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Investigation on the chemoenzymatic synthesis of *threo*- and *erythro*-β-hydroxy-L-glutamic acid derivatives

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ABSTRACT

A derivative of the malonic semialdehyde was transformed by means of a bioconversion catalyzed by the enzyme L-threonine aldolase from *E. coli* into a 6:4 epimeric mixture of two precursors of β-hydroxy glutamic acid. The enzyme was selective for the formation of the (*S*)-configuration at C-2, whereas the configuration at C-3 was not controlled. The two epimers were separated exploiting a diastereoselective acylation in organic solvent catalyzed by lipase PS. The relative and absolute configurations of the products were preliminarily assigned on the base of the model proposed by Kazslauskas for the stereopreference of lipase PS and by comparison of the chemical shifts of the H-2 and H-3 protons of the two homologues. The possibility of transforming the obtained products into β-hydroxy glutamic acid derivatives by conventional chemical reactions was demonstrated.

1. Introduction

L-Glutamic acid (Glu) is a nutrient involved in important biochemical pathways (gluconeogenesis and ammonia detoxification) [1]. In the mammalian central nervous system (CNS) it is considered to be the major mediator of excitatory signals, contributing to brain development and to physiological functions such as cognition, memory, learning and nociception [2–7].

Due to its potential neurotoxicity when accumulating in the synaptic cleft, the extracellular concentration of glutamate is controlled under physiological conditions by membrane-bound proteins which act as high affinity excitatory amino acid transporters (EAATs) [6–8]. In pathological conditions (e.g. ischemia, neurotrauma, neurodegenerative diseases), EAATs release additional Glu in the synapsis through the reversed mode of operation, leading to an overstimulation of glutamate receptors which causes a massive calcium influx responsible for neuronal cell death [8].

In this frame EAATs are considered as molecular targets for the treatment of cerebral ischemia and neurodegenerative pathologies. In particular, attention is focused on EAAT non-transportable inhibitors (blockers) which, in pathological conditions, could reduce EAAT-mediated glutamate release into the synaptic cleft, thus preventing excitotoxicity and the subsequent cell death [9–11]. Initial synthetic and biochemical studies, focusing on the modification of the structure of the endogenous substrates, namely aspartate (Asp) and glutamate (Glu), led to the identification of potent substrate inhibitors and uptake blockers [12,13]. The strategies used to find out EAAT inhibitors were based on two approaches: (i) the conformational blockade of the neurotransmitters skeleton [12] and (ii) the insertion into their structure of further functional groups [13]. The outcome of these investigations is exemplified by 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic acid (HIP-A, **1**) and 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid (HIP-B, **2**), which are non-competitive inhibitors, and L-*threo*-β-benzyloxyaspartate (L-TBOA, **3**). In particular, L-TBOA, a non-transportable blocker of EAATs, is widely recognized as the gold standard compound for the biological screening of novel derivatives as well as the tool to investigate the physiological roles played by the different subtypes of this set of proteins [13].

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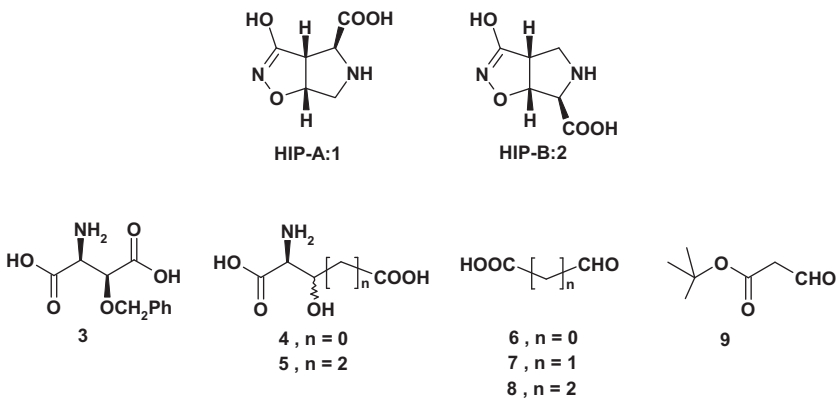

Formulae 1-9

Structural modification of glutamate chain also produced intriguing biological results [14]. Particularly, in a recent work, β- and γ-benzyloxy-L-glutamic acid derivatives have been synthesized and their activity as EAAT blockers has been evaluated [15].

In this frame we became interested in investigating a biocatalytic approach to obtain β-hydroxy-L-glutamic acid derivatives, specifically by exploiting the lyase L-threonine aldolase (L-TA) from *E. coli* [16]. The advantages of an enzymatic strategy over conventional methodologies rely on the mild conditions and the minimal need for substrate protection [17].

L-Threonine aldolases (L-TA) are pyridoxal-5-phosphate (PLP)-dependent enzymes that catalyze either the cleavage of L-threonine (or L-*allo*-threonine) into glycine and acetaldehyde, or the reverse aldol reaction [18,19]. There is a strict requirement for glycine as a donor, but a wide range of aliphatic and aromatic aldehydes as electrophilic substrates can be accepted [20,21]. The aldol reaction proceeds with complete control of the stereoselectivity at the α-carbon, since the outcome is the sole (L)-epimer. On the other hand, the stereochemistry at the β-carbon is not totally controlled and a mixture of diasteroisomers is generally formed (Fig. 1).

In a previous work [22] we have demonstrated that a mixture of stereoisomeric β-hydroxy-L-aspartates (**4**) and 2-amino-3-hydroxyhexanedioic acids (**5**) can be obtained by L-TA-catalyzed condensation of glycine with the commercially available glyoxylic acid (**6**) and succinic semialdehyde (**8**), respectively. In this paper we report the results of the investigation on the related synthesis of β-hydroxy-L-glutamic acid derivatives through the biocatalytic condensation of glycine with analogues of the malonic semialdehyde **7**.

2. Results and discussion

At variance with compounds **6** and **8**, aldehyde **7** is not commercially available, probably due to its instability. Taking into account its tendency to decarboxylate to acetaldehyde, it became necessary to develop a synthetic strategy to prepare a suitable derivative in which the carboxylic moiety, if present, was masked. Aldehyde **9**, carrying a *tert*-butyl ester, was identified as a suitable target since we had previously verified that, due to its steric hindrance, this alkyl moiety was not recognized by the hydrolases present as contaminants in our aldolase preparation [23]. Moreover, *tert*-butyl esters, if needed, can generate the corresponding carboxylate group under nonracemizing mild acidic conditions.

