Tetrahedron 64 (2008) 5079–5084

Contents lists available at ScienceDirect

Tetrahedron

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

Enzymatic synthesis of ω-carboxy-β-hydroxy-(L)-α-amino acids

Francesca Sagui [a,b], Paola Conti [b], Gabriella Roda [b], Roberto Contestabile [c], Sergio Riva [a,*]

[a] Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milano, Italy
[b] Istituto di Chimica Farmaceutica e Tossicologica, Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milano, Italy
[c] Dipartimento di Scienze Biochimiche, Università di Roma 'La Sapienza', Piazzale A. Moro 5, 00185 Roma, Italy

ARTICLE INFO

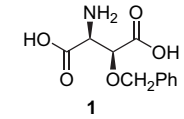
Article history:
Received 13 December 2007
Received in revised form 25 February 2008
Accepted 19 March 2008
Available online 22 March 2008

ABSTRACT

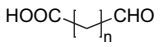
Commercially available ω-carboxy-aldehydes and glycine have been subjected to the catalytic action of an L-threonine aldolase from *Escherichia coli* to give the corresponding β-hydroxy-α-(L)-amino acids as a mixture of *erythro*/*threo* epimers.
Specifically, the reaction with glyoxylic acid (**2**) gave the epimeric β-hydroxy-(L)-aspartates **(*t*,*e*)-9** that could be isolated by ion-exchange chromatography in 67% yield. Following esterification and *N*-Boc protection, the two epimers could be isolated as pure compounds.
Similarly, the aldolase-catalyzed addition of glycine to succinic semialdehyde (**4**) gave the expected mixture of β-hydroxy-L-α-aminoadipic acids **(*t*)-12** and **(*e*)-12** in 34% yield.

1. Introduction

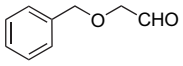
The amino acid L-glutamate (Glu) is considered to be the major mediator of excitatory signals in the mammalian central nervous system (CNS) and is probably involved both in brain development and in most aspects of its normal function including cognition, memory, and learning.[1–6] Due to its potential neurotoxicity, the extracellular concentration of glutamate is tightly controlled by high-affinity Na^+-dependent excitatory amino acid transporters (EAATs) that maintain Glu concentration below the excitotoxic level.[5,7] A number of pharmacological agents are capable of inhibiting glutamate transport and most of them act as competitive substrates. At present, attention is focused on non-transportable inhibitors (or blockers), compounds that, in pathological conditions, could counteract EAAT-mediated glutamate release in the synaptic cleft. As reported in the literature,[8] derivatives of D,L-*threo*-β-hydroxyaspartate (D,L-THA) possessing an ethereal bulky substituent, such as a benzyl or a naphthyl group, function as blockers at all subtypes of EAATs. In particular, D,L-*threo*-β-benzyloxy-aspartate (D,L-TBOA) is a non-transportable blocker of the glutamate transporters, widely recognized as a reference compound for biological screenings on the physiological roles of these proteins. The L-isomer is more efficacious than the D-enantiomer and the *threo* isomer (**1**) is more potent as a blocker than its *erythro* counterpart.[8]



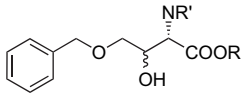
1



2 , n = 0
3 , n = 1
4 , n = 2



5



(*t*,*e*)-**6** : R = H ; R' = H_2
(*t*,*e*)-**7** : R = Me ; R' = H_3^+ Cl^-
(*t*,*e*)-**8** : R = Me ; R' = HBoc

Generally speaking, β-hydroxy-α-amino acids (i.e., **1**) are a group of interesting compounds that can be found in a number of complex natural products possessing a wide range of biological activities, i.e., they can act as antibiotics and immunosuppressants.[9] In addition, these amino acids are useful building blocks in synthetic, combinatorial, and medicinal chemistries.[10] A number of reports describe the synthesis of these compounds, both as racemates and as single enantiomers.[11] Specifically, optically pure β-hydroxy-aspartate derivatives (like **1**) were prepared with protocols that required several synthetic steps and the use of toxic reagents, such as OsO_4.[8]

The biocatalyzed preparation of these compounds has been suggested as a complement to the existing chemical methods; the advantages of an enzymatic strategy, usually performed in a single step process, being the mild conditions and the minimal need for substrate protection.[12] Accordingly, in recent years, L- and D-threonine aldolases have been studied for the non-physiological synthesis of β-hydroxy-α-amino acids using various aldehydes as

* Corresponding author. Tel.: +39 02 2850 0032; fax: +39 02 2890 1239.
E-mail address: sergio.riva@icrm.cnr.it (S. Riva).

