* = 6.9 Hz, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$): 207.3 (C=O), 181.3 (C=O), 46.3 (CH_2), 34.6 (CH), 29.9 (CH_3), 16.8 (CH_3); MS (70 eV, EI): *m/z* (%) 130 (1.6), 115 (3), 112 (11), 87 (7), 70 (14), 61 (15), 58 (7), 45 (15), 43 (100), 42 (13), 41 (10), 39 (5), CG t_R [(*S*)-**6**] = 44.92 min, Chirasil-DEX CB β-cyclodextrin (25 m × 0.25 mm, 25 μm), temperature gradient: 50 °C (14 min iso), 0.5 °C min^{-1} to 67 °C, 10 °C min^{-1} to 160 °C, R_f 0.52 (AcOEt/MeOH, 3:2).

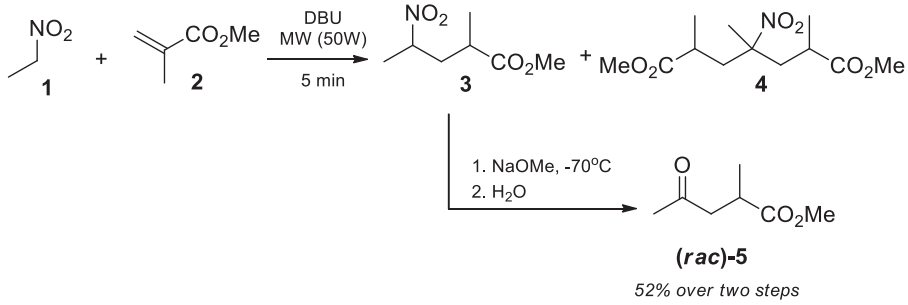

Figure 2. Synthesis of racemic keto ester 5.

Preparation of methyl (2S)-(–)-2-methyl-4-oxopentanoate (S)-5

The same methodology as used for (*S*)-**6** was applied here. To a 25 mL one neck round-bottomed flask, a DMF (10 mL) solution of (2*S*)-(–)-2-methyl-4-oxopentanoic acid (*S*)-**6** (0.52 g, 4.0 mmol) was cooled to 0 °C. Anhydrous potassium carbonate (1.38 g, 10 mmol) was added to this solution portion-wise and after addition, the mixture was allowed to react for 15 min before addition of methyl iodide (16 mmol, 1.00 g, 0.44 mL) in one portion. The reaction mixture was stirred at 0 °C for 30 min and then for more 1.5 h at room temperature. Next, water was added until complete dissolution of K_2CO_3, and this solution was extracted with ethyl acetate as much as necessary to extract all γ-keto ester. (The extraction was monitored by TLC using 50% ethyl acetate as the eluent). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford (2*S*)-(–)-methyl 2-methyl-4-oxopentanoate (*S*)-**5** as a colorless oil in 80% yield (0.46 g). The enantiomeric excess was unchanged as determined by chiral gas chromatography analysis; CG t_R [(*S*)-**5**] = 44.92 min, Chirasil-DEX CB β-cyclodextrin (25 m × 0.25 mm, 25 μm), temperature gradient: 50 °C (14 min iso), 0.5 °C min^{-1} to 67 °C, 10 °C min^{-1} to 160 °C, $[\alpha]_D^{25}$ = –12.9 (c 0.8, $CHCl_3$).