As shown in Fig. 2, the synthesis of substrate **9** was carried out starting from Meldrum's acid **10** which, after a condensation with triethyl orthoformate followed by a treatment of the intermediate **11** with hydrochloric acid, gave aldehyde **12**, depicted in Fig. 2 as the enol tautomer [24]. The protected semimalonic aldehyde **9** was then obtained by reacting derivative **12** with *tert*-butyl alcohol and subsequently purified by distillation. It was mixed with an excess glycine and submitted to the action of the L-TA from *E. coli*, produced and purified as previously described [22]. To our disappointment, no significant product formation was detected by TLC. A conceivable explanation could be related to the presence in the aqueous solution of a substantial percentage of the enolic form **9a** which is unsuitable to undergo a nucleophilic attack (Fig. 2).

A different synthetic strategy based on aldehyde **13**, an homologous of benzyloxyacetaldehyde known to be a good substrate for L-TA [20–22] was subsequently investigated.

Compound **13** could be easily obtained by oxidation with TEMPO, NaOCl and NaBr of the corresponding commercially available alcohol **14** (Fig. 3). The L-TA-catalyzed condensation of aldehyde **13** with glycine gave a product of intermediate polarity, whose TLC spot was positive to the ninhydrin test. This compound was purified with an hydrophobic ion exchange resin (SEPHABEADS SP20755-Resindion) suitable to separate amino acids on the base of their different hydrophobicity, retaining the more lipophilic ones. The excess of glycine could be easily washed out, since it did not interact with the resin, whereas the expected product was retained and it was eluted by increasing the percentage of ethanol in the solvent.

As expected, the enzymatic condensation showed complete stereocontrol in the formation of the α-carbon centre but it was not stereoselective at the β-carbon. The isolated compound, tagged as **(*t*,*e*)-15**, was a mixture of diastereomers (L-absolute configuration, >98% e.e.), as evident from the NMR spectrum of the crude product. The *threo* isomer **(*t*)-15** was characterized by a doublet at 3.11 ppm (H-2) and a multiplet at 3.95 ppm (H-3), both integrating 0.6 H, while the *erythro* isomer **(*e*)-15** showed a doublet at 3.14 ppm (H-2) and a multiplet at 3.90 ppm (H-3), both integrating 0.4 H (Fig. 4).

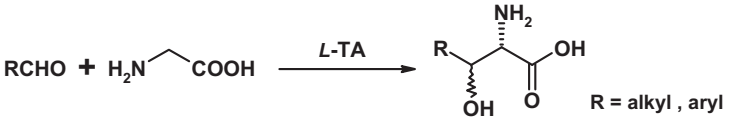

Fig. 1. L-TA-catalyzed condensation of a generic aldehyde and glycine to give β-hydroxy-α-L-amino acids.

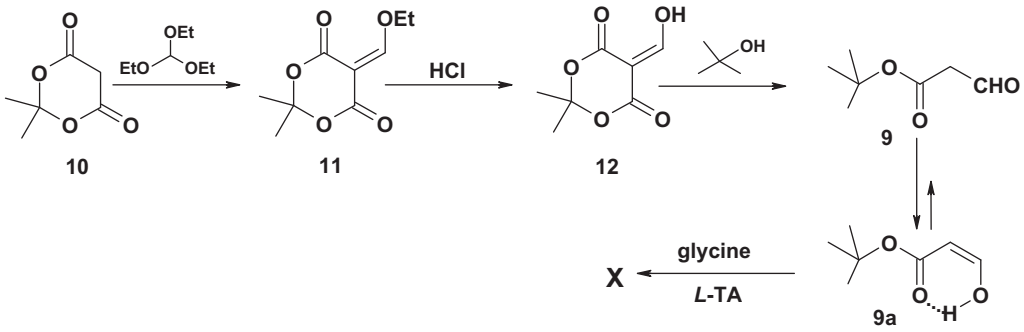

Fig. 2. Synthesis of the malonic semi-aldehyde *tert*-butyl ester **9** and its attempted L-TA-catalyzed condensation with glycine.

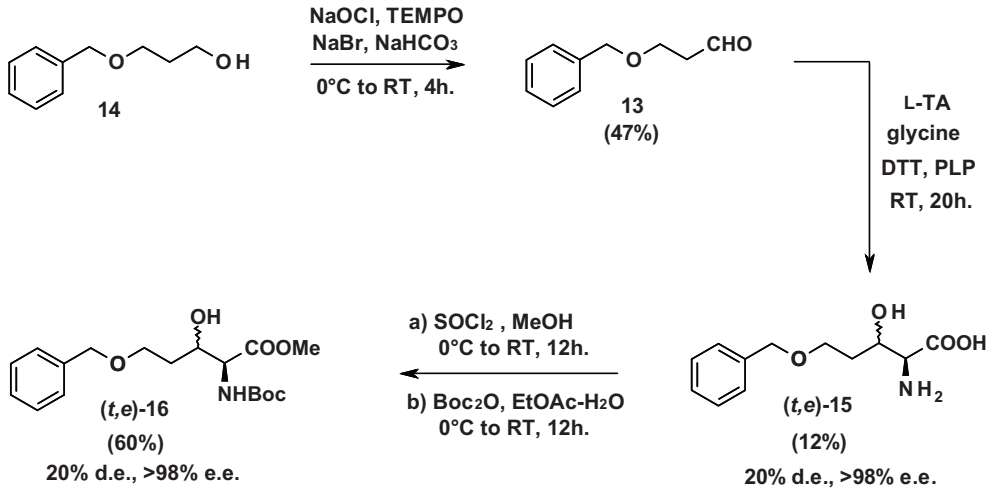

Fig. 3. Chemoenzymatic synthesis of (***t,e***)**-16**. Yields refer to isolated products after chromatographic purification.

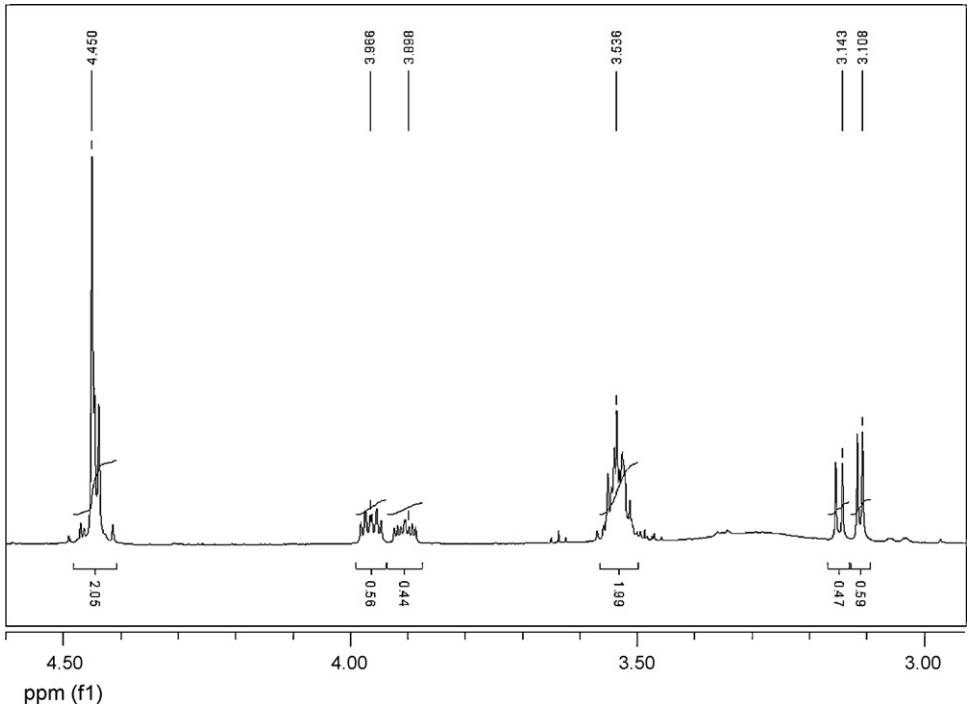

Fig. 4. Portion of the ^{1}H NMR spectrum of (***t,e***)**-15**.