0040-4020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2008.03.070

acceptors and glycine as a donor.[13] In order to extend this methodology to the preparation of ω-carboxy-β-hydroxy-α-amino acids (analogues of **1**), we have studied the performances of an L-threonine aldolase (L-TA) from *Escherichia coli*[14] with structurally different aldehydes, and in the following we present the preliminary results obtained.

2. Results and discussion

Threonine aldolases (TAs) are pyridoxal-5′-phosphate (PLP)-dependent enzymes that catalyze the reversible cleavage of threonine (or *allo*-threonine) into glycine and acetaldehyde.[15] Genes encoding for these proteins have been found in plants, vertebrates, and several bacteria, yeasts, and fungi.[16]

Two types of TAs exist, depending on the configuration of the newly formed stereocenter at the α-carbon. Both L- and D-TA have a strict requirement for glycine as a donor, but can accept a wide range of aliphatic and aromatic aldehydes as electrophilic substrates.[13,17] As shown in Figure 1 for an L-TA-catalyzed condensation, the reactions proceed with complete stereoselective control at the α-carbon and the outcome is the sole (L)-epimer. On the other hand, these enzymes are usually unable to control the stereochemistry at the β-carbon and produce a mixture of diastereoisomers in moderate yields. Quite recently, both these drawbacks have been overcome by means of a bi-enzymatic dynamic kinetic asymmetric transformation based on the consecutive catalysis of a low-specific L-threonine aldolase and of a highly selective L-tyrosine decarboxylase.[18] This methodology allowed the synthesis of a number of enantiopure amino alcohols.

To the best of our knowledge, threonine aldolases have never been tested with aldehydes carrying a carboxylic substituent (e.g., glyoxylic acid **2**, malonic semialdehyde **3**, and succinic semialdehyde **4**), despite the fact that the enzyme-catalyzed condensation of glycine with these compounds could offer a direct approach to epimeric β-hydroxy-aspartates, β-hydroxy-glutamates, and higher homologues.

Threonine aldolases are still not commercially available, and therefore our investigation started from the production of the enzyme, fermenting an *E. coli* strain overexpressing an L-TA and partially purifying it via ammonium sulfate precipitation and ion-exchange chromatography (for details see Section 4).

In order to optimize the reaction conditions and the product recovery, the biocatalyzed reaction was initially tested on benzyloxyacetaldehyde (**5**), a compound that has been reported to be a good substrate for these enzymes.[13c] L-TA-catalyzed condensation of glycine with **5** gave a product of intermediate polarity (**(*t*,*e*)-6**, detected by TLC using the ninhydrin reagent). The reaction mixture was extracted with Et_2O to remove the unreacted aldehyde and lyophilized. The crude residue was further elaborated to new derivatives that could be more easily isolated and characterized. This aspect was not fully investigated in previous articles as, quite often, the reported reaction yields and *threo*/*erythro* ratios were only evaluated on the bases of the ^1H NMR spectra of the product mixtures (with or without unreacted glycine). The derivatization reactions were quite straightforward and consisted in the esterification of the carboxylic acid moiety and *N*-Boc protection of the amino group. The latter step, however, required a careful optimization of the reaction conditions, since β-hydroxy-α-amino acids are quite prone to give retroaldolic reactions. The problem was solved by performing the reaction in a biphasic system in which the substrate (**(*t*,*e*)-7**) was dissolved in water and the Boc_2O confined in the organic phase. Dropwise addition of a solution of sodium bicarbonate to the water phase neutralized the hydrochloride **7**, which was transferred into the organic phase to react with $(Boc)_2O$ producing the expected *N*-protected derivatives **(*t*,*e*)-8**. The excess of glycine present in the crude mixture was similarly derivatized and the new mixture could be easily separated by flash chromatography on silica gel, allowing the separation of the *threo* and *erythro* β-hydroxy-amino acid derivatives **(*t*,*e*)-8** from *N*-Boc-glycine methyl ester, giving a mixture of the two β-epimers in 16% isolated yields. The obtained products were characterized by ^1H NMR, which showed the expected presence of the two *threo* and *erythro* diastereoisomers in an approximately 1:1 ratio.

Glyoxylic acid (**2**) was the first carboxy-aldehyde investigated, as the *threo* product that could be obtained by L-TA-catalyzed aldol condensation is a direct precursor of the previously cited L-TBOA (**1**).[8] A methodology capable of producing stereoisomerically pure L-β-hydroxyaspartate in a single step could be very useful to generate a library of derivatives, differently substituted at the alcoholic moiety.