Preparation of (2R)-(+)-2-methyl-4-oxopentanoic acid (R)-6

To a 50 mL one neck round-bottomed flask, a solution of (2*R*)-(+)-methyl-2-methyl-4-oxopentanoate (*R*)-**5** (0.288 g, 2 mmol) was prepared in THF:H_2O (10 mL:3 mL). To this solution was added LiOH (0.09 g, 2.1 mmol) with stirring for 90 min. THF was removed under reduced pressure and an additional 10 mL of water was added. The pH was adjusted to 1 and extracted with AcOEt (4×). The organic phases were reunited and washed with saturated NaCl, dried with $MgSO_4$, and the solvent removed under reduced pressure to afford 0.19 g of (2*R*)-(+)-2-methyl-4-oxopentanoic acid (*R*)-**6** in 72% yield; CG t_R [(*R*)-**6**] = 44.21 min, Chirasil-DEX CB β-cyclodextrin (25 m × 0.25 mm, 25 μm), temperature gradient: 50 °C (14 min iso), 0.5 °C min^{-1} to 67 °C, 10 °C min^{-1} to 160 °C, $[\alpha]_D^{25}$ = +17.6 (c 0.8, $CHCl_3$).

Results and discussion

Preparation of racemic substrates

The title compound was prepared via a two reactions sequence starting from the condensation of methyl methacrylate and nitroethane followed by a Nef reaction of the intermediate γ-nitroester (Figure 2). The first reaction was a DBU-catalyzed Michael addition of the nitroethane (**1**) to neat methyl methacrylate (**2**) under microwave irradiation. This resulted in a mixture of the γ-nitro ester (**3**) and dimethyl 2,4,6-trimethyl-4-nitro-heptanedionate (**4**) (Escalante and Díaz-Coutiño 2009). The second step was adapted from literature (Ballini 1993) where the γ-nitro ester (**3**) was subjected to a Nef reaction to afford the desired racemic methyl 2-methyl-4-oxopentanoate (**5**) in good overall yield. The Nef reaction was carried out using the crude mixture of **3** and **4** because the diester **4** is unreactive toward Nef reaction conditions and is thus easily separated from **5**. The amount of base used to perform the Nef reaction had to be increased 10-fold to achieve moderate to good yields. The desired keto-ester **5** was easily separated from **4** by a horizontal distillation.

Screening of hydrolases for best resolution of (rac)-5

Using the keto ester (*rac*)-**5**, a series of lipases and esterases was used in standard hydrolysis conditions (Delinck and Margolin 1990; Thodi et al. 2009) to screen the best hydrolysis enantioselectivities (Figure 3, Supporting Information Table S21). Although the short reaction time invariably contributed to the

observed low *E*-values, a close inspection on the obtained results revealed two potential enzymes – porcine pancreas lipase (PPL) and the esterase from horse liver. In our adopted approach, (*rac*)-5 was more quickly hydrolyzed by the horse liver esterase (Supporting Information Table S21, entry 7), however moderate enantioselectivity in favor of the acid (*S*)-**6** was observed. The evaluation of the enzyme catalyzed hydrolyses were made by chiral gas-chromatography as indicated in the experimental section. Only CAL-B showed the opposite enantioselectivity for the hydrolysis as observed by the chromatograms. In contrast, the PPL showed better performance, a better *E*-value and a good ratio for the enantiomeric excess of the acid and the ester (Supporting Information Table S21, entry 3). Although only *Candida antarctica* lipase B showed an opposite selectivity for the hydrolysis of (*rac*)-**5** (Supporting Information Table S21, entry 1), the poor conversion led us to discard this enzyme for further studies.

Study of reaction conditions on the hydrolysis of (rac)-5 promoted by PPL

The influence of other reaction conditions in PPL-catalyzed hydrolysis was examined to improve the hydrolysis stereoselectivity of racemic-**5** in an extended reaction time of 4 h. This included the pH of the 0.1 mol L^{-1} phosphate buffer. We used values of 6.2, 7.2 and 8.0 (see Supporting Information Table S22). The enantioselectivity in pH 7.2 was slightly better both after 2 and 4 h of reaction. In this case, the enantiomeric excess of the acid (*S*)-**6** was 62% after 4 h of reaction.