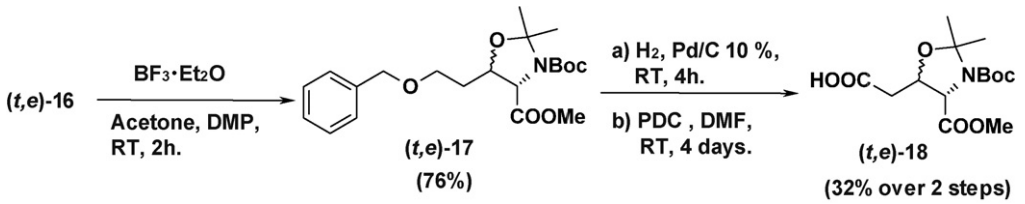

Fig. 5. Synthesis of (***t,e***)**-18**.

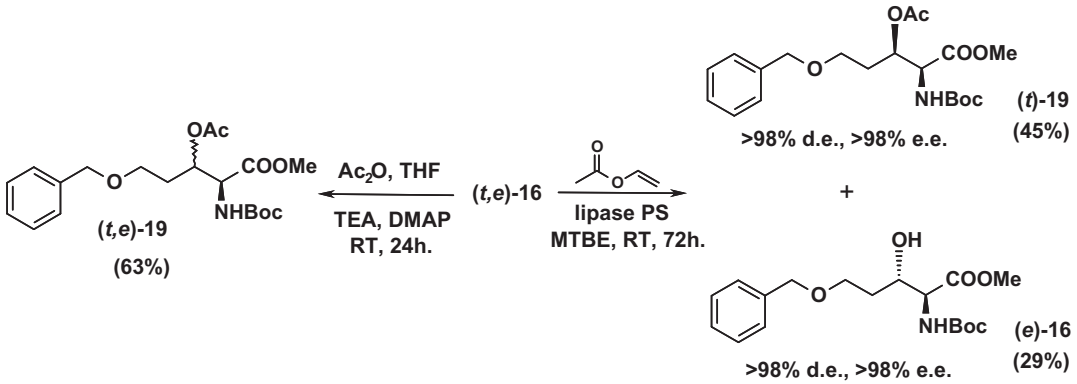

Fig. 6. Aspecific chemical acetylation and enzymatic diastereoselective acetylation of (***t,e***)**-16**. Yields refer to isolated products after chromatographic purification.

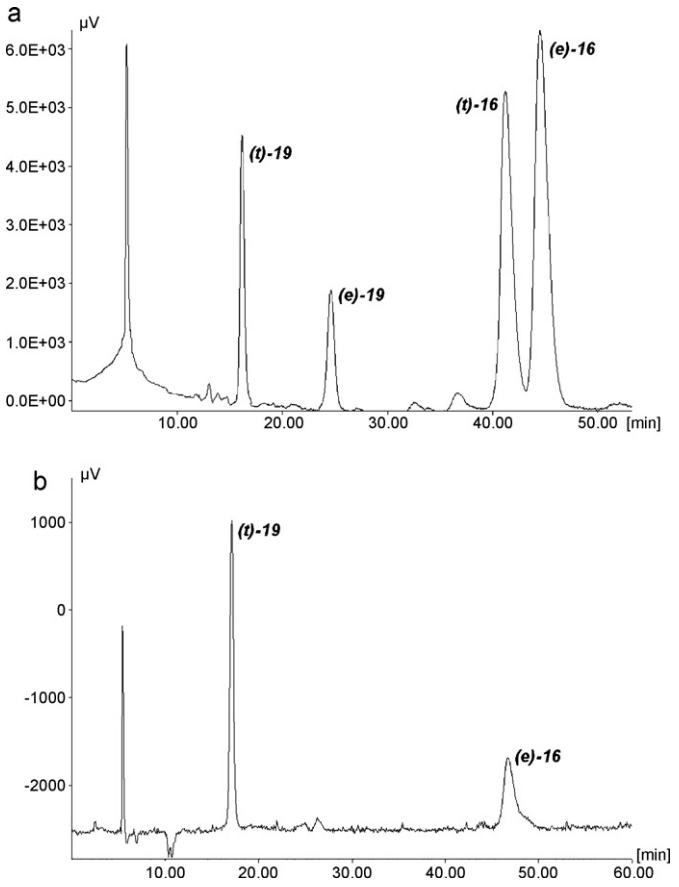

Fig. 7. (a) Chiral HPLC separation of (***t,e***)**-19** and (***t,e***)**-16** on a CHIRALPAK IA column; (***t***)**-19**: 16.167 min, (***e***)**-19**: 24.608 min, (***t***)**-16**: 41.167 min, and (***e***)**-16**: 44.475 min. (b) Chromatogram obtained after the enzymatic kinetic resolution of (***t,e***)**-16**.

In order to evaluate the biological activity of the target compounds, it is necessary to synthesize them in an enantiomerically and diastereomerically pure form. Since in a previous work [22] we were able to separate the two epimers of **4** by a simple silica gel chromatography of their *N*-Boc methyl ester derivatives, the same approach was attempted with the epimeric mixture (***t,e***)-**15**. Accordingly, they were transformed into the corresponding derivatives (***t,e***)**-16** (Fig. 3), which, however, turned out to be unseparable by silica gel chromatography.

In a further attempt the mixture (***t,e***)**-16** was transformed into the corresponding acetonides (***t,e***)**-17**, which subsequently underwent a catalytic hydrogenation to remove the benzyl group and an oxidation by means of the Corey–Schmidt reagent (PDC in DMF) to give a mixture of the protected glutamate derivatives (***t,e***)**-18** (Fig. 5). Unfortunately, even this strategy did not allow the separation of the two epimeric derivatives by silica gel chromatography, despite the fact that they had been transformed into more rigid and polar derivatives (***t,e***)**-17** or (***t,e***)**-18**.