Following the previously discussed protocol, glyoxylic acid (**2**) was used as a donor in the presence of glycine and, to our satisfaction, the formation of a more polar product was clearly detected by TLC. In order to increase the condensation yields, the reaction was then performed in the presence of an excess (5 equiv) of glycine and stopped, after 48 h, by snap freezing and lyophilized. The separation of the amino acids from the enzyme and the excess of glycine present in the crude residue became possible using a strong ion-exchange resin, by taking advantage of their different pK_a values. The reaction mixture, dissolved in water at neutral pH, was loaded on a strongly basic ion-exchange column. Glycine was not kept by the resin and was eluted in the void volume, while, conversely, the desired products **(*t*,*e*)-9** were eluted with a 0.1 M formic acid solution and, following lyophilization, were isolated in 67% yields (Fig. 2).

^1H and ^{13}C NMR analyses confirmed the proposed structures and allowed us to evaluate as 1:1 the *threo*/*erythro* ratio of the products, thus confirming the absence of any β-stereoselectivity in

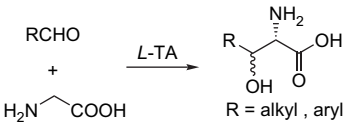


Figure 1. L-TA-catalyzed condensation of a generic aldehyde and glycine to give β-hydroxy-α-L-amino acids.

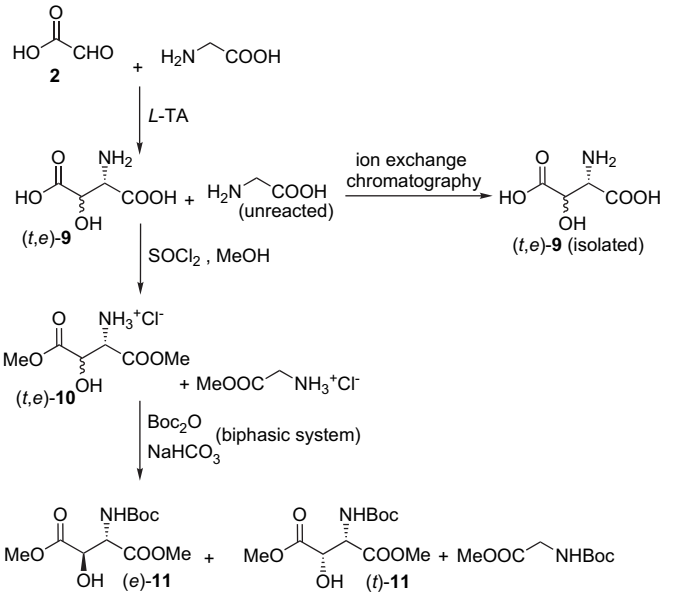


Figure 2. L-TA-catalyzed condensation of glyoxylic acid (**3**) and glycine, and subsequent chemical elaboration of the products.

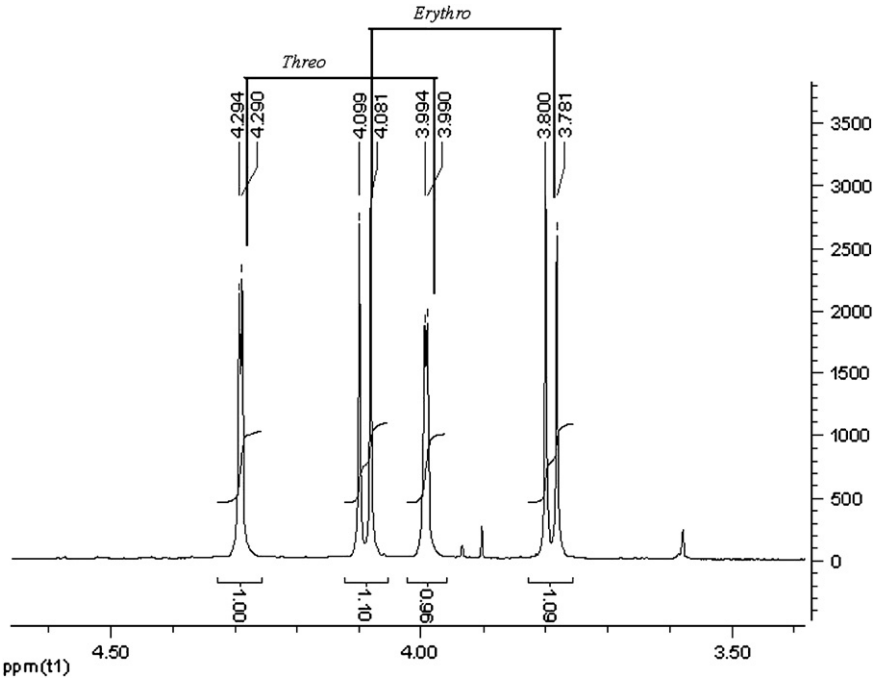


Figure 3. ^{1}H NMR spectrum of **(*t*,*e*)-9** in DMSO-d_6.