Co-solvents normally improve the solubility of water-immiscible substrates for enzyme-mediated transformations (Carrea and Riva 2000; Adamczak and Krishna 2004; Shen et al. 2008). Therefore, we examined the influence of the co-solvents in the enantioselective hydrolysis of *rac*-**5**. Six organic solvents were evaluated using 10% of their content in 0.1 mol L^{-1} phosphate buffer (pH 7.2). No co-solvent could improve the reaction performance (see Supporting Information Table S23). The solvents led to a decrease in the enantioselectivity factor, conversion and the enantiomeric excess of (*S*)-**6**. This is likely a dilution effect for water immiscible solvents. Nevertheless, water soluble solvents promote both enzyme denaturation and a decrease in the oil–water interface extension. Such effects are related to the well-known interfacial action of PPL (Schmid and Verger 1998; Kapoor and Gupta 2012).

Salt effects on PPL-catalyzed hydrolysis enantioselectivity

As this kinetic resolution reaction was not optimized yet, we decided to examine the effect of the presence of salts in the reaction media due to the interfacial mechanism of lipase catalysis. Kosmotropic salts modulate water surface tension by increasing the salting-out of hydrophobic substances and decreases protein solubility. This behavior would be positive for the studied reaction (Andrade et al. 2010). Such effects on solute–solvent interactions biased by salts, known as Hofmeister effect are seldom considered in biocatalytic reactions for synthetic purposes. Thus, we decided to explore this approach using two kosmotropic salts, sodium chloride and sodium sulfate which led to a positive process improvement (Table 1). The addition of salts increased the ionic strength of medium and suppressed the effect of HPO_4^{2-} however, the same concentration of sodium sulfate doubled the conversion and increased the enantiomeric excess of **(*S*)-6** and the enantioselectivity factor in a two-fold factor.

These results may be explained by the differences in the reaction medium in both cases as can be noticed by the ionic strengths' of each reaction as long the dynamics of substrate and enzyme partitions also changes. Although the concentration of sodium sulfate was not further increased due to the limit of its solubility in the phosphate buffer, the concentration decrease of this salt led to an adverse effect in kinetic resolution efficiency.

Next, we performed the reaction using potassium sulfate as a salt additive. In this case, the increase regarding the conversion from 2–4 h was meager. This result prompted us to discard this salt. Interestingly, the cation exchange from sodium to potassium led to a noticeable decrease in reaction rate which may be explained by the increased chaotropic character of the softer potassium ion. If our initial hypothesis were correct, an enhanced chaotropic salt would impair the reaction efficiency. In fact, we selected potassium fluoride as a negative control which sternly

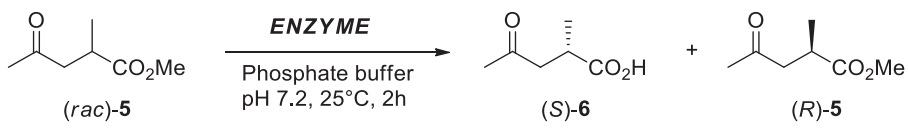

Figure 3. Enzymatic hydrolysis of γ-keto ester (*rac*)-**5**.

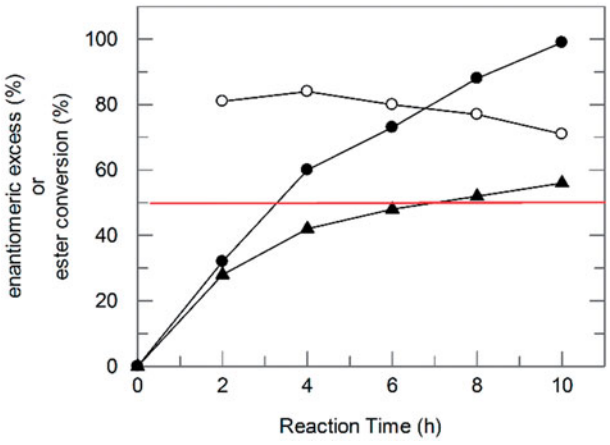

Figure 4. Time course of PPL-catalyzed (*rac*)-**5** hydrolysis in preparative scale. (●: (*R*)-**5** ee; ○: (*S*)-**6** ee; ▲: ester conversion).