In a third attempt to separate the two epimers (***t,e***)**-15**, it was then investigated the well-known ability of lipases to catalyze stereoselective acylations in organic solvents [17d,25]. Preliminarily the mixture (***t,e***)**-16** was transformed into the corresponding

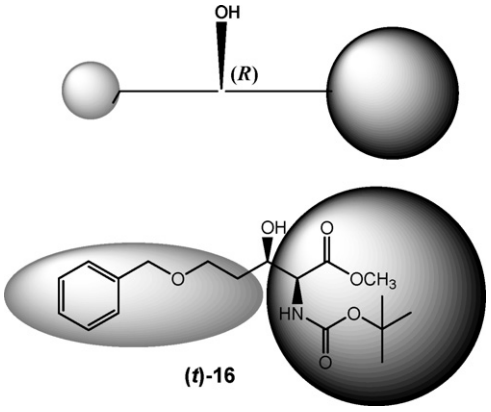

Fig. 8. Stereopreference of lipase PS (Kaslauskas's rule) [26].

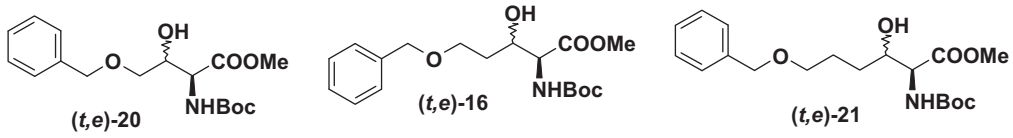

Fig. 9. "Inferior" **(*t*,*e*)-20** and "superior" **(*t*,*e*)-21** homologues of **(*t*,*e*)-16**.

acetyl derivatives **(*t*,*e*)-19** by a standard procedure (Fig. 6). The mixtures **(*t*,*e*)-16** and (*t*,*e*)-**19** were analyzed by chiral HPLC and it was found that both the epimers **(*t*,*e*)-16** and (*t*,*e*)-**19** could be separated with a CHIRALPAK IA (Daicel) column; the acetylated derivatives **(*t*,*e*)-19** being the first to be eluted at 16.1 and 24.6 min versus 41.2 and 44.5 of **(*t*,*e*)-16** (Fig. 7a).

Seven commercially available lipases preparations were tested for their ability to catalyze the diastereoselective acetylation of **(*t*,*e*)-16** in methyl *tert*-butyl ether as a solvent and in the presence of vinyl acetate. Three lipases were able to accept this epimeric mixture as a substrate, but only a lipase from *Pseudomonas* resulted to be diastereoselective.

The preparative enzymatic resolution of **(*t*,*e*)-16** was then carried out and, after 72 h the non-acetylated epimer corresponding to the peak at 41.2 min was completely transformed into the fastest eluted acetyl derivative with a retention time of 16.1 min (conversion **(*t*)-16** → **(*t*)-19** was observed to be >98%). The acetyl derivative and the residual alcohol were separated by silica gel chromatography and isolated in 45% and 29% yield, respectively. Their diastereomeric excesses, evaluated by chiral HPLC analysis, resulted to be >98%. ^{1}H NMR spectra of the two compounds confirmed, in both cases, the presence of a single epimer.

Relative and absolute configurations at the stereocenters of the two isolated epimeric compounds were preliminarily assigned on the basis of the well-known chiral preference of lipase from *Pseudomonas*. A model proposed by Kazslauskas [26] assesses that in the case of a secondary alcohol with two substituents of different steric hindrances, this enzyme preferentially acylates the stereoisomer with a *R* configuration. As shown in Fig. 8, by applying this rule to our substrates, and by considering that L-TA leads exclusively to the formation of the 2*S* isomers, we propose the structure of **(*t*)-19** and **(*e*)-16** for the two isolated products, with the absolute configurations 3*R*,2*S* and 3*S*,2*S*, respectively. Additionally, for the sake of comparison, **(*e*)-16** could be easily acetylated to the corresponding **(*e*)-19**.

The proposed relative configuration of the isolated **(*e*)-16** and **(*t*)-16** could be confirmed by comparison of their ^{1}H NMR spectra with the literature data reported for their one carbon lower homologues, **(*t*,*e*)-20** [17,27] and one carbon higher homologues, **(*t*,*e*)-21** [28,29] (Fig. 9). As regards the signals of the H-3 proton in this series, it could be noted that in the *erythro* epimers these signals resulted upshielded with respects to the corresponding signals of the *threo* counterpart. In fact in **(*t*,*e*)-21** the H-3 signal of the *erythro* isomer fell at 3.89 ppm, while in the *threo* isomer it was found at 4.05 ppm. Similarly, in **(*t*,*e*)-20** the H-3 proton of the *erythro* isomer was at 4.25 ppm, whereas in its *threo* counterpart it resonated around 4.40 ppm. In analogy, it sounds reasonable to propose that even in the case of the mixture **(*t*,*e*)-16**, the H-3 signal at higher fields (4.08 ppm) due to the isomer that was not acylated by lipase PS, could be associated to (3*S*,2*S*)-*erythro* epimer, thus confirming the assignments based on the Kaslauskas' rule.

3. Conclusions

Two enantiomerically and diastereomerically pure precursors of β-hydroxy glutamic acid were synthesized exploiting two selective enzymatic transformations: an aldol reaction catalyzed by a L-threonine aldolase, which was completely stereoselective in the formation of the (*S*)-stereocentre in position C-2 and gave two C-3 epimers in a 6:4 ratio, followed by an esterification catalyzed by a lipase from *Pseudomonas*, which selectively acylated the 3*R*-epimer. The relative and absolute configurations of the products were preliminarily assigned considering the model proposed by Kazslauskas on the stereopreference of lipase PS and confirmed by ^{1}H NMR. The possibility to transform the derivatives obtained with the present enzymatic methodologies into stereomeric β-hydroxy glutamic acids by means of conventional chemical reactions should contribute to deepen their intriguing pharmacological profile.

4. Experimental part

4.1. Materials and methods

All the reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise stated. The coding sequence of the *ltaE* gene, encoding the *E. coli* low-specificity L-threonine aldolase (eTA) was previously obtained from the genomic DNA of the *E. coli* K-12 strain as described in the literature [16]. The host strain for recombinant expression was HMS174(DE3) [F^- *recA1 hsdR*(r_{K12}^- m_{K12}^+) (DE3) (Rif^R)].

Lipase form *Candida rugosa*, porcine pancreatic lipase and subtilisin were purchased from Sigma–Aldrich, lipase PS from Amano, Novozym 435 and lipozyme were from Novozymes, lipase from *Chromobacterium viscosum* from Finnsugar.