the L-TA-catalyzed condensation. The assignment of the signals due to the two epimers **(*t*,*e*)-9** was made possible by a comparison of the ^{1}H NMR spectrum of the mixture with that due to the commercially available racemic mixture of (D,L)-*threo*-β-hydroxy aspartic acid. The ^{1}H NMR of **(*t*,*e*)-9** (in DMSO-d_6) is reported in Figure 3. The protons of the *threo* isomer were downfield shifted and characterized by a smaller spin-coupling constant (J_{threo}= 1.2 Hz) in comparison to the corresponding signals of its epimer ($J_{erythro}$=8.0 Hz).

As this protocol of purification did not allow the separation of the two epimers, we transformed the mixture into the corresponding *N*-Boc-β-hydroxyaspartate methyl esters, **(*t*)-11** and **(*e*)-11** (Fig. 2), which could be separated by flash chromatography and characterized by NMR and mass analyses (see Section 4). The assignment of the absolute configurations of the two products was made possible by comparison with literature values.[8]

Since β-hydroxy-L-α-aminoadipic acids **(*t*)-12** and **(*e*)-12** have never been tested as potential glutamate transporter inhibitors, we applied the L-TA-catalyzed condensation of glycine to succinic semialdehyde **4**, which is commercially available. The biotransformation was carried out following the usual protocol, using an excess (5 equiv) of glycine. The reaction mixture was lyophilized and the crude residue purified from the excess of glycine by ion-exchange chromatography to give the expected mixture of products **(*t*,*e*)-12** in 34% yields.

The structural characterization of these compounds was quite troublesome, as the formed ε-carboxy-β-hydroxy-α-amino acids **(*t*,*e*)-12** gave under slightly acidic conditions a mixture of the lactones **13** and of other isomers like the epimeric lactams **(*a*)-14** and **(*e*)-14**, a phenomenon that was already described in the literature.[19] This problem could be avoided by making the solution of the recovered products alkaline before lyophilization (see Section 4). In this way, it was possible to confirm by ^{1}H NMR the structure of compounds **(*t*,*e*)-12**, and the usual lack of β-selectivity (Fig. 4) was observed.

The ratio between the *threo* and *erythro* epimers could be easily evaluated by comparing the area of the peaks due to the α-protons of the two isomers, two doublets resonating at 3.29 and 3.16 ppm.

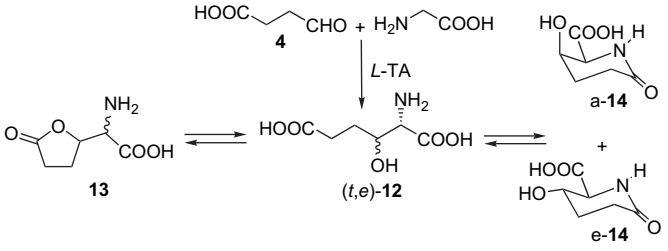


Figure 4. L-TA-catalyzed condensation of succinic semialdehyde (**4**) and glycine, and subsequent chemical elaboration of the products.

Specifically, in this case the *threo* isomer was formed in slightly higher amounts, the *threo*/*erythro* ratio being 3:2. The assignment of the absolute configuration was done by comparing the chemical shifts and the coupling constants of the signals due to these epimers with those of the β-hydroxy-aspartic acid (**(*t*,*e*)-9**). The structures of the two lactams **(*a*)-14** and **(*e*)-14**, formed when the purified mixture of **(*t*,*e*)-9** was kept at acid pH, could also be assigned by mono- and bi-dimensional ^{1}H NMR.

3. Conclusions

We have shown that ω-carboxy-aldehydes can be used as substrates in L-TA-catalyzed aldolic condensations, giving—in one-step—ω-carboxy-β-hydroxy-L-α-amino acids as *threo*/*erythro* mixtures. The epimeric products could be isolated by ion-exchange column chromatography and, in the case of the β-hydroxy-aspartates, the two epimers could be also separated after their transformation into more lipophilic derivatives. These compounds can be used as a starting material for the synthesis of a wide range of molecules carrying different substituents at the hydroxylic moiety, which could perform as inhibitors of glutamate transporters.