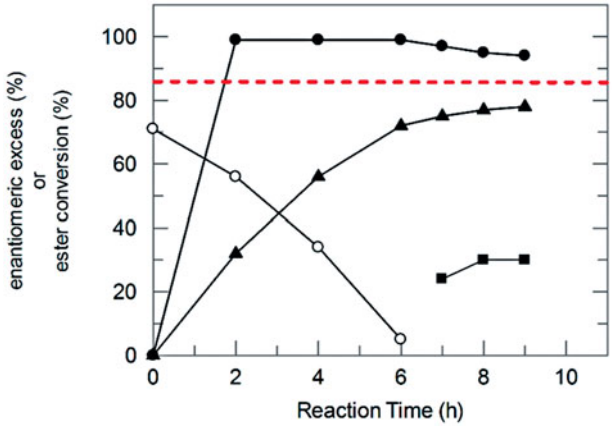

Figure 5. Time course of PPL-catalyzed hydrolysis of enantioenriched (*S*)-**5** in preparative scale. The starting ee for (*S*)-**5** was 71%. The red dotted line indicates the maximum amount of acid (*S*)-**6** starting that can be obtained from enantioenriched (*S*)-**5** with 71% ee; this is 85.5%. (○: (*S*)-**5** ee; ■: (*R*)-**5** ee; ●: (*S*)-**6** ee; ▲: ester conversion).

handicapped both the reaction conversion and the enantioselectivities as seen in Table 1.

Temperature effect on optimized PPL-catalyzed hydrolysis of (rac)-5

The temperature effect was also studied, and it showed a slight increase in the enantiomeric ratio (*E*) at 32 °C (Table 2). Despite the expected increase in the reaction conversion as the temperature rises to 40 °C, the enantiomeric excess of the desired acid decreased. Thus, the optimal reaction temperature was 32 °C. The *E*-value of this condition (21.1) was the best so far and presented good conversion of racemic ester (44%).

Enzymatic kinetic resolution of methyl 2-methyl-4-oxopentanoate

With the optimized reaction conditions determined, the PPL-catalyzed enantioselective hydrolysis of (*rac*)-**5** was carried out and monitored for 10 h. All efforts were made to increase the enantiomeric excess of the (2*S*)-2-methyl-4-oxopentanoic acid (*S*)-**6**, but it was not possible to increase its enantiomeric excess over 95%, desired for synthetic purposes. Alternatively, the hydrolysis reaction was allowed to surpass the 50% conversion where a decrease on (*S*)-**6** enantiomeric excess was expected. Despite this, a higher enantiomeric excess of the less PPL-reactive ester enantiomer, (*R*)-**5**, was >99% after 10 h (Figure 4).

At this point, the enantiomeric excess of the (2*S*)-2-methyl-4-oxopentanoic acid, (*S*)-**6**, was 71% and its enantiopurity was improved after a second PPL-catalyzed kinetic resolution reaction using the enantioenriched methyl ester. It took 6 h to provide the