Fermentations were performed with a 1 L fermentor Inova 4230, sterilizations were performed using autoclave Steristeam CDL. Enzymatic activities were monitored using a Jasco V-530 UV/vis spectrophotometer. Enzyme purification and enantiomeric excess determinations were performed by HPLC, using a JASCO 880-PU instrument equipped with a Jasco 870 UV detector. Measurements of pH were carried out with a pH-meter Crison micropH 2000. TLC: silica plates (Merck 60 F_{254}) or reverse phase silica plates (Merck RP-18_{254}). Substrates and products were visualized at 254 nm and/or by treatment with the ninhydrin reagent (ninhydrin, 2 g, in 100 ml MeOH). Flash chromatography silica gel: Merck 60, 40–63 μm.

^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 (300 MHz) or on a Bruker AC400 (400 MHz) or on a Bruker AC500 (500 MHz). MS spectra were recorded on Bruker Esquire 3000 Plus spectrometer.

4.2. Production and purification of L-TA from E. coli

L-TA was expressed from *E. coli* HMS 174 (DE3)/pET 22 b(+)(L-TA) constructs containing the DNA sequence. The cells were cultivated on LB media (Trypton 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with 100 mg/L ampicillin and 30 mg/L of B_6 vitamins. Overnight cultures (100 mL) in 500 ml flasks were inoculated with single colonies and grown at 37 °C. The 500 mL main cultures in 2000 mL flasks were inoculated with 3 ml of the preculture and grown at 37 °C to an OD_{600} of 0.3 and then induced with IPTG at a final concentration of 0.05 mM. Cultivation was continued overnight at 37 °C, and the cells were harvested by centrifugation for 20 min at 4500 rpm. After resuspension of the pellets (10 mL/g cell) in a lysis buffer (10 mM Tris/HCl, 1 mM NaCl, 1 mM

EDTA, pH 7.6), the cells were disrupted using a solution of lysozyme (1 mg/g cell) followed by the precipitation of the DNA by adding streptomycine sulfate (10 g/L dissolved in the minimum amount of the same buffer) and leaving the suspension at 0 °C for 20 min. The crude lysate was cleared by centrifugation at 12,000 rpm for 30 min. The enzyme was precipitated from the supernatant (cell free extract) by adding ammonium sulfate (80% saturation) and partially purified using an ion-exchange column (DEAE 650-S, Merck) where the dissolved pellet was loaded in a phosphate buffer 20 mM pH = 7.5 and the enzyme eluted with a linear gradient of NaCl, up to 0.4 M. The partially purified enzyme was stored as a precipitate in ammonium sulfate at 4 °C.

In order to improve the yields of recovered L-TA, cell growth was performed in a 1 L capacity fermentor (INFORS AG CH-4103), following the same protocol developed for the suspended cell systems, monitoring and actively controlling oxygen supply, pH, temperature, and stirring at 220 rpm. This experiment gave satisfactory results, as – following cells lyses, ammonium sulfate precipitation and subsequent dialysis – 688 U of L-TA were obtained, to be compared with the 125 U obtained with the previously described flask cultures.

4.3. L-*TA activity assay*

L-*allo*-Threonine (50 mM), sodium phosphate buffer (20 mM, pH 7), PLP (50 μM), NADH (200 μM), yeast alcohol dehydrogenase (32 U) were added in cuvette to a final volume of 990 μL. The reactions were started by adding 10 μL of purified L-TA and monitored by measuring the decrease of absorbance at 340 nm in a spectrophotometer ($\varepsilon = 6.2 \times 10^3\ l \times mol^{-1} \times cm^{-1}$) at 30 °C.

One unit of L-TA is the amount of enzyme that catalyzes the formation of 1 μmol of acetaldehyde (1 μmol of NADH oxidized) per minute at room temperature.

4.4. *Synthesis of 5-(ethoxymethylene)-2,2-dimethyl-1,3-dioxan-4,6-dione* ***(11)***

A mixture of Meldrum's acid **10** (2,2-dimethyl-1,3-dioxan-4,6-dione, 4.0 g, 27.77 mmol) and triethylorthoformate (13.35 g, 90.08 mmol) was stirred at 85 °C for 2 h. When TLC analysis ($CHCl_3$/MeOH 8:2; R_f: **11** = 0.67) indicated the complete consumption of Meldrum's acid the solvent was evaporated *in vacuo* to obtain the crude product **11** as a viscous yellow oil.

The product was used for the following reaction without any further purification and characterization.

4.5. *Synthesis of 5-(hydroxymethylene)-2,2-dimethyl-1,3-dioxan-4,6-dione* ***(12)***

The crude compound **11** (695 mg, 3.47 mmol) was stirred for 30 min in a solution of HCl 2 N (12 mL) until an orange precipitate was formed. The suspension was then dissolved in brine and the water phase extracted with diethyl ether (3× 10 mL). The organic phase was washed with brine (1× 10 mL), dried over Na_2SO_4 and the solvent removed *in vacuo* to give the product **12** as a yellow solid in 69% yield (793 mg). The formation of **12** was followed by TLC ($CHCl_3$/MeOH 97:3; R_f: **12** = 0.23).

^{1}H NMR (400 MHz, dmso-d_6) δ (ppm): 1.51 (s, 6H, CH_3); 8.53 (s, 1H, C–H); 12.72 (bs, 1H, C–OH).

^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 27.52 (CH_3); 95.71 (C=C–OH); 107.38 (C–CH_3); 168.32 (C=O); 171.75 (C=O); 177.31 (C=C–OH).

4.6. *Synthesis of tert-butyl 3-oxopropanoate* ***(9)***

A solution of formyl Meldrum's acid (**12**, 2.0 g, 11.6 mmol) and *tert*-butyl alcohol (1.3 mL) in dry benzene (23.2 mL) was refluxed for 1 h under inert atmosphere. The solvent was evaporated *in vacuo* at room temperature. Distillation of the residue under reduced pressure gave a mixture of products **9** and **9a** in 28% yield (478 mg). The formation of **9** was followed by TLC ($CHCl_3$/MeOH 97:3; R_f: **12** = 0.23; **9** = 0.37).

^{1}H NMR **9** (300 MHz, dmso-d_6) δ (ppm): 1.45 (s, 9H, CH_3); 3.28 (br s, 2H, CH_2COO); 9.61 (br s, 1H, HCO). ^{1}H NMR **9a** (300 MHz, dmso-d_6) δ (ppm): 1.45 (s, 9H, CH_3); 5.02 (d, 1H, CHCOO, J = 12.2 Hz); 7.04 (m, 1H, HOC<u>H</u>=); 11.51 (d, 1H, <u>H</u>OCH=, J = 7.8 Hz).

4.7. *Synthesis of 3-(benzyloxy)propanal* ***(13)***

A solution of $NaHCO_3$ (9.87 g) in NaOCl aq (2.5% in water, 130 mL) was added dropwise (3 mL/min) at 0 °C to a stirred mixture of 3-(benzyloxy)propan-1-ol (**14**, 4.3 ml, 27 mmol), NaBr (2.77 g, 27 mmol) and TEMPO (0.8 g, 5.2) in AcOEt, toluene and water (150 mL, 4:4:1). After the addiction, the mixture was stirred at room temperature for 4 h and the reaction was monitored by TLC (petroleum ether/EtOAc 8:2; R_f: **13** = 0.42). After the reaction, the two phases were separated, the aqueous phase was extracted with EtOAc (3× 15 mL) and the collected organic layers were washed firstly with a solution (2× 15 mL) of KI (10%) in $KHSO_4$ (aqueous saturated solution), then with a solution (2× 15 mL) of $Na_2S_2O_3$ (10% in water), then with phosphate buffer (pH 7, 2× 15 mL) and finally with brine (2× 15 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was then purified by flash silica gel column chromatography (petroleum ether/EtOAc 8:2) obtaining the aldehyde **13** (2.07 g) in 47% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 2.62 (dt, 2H, J = 1.8, 6.1 Hz, H-2); 3.74 (t, 2H, J = 6.1, 6.1 Hz, H-3); 4.46 (s, 2H, H-4); 7.21–7.32 (m, 5H, ArH); 9.73 (t, 1H, J = 1.8, 1.8 Hz, CHO).

4.8. *Synthesis of (2S)-3(t,e)-2-amino-5-(benzyloxy)-3-hydroxypentanoic acid* ***((t,e)-15)***

To a solution of glycine (2.57 g, 34.3 mmol), dithiothreitol (DTT, 79 mg), pyridoxal phosphate (PLP, 9 mg, 0.125 mM) in water (35 mL), NaOH (1 M) was added up to pH = 7.5. Then, L-TA (46 U) and aldehyde **13** were added and the reaction mixture was stirred at r.t. for 20 h and monitored by TLC (*n*-BuOH/H_2O/AcOH 4:2:1; R_f: **15** = 0.5, detection was carried out with ninhydrin reagent). The mixture was extracted with EtOAc (60 mL) and the aqueous phase was loaded on a hydrophobic chromatographic column [regenerated (15%, v/v MeOH) phenylic resin: Sepabeads SP20755 Resindion]. The resin was initially eluted with water to remove the excess of glycine and then washed with solutions of ethanol in water (up to 30%) to recover the product. The fractions containing the product were collected and lyophilised to give the epimeric mixture ***(t,e)*-15** (324 mg) in 12% yield. The product was characterized by ^{1}H NMR which also allowed the evaluation of the diastereoisomeric *threo*/*erythro* ratio (6:4) of ***(t,e)*-15** (with a d.e. – value of 20% in favor of ***(t)*-15**; e.e. – values resulted to be >98% for both epimers by chiral HPLC analysis).

^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 1.50–1.65 (m, 1H, H-$4_{erythro/threo}$); 1.74 (m, 0.4H, H-$4_{erythro}$); 1.85 (m, 0.6H, H-4_{threo}); 3.11 (d, 0.6H, J = 4.5 Hz, H-2_{threo}); 3.14 (d, 0.4H, J = 5.8 Hz, H-$2_{erythro}$); 3.53 (m, 2H, H-5); 3.90 (m, 0.4H, H-$3_{erythro}$); 3.95 (m, 0.6H, H-3_{threo}); 4.44 (s, 2H, H-6) 7.25–7.32 (m, 5H, ArH).

4.9. *Synthesis of tert-butyl (S)-1-(methoxycarbonyl)-4-(benzyloxy)-2-hydroxybutylcarbamate* ***((t,e)-16)***

(A) Epimeric mixture ***(t,e)*-15** (230 mg, 0.96 mmol) was added portionwise at 0 °C to a solution of $SOCl_2$ (0.21 mL, 2.88 mmol) in methanol (2.5 mL) under nitrogen. After 1 h at 0 °C, the reaction

mixture was stirred at r.t. overnight and monitored by TLC (*n*-BuOH/H_2O/AcOH 4:2:1; R_f: **(*t,e*)-15a** = 0.33). The mixture was then concentrated under reduced pressure and the obtained crude material **(*t,e*)-15a** was used in the next step without further purification.

(B) To a biphasic solution of **(*t,e*)-15a** (230 mg, 0.962 mmol) in water (1 mL) and EtOAc (4 mL) at 0 °C under stirring, Boc_2O (252. mg, 1.154 mmol) was added. The pH of the mixture was monitored and adjusted to a value ≥7 by adding a few drops of a saturated solution of $NaHCO_3$. After 2 h at 0 °C the reaction mixture was stirred at r.t. overnight and monitored by TLC (petroleum ether/EtOAc 6:4; R_f: **(*t,e*)-16** = 0.41). After the reaction, the aqueous phase was separated and extracted with EtOAc (3× 2 mL), the collected organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography (CH_2Cl_2/MeOH 9.9:0.1) obtaining **(*t,e*)-16** (203 mg) in 60% yield over two steps (starting from **(*t,e*)-15**). HPLC: Column CHIRALPAK IA (Daicel); eluent: petroleum ether, isopropanol 9.5/0.5, flow: 0.6 mL/min; λ = 254 nm. **(*t*)-16**: 41.167 min, **(*e*)-16**: 44.475 min; the mixture showed a d.e. – value of 20% in favor of **(*t*)-16**; e.e. – values resulted to be >98% for both epimers.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.44 (s, 5.5H, CH_3Boc_{threo}); 1.45 (s, 3.5H, $CH_3Boc_{eryithro}$); 1.74–1.98 (m, 2H, H-$4_{erythro/threo}$); 2.90 (bs, 1H, OH); 3.61–3.74 (m, 2H, H-$5_{erythro/threo}$); 3.75 (s, 2H, OCH_{3threo}); 3.76 (s, 1H, $OCH_{3erythro}$); 4.08 (m, 0.4H, H-$3_{erythro}$); 4.28 (bs, 0.6H, H-3_{threo}); 4.32–4.38 (m, 1H, H-$2_{erythro/threo}$) 4.51 (2s, 2H, H-$6_{erythro/threo}$); 5.37 (bs, 0.6H, NH_{threo}); 5.45 (bs, 0.4H, $NH_{erythro}$); 7.23–7.36 (m, 5H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 28.62 ($CH_3Boc_{erythro/threo}$); 33.42 (C-$4_{erythro}$); 33.49 (C-4_{threo}); 52.63 ($_{erythro}$); 52.74 ($_{threo}$); 58.29 ($_{threo}$); 58.70 ($_{erythro}$); 68.81 ($_{erythro}$); 69.36 ($_{threo}$); 72.34 ($_{threo}$); 72.65 ($_{erythro}$); 73.70 ($_{erythro}$); 73.85 ($_{threo}$); 80.23 ($_{threo}$); 80.55 ($_{erythro}$); 128.00 (C–$Ar_{erythro}$); 128.05 (C–Ar_{threo}); 128.11 (C–$Ar_{erythro}$); 128.22 (C–Ar_{threo}); 128.78 (C–$Ar_{erythro}$); 128.83 (C–Ar_{threo}); 137.85 (C–ipso$_{threo}$); 138.10 (C–ipso$_{erythro}$); 156.04 (C–$O_{erythro}$); 156.43 (C–O_{threo}); 171.37 (C–$O_{erythro}$); 171.96 (C–O_{threo}). *m*/*z* (ESI) = 376 Da (M+Na^+), 354 Da (M+H^+).