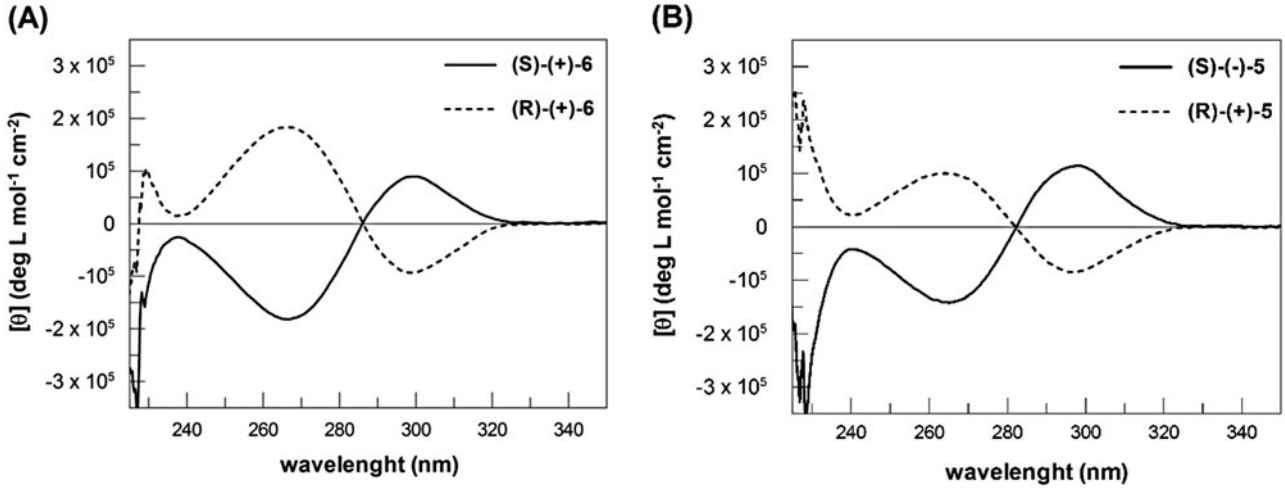

Figure 6. CD spectra of enantiomerically pure acids(*R*)-(+)-**6** and(*S*)-(–)-**6** and esters(*R*)-(+)-**5** and (*S*)-(–)-**5**.

carboxylic acid with high enantiomeric excess (>99%) (Figure 5). After this period, the reaction rate was slower, and the most abundant ester enantiomer is the enantiomer (*R*)-**5** (see Supporting Information for the complete dataset).

The chiroptical properties of both enantiomers of 2-methyl-4-oxopentanoic acid (**6**), and 2-methyl-4- oxopentanoate (**5**) were fully characterized by polarimetry and by circular dichroism. The *R* enantiomers of **5** and **6** were levorotatory, and their enantiomers were dextro rotatory. In chloroform, enantiomerically pure acid (*R*)-(+)-**6** showed first and second positive Cotton effects at 230 and 266 nm, respectively, but the corresponding third Cotton effect was negative at 299 nm. The circular dichroism spectrum of enantiomeric (*S*)-(–)-**6** in chloroform was symmetrical to those of the acid (*R*)-(+)-**6** (Figure 6(A)). The CD spectra of enantiomeric esters were acquired in chloroform solution. The ester (*R*)-(+)-**5** showed first and second positive Cotton effect at 228 and 264 nm, and the third Cotton effect was negative at 297 nm; not surprisingly the spectrum of ester (*S*)-(–)-**5** was symmetrical to those of its enantiomer (Figure 6(B)).

Conclusions

Taken together, the results in this work showed that sodium sulfate, a kosmotropic salt, exerted a positive effect in the performance of a porcine pancreas lipase, an interfacial biocatalyst, in the kinetic resolution reaction of the methyl ester of 2-methyl-levulinic acid. Thus, the method studied herein allowed access to both enantiomers of synthetically valuable 2-methyl-levulinic acid derivatives in high optical purities in a two-step biocatalytic and sustainable process; it complements the previously reported stereospecific biocatalytic reaction which uses old yellow protein and related ene-reductases for the same purpose. Also, the approaches to improve biocatalytic reactions related to reaction media variables, salt effects are seldom explored. The addition of salts is an affordable approach to modulate biocatalysts action to improve processes. Nonetheless, the exact mechanism of the salts, i.e. Hofmeister effect, is still a complex subject, since it modulates both the dynamics between reagents and the macromolecular catalysts leading to improved reaction conditions and thus it is worth to be studied more systematically when one designs new enzyme-catalyzed reactions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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