*4.10. Synthesis of (S)-3-tert-butyl 4-methyl 5-(2-(benzyloxy)ethyl)-2,2-dimethyloxazolidine-3,4-dicarboxylate **((t,e)-17)***

To a solution of **(*t,e*)-16** (140 mg, 0.396 mmol) in acetone (2 mL) and 2,2-dimethoxypropane (DMP, 0.43 mL), $BF_3{\cdot}Et_2O$ (3 μL) was added. The resulting solution was stirred at r.t. for 2 h and monitored by TLC (petroleum ether/EtOAc 6:4; R_f: **(*t,e*)-17** = 0.82). The solvent was removed under reduced pressure, the residual oil taken up in CH_2Cl_2 (20 mL) and the resulting solution washed with a mixture of a saturated $NaHCO_3$ solution and water (1:1, 10 mL), then brine (10 mL), dried (Na_2SO_4) and the solvent evaporated *in vacuo* to give the product **(*t,e*)-17** as a pale yellow oil (118 mg) in 76% yield, used in the next step without any further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.32–1.40 (2s, 9H, CH_3Boc); 1.42–1.54 (4s, 6H, CH_3); 1.90 (m, 2H, H-4); 3.54 (m, 2H, H-5); 3.65 (s, 3H, OCH_3); 4.07–4.34 (m, 2H, H-2, H-3); 4.43 (s, 2H, H-6); 7.23–7.36 (m, 5H, ArH). *m*/*z* (ESI) = 416 Da (M+Na^+), 394 Da (M+H^+).

*4.11. Synthesis of (S)-3-tert-butyl 4-methyl 5-(2-(hydroxyethyl)-2,2-dimethyloxazolidine-3,4-dicarboxylate **((t,e)-17a)***

To a solution of **(*t,e*)-17** (120 mg, 0.3 mmol) in MeOH (15 mL), Pd/C-10% (40 mg) as catalyst was added. The resulting suspension was stirred under H_2 (1 atm) at r.t. for 4 h and the reaction was monitored by TLC [petroleum ether/EtOAc 7:3; R_f: **(*t,e*)-17a** = 0.28]. The mixture was filtered on celite and the filtrate concentrated *in vacuo*. The crude residue was then purified by flash silica gel column chromatography ($CHCl_3$/MeOH 9.5:0.5) obtaining **(*t,e*)-17a** (77 mg) in 85% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.41–1.49 (2s, 9H, CH_3Boc); 1.52–1.73 (4s, 6H, CH_3); 1.85–2.06 (m, 2H, H-4); 3.76–3.77 (2s, 3H, OCH_3); 3.82 (t, 2H, *J* = 6.5 Hz, H-5); 4.08 (d, 1H, *J* = 7.7 Hz, H-2); 4.23 (m, 1H, H-3). *m*/*z* (ESI) = 326 Da (M+Na^+).

*4.12. Synthesis of 2-((S)-3-(tert-butoxycarbonyl)-4-(methoxycarbonyl)-2,2-dimethyloxazolidin-5-yl)acetic acid **((t,e)-18)***

To a stirred solution of pyridinium dichromate (PDC, 400 mg, 1.06 mmol) in DMF (2 mL) **(*t,e*)-17a** (25 mg, 0.0824 mmol) was added and the mixture was stirred at r.t. for 4 days. The reaction was monitored by TLC ($CHCl_3$/MeOH 9.5:0.5; R_f: **(*t,e*)-18** = 0.52) and when no starting material could be detected, $NaHCO_3$ saturated aqueous solution (5 mL) and petroleum ether (5 mL) were added to the reaction. The organic phase was separated. The aqueous phase was washed with EtOAc (5 mL), carefully acidified with HCl to a value of pH ≤ 3, and extracted with EtOAc (3× 5 mL). The combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by flash silica gel column chromatography ($CHCl_3$/MeOH, 9.5:0.5) giving **(*t,e*)-18** (10 mg) in 38% yield.

^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 1.40–1.49 (2s, 9H, CH_3Boc); 1.51–1.60 (4s, 6H, CH_3); 2.50 (2d, 1H, *J* = 4.3, 6.6 Hz, H-4); 2.69 (d, 1H, *J* = 6.6 Hz, H-4); 3.74 (2s, 3H, OCH_3); 4.14 (m, 1H, H-2); 4.43 (m, 1H, H-3). *m*/*z* (ESI) = 340 Da (M+Na^+), 318 Da (M+H^+).

*4.13. Synthesis of tert-butyl (2S)-1-(methoxycarbonyl)-2-acetoxy-4-(benzyloxy)butyl carbamate **((t,e)-19)***

To a solution of **(*t,e*)-16** (10 mg, 0.028 mmol) in THF (0.5 mL), triethylamine (TEA, 0.04 mL, 0.238 mmol), acetic anhydride (0.016 mL, 0.283 mmol) and dimethylaminopyridine (DMAP, 0.35 mg, 0.002 mmol) were added. The reaction was stirred at r.t. for 24 h and monitored by TLC (petroleum ether/EtOAc 6:4: R_f: **(*t,e*)-19** = 0.69). The mixture was then concentrated *in vacuo* and the residue taken up in EtOAc and washed (3× 0.5 mL) with HCl (1 M). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was purified by flash silica gel column chromatography (petroleum ether/EtOAc 7:3) giving **(*t,e*)-19** (7 mg) in 63% yield. HPLC [column: Chiralpack IA, eluent: petroleum ether/*i*-PrOH 9.5:0.5, flow: 0.6 mL/min, λ = 254 nm, retention times: **(*t*)-19** = 16.167 min; **(*e*)-19** = 24. 608 min].

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 1.44 (s, 3.5H, $CH_3Boc_{erythro}$); 1.45 (s, 5.5H, CH_3Boc_{threo}); 1.86–1.99 (m, 2H, H-$4_{erythro/threo}$); 1.97 (s, 1.8H, CH_3CO_{threo}); 1.98 (s, 1.2H, $CH_3CO_{erythro}$); 3.44–3.55 (m, 2H, H-$5_{erythro/threo}$); 3.72 (s, 1.8H, OCH_{3threo}); 3.74 (s, 1.2H, $OCH_{3erythro}$); 4.42–4.51 (m, 2.6H, H-2_{threo}, H-$6_{erythro/threo}$); 4.65 (bs, 0.4H, H-$2_{erythro}$); 5.18 (d, 0.6H, *J* = 9.5 Hz, NH_{threo}); 5.28 (m, 0.4H, H-$3_{erythro}$); 5.41 (bs, 0.4H, $NH_{erythro}$); 5.54 (m, 0.6H, H-3_{threo}); 7.27–7.35 (m, 5H, ArH). *m*/*z* (ESI) = 418 Da (M+Na^+), 396 Da (M+H^+).

*4.14. Screening of commercially available lipase preparations for the diastereoselective acetylation of **(t,e)-16***

To a solution of **(*t,e*)-16** (5 mg, 0.041 mmol) in methyl *tert*-butyl ether (MTBE, 1 mL) and vinyl acetate (100 μL) seven commercially available lipase preparations (lipase PS from *Pseudomonas*, lipase from *Chromobacterium viscosum*, lipase from *Candida rugosa*, porcine pancreatic lipase, lipozyme, subtilisin and Novozym 435) were added. The reaction was shaken at 45 °C and 250 rpm and monitored by TLC and by HPLC (column: Chiralpack IA, eluent: petroleum ether/*i*-PrOH 9.5:0.5, flow: 0.6 mL/min). Lipase from *Pseudomonas*, lipase from *Candida rugosa* and Novozym435 were able to accept the epimeric mixture as a substrate, but only the

lipase from *Pseudomonas* resulted to be diastereoselective transforming the epimer at 41.176 min of **(*t,e*)-16** into the acetylated derivative with a retention time of 16.167 min.

4.15. Immobilization of lipase from Pseudomonas

The enzyme (Lipase PS from Amano, 3 g) was carefully mixed with celite (HyfloSuperCel, 10 g) and phosphate buffer 0.1 M pH = 7 was added (10 mL). The mixture was vigorously stirred and air dried at room temperature for three days.

4.16. Preparative kinetic resolution of (*t,e*)-16

To a solution of **(*t,e*)-16** (100 mg, 0.283 mmol) in methyl *tert*-butyl ether (MTBE, 18 mL) vinyl acetate (2 mL) and immobilized (on celite) lipase from *Pseudomonas* (2 g) were added. The reaction was shaken at 45 °C and 250 rpm for 4 days and monitored by TLC (petroleum ether/EtOAc 6:4; R_f: **(*t,e*)-16** = 0.41, **(*t*)-19** = 0.69] and by HPLC (column: Chiralpack IA, eluent: petroleum ether/*i*-PrOH 9.5:0.5, flow: 0.6 mL/min, λ = 254 nm, retention times: **(*t*)-19** = 16.167 min, **(*e*)-16** = 44.475 min). Conversion **(*t*)-16** → **(*t*)-19** resulted to be >98% by HPLC analysis. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The crude residue was then purified by flash silica gel column chromatography (petroleum ether/EtOAc 8:2) giving **(*t*)-19** (45 mg, d.e. and e.e. >98%) in 45% yield and **(*e*)-16** (29 mg, d.e. and e.e. >98%) in 29% yield.

^{1}H NMR **(*e*)-16** (500 MHz, $CDCl_3$) δ (ppm): 1.44 (s, 9H, CH_3); 1.80–1.92 (m, 2H, H-4); 3.63–3.73 (m, 2H, H-5); 3.75 (s, 3H, OCH_3); 4.08 (m, 1H, H-3); 4.34 (bs, 1H, H-2); 4.51 (s, 2H, H-6); 5.45 (bs, 1H, NH); 7.28–7.35 (m, 5H, ArH).

^{13}C NMR **(*e*)-16** (125 MHz, $CDCl_3$) δ (ppm): 28.62 (CH_3Boc); 33.42 (C-4); 52.63; 58.70; 68.81; 72.65; 73.70; 80.55; 128.00 (C–Ar); 128.11 (C–Ar); 128.78 (C–Ar); 138.10 (C–ipso); 156.04 (C–O); 171.37 (C–O).

^{1}H NMR **(*t*)-19** (500 MHz, $CDCl_3$) δ (ppm): 1.45 (s, 9H, CH_3); 1.93–1.96 (m, 2H, H-4); 1.97 (s, 3H, CH_3CO); 3.48–3.55 (m, 2H, H-5); 3.72 (s, 3H, OCH_3); 4.45–4.51 (m, 3H, H-2, H-6); 5.18 (d, 1H, *J* = 9.5 Hz, NH); 5.54 (m, 1H, H-3); 7.27–7.35 (m, 5H, ArH).

^{1}H NMR **(*t*)-19** (500 MHz, d_6-benzene) δ (ppm): 1.41 (s, 9H, CH_3); 1.54 (s, 3H, CH_3CO); 1.83–1.95 (m, 2H, H-4); 3.22–3.37 (m, 2H, H-5); 3.30 (s, 3H, OCH_3); 4.19–4.27 (2d, 2H, *J* = 12.1 Hz, H-6); 4.75 (dd, 1H, *J* = 1.4, 9.7 Hz, H-2); 5.38 (d, 1H, *J* = 9.7 Hz, NH); 5.80 (m, 1H, H-3); 7.06–7.27 (m, 5H, ArH).

4.17. Synthesis of tert-butyl (1S,2S)-1-(methoxycarbonyl)-2-acetoxy-4-(benzyloxy)butyl carbamate ((*e*)-19)

To a solution of **(*e*)-16** (10 mg, 0.028 mmol) in THF (0.5 mL), triethylamine (TEA, 0.04 mL, 0.238 mmol), acetic anhydride (0.016 mL, 0.283 mmol) and dimethylaminopyridine (DMAP, 0.35 mg, 0.002 mmol) were added. The reaction was stirred at r.t. for 24 h and monitored by TLC (petroleum ether/EtOAc 6:4: R_f: **(*e*)-19** = 0.69). The mixture was then concentrated *in vacuo* and the residue taken up in EtOAc and washed (3× 0.5 mL) with HCl (1 M). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was purified by flash silica gel column chromatography (petroleum ether/EtOAc 7:3) giving **(*e*)-19** (7 mg) in 63% yield. HPLC (column: Chiralpack IA, eluent: petroleum ether/*i*-PrOH 9.5:0.5, flow: 0.6 mL/min, λ = 254 nm, retention times: **(*e*)-19** = 24.608 min).

^{1}H NMR **(*e*)-19** (500 MHz, $CDCl_3$) δ (ppm): 1.44 (s, 9H, CH_3); 1.86–1.99 (m, 2H, H-4); 1.98 (s, 3H, CH_3CO); 3.44–3.54 (m, 2H, H-5); 3.74 (s, 3H, OCH_3); 4.42–4.50 (2d, 2H, *J* = 11.9, 11.9 Hz, H-6); 4.65 (bs, 1H, H-2); 5.28 (m, 1H, H-3); 5.41 (bs, 1H, NH); 7.28–7.35 (m, 5H, ArH).

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