Work is in progress to extend this protocol to the not commercially available malonic semialdehyde **3** and the results will be reported in due course.

4. Experimental part

4.1. Materials and methods

Aldehydes **2**, **4**, and **5** were purchased from Sigma–Aldrich. All the other reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise stated. *E. coli* transfected with the gene encoding for L-threonine aldolase was provided by Prof. R. Contestabile, University of Rome.

Fermentations were performed with a 1 l fermentor Inova 4230 and sterilizations were performed using autoclave Steristeam CDL. Enzymatic activities were monitored using a Jasco V-530 UV/VIS spectrophotometer. Enzyme purification was partially 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). Mass spectra were recorded with an FT-ICR (Fourier Transfer Ion Cyclotron Resonance) APEX™ II model (Bruker Daltonics) equipped with a 4.7 T crio-magnet (Magnex).

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 (Tripton 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) supplemented with 100 mg/l ampicillin and 30 mg/L of vitamin B_6. 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 pre-culture and grown at 37 °C to an OD_{600} of 0.3 and then induced with IPTG at a final concentration of 0.05 M. 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 DNA by adding streptomycin sulfate (10 g/l dissolved in 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 growing was performed in a 1 l capacity fermentor (INFORS AG CH-4103), following the same protocol developed for the suspended cell systems, monitoring oxygen supply, pH, temperature, and stirring. 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), and yeast alcohol dehydrogenase (32 U) were added into a 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 ($\varepsilon=6.2\times10^3$ l/mol cm) 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 2-(*S*)-amino-3-(*R,S*)-hydroxy-4-benzyloxy-butanoic acid (*t,e*)-6

To a solution of benzyloxyacetaldehyde (**5**, 685 μl, 5.3 mmol), glycine (400 mg, 5.3 mmol), pyridoxal phosphate (PLP, 1.7 mg, 0.125 mM), and dithiothreitol (DTT, 24.6 mg, 3 mM) in 50 ml of H_2O, NaOH (1 M) was added up to pH=7.5. L-TA (25 U) was recovered by centrifugation from the stored suspension, solubilized in 3 ml of water, and added to the reaction mixture, which was stirred for 48 h at 45 °C and monitored by TLC (eluent: BuOH–H_2O–AcOH 4:2:1; R_f: **(*t,e*)-6**=0.48, glycine=0.18, **5**=0.90; detection was carried out with the ninhydrin reagent).

The reaction mixture was then extracted with Et_2O (2×150 ml) to remove the unreacted **5** and the water phase lyophylized to give a yellow powder. The excess of glycine was precipitated by adding cold MeOH (100 ml), the suspension was filtered, the solvent dried over Na_2SO_4, and evaporated to give the epimeric mixture of the products (**(*t,e*)-6**, 146 mg, 13% yields) as a white solid.

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 3.25 (d, J=6.0 Hz, H-2_{threo}), 3.27 (d, J=3.7 Hz, H-$2_{erythro}$): total 1H; 3.49 (dd, J_1=6.0 Hz, J_2=10.1 Hz, H-$4_{a,threo}$) and 3.53 (dd, J_1=5.8 Hz, J_2=10.1 Hz, H-$4_{a,erythro}$): total 1H; 3.57 (dd, J_1=4.1 Hz, J_2=10.1 Hz, H-$4_{b,threo}$) and 3.59 (dd, J_1=5.8 Hz, J_2=10.1 Hz, H-$4_{b,erythro}$): total 1H; 3.94 (dt, J_1=6.0 Hz, J_2=4.1 Hz, H-3_{threo}) and 4.13 (dt, J_1=3.7 Hz, J_2=5.8 Hz, H-$3_{erythro}$): total 1H, H-3; 4.53 (2H, s, $ArCH_2$); 7.10–7.40 (5H, m, H–Ar).

4.5. Synthesis of methyl 2-(*S*)-*tert*-butoxycarbonylamino-3-(*R,S*)-hydroxy-4-benzyloxy-butanoate (*t,e*)-8

(a) The crude residue obtained from the previous biocatalyzed condensation was subjected to the following reaction without preliminary purification in order to avoid product loss during the methanolic precipitation of the excess of glycine.

A solution of thionyl chloride (507 μl, 7 mmol) in MeOH (1.1 ml) was cooled to 0 °C and crude **(*t,e*)-6** (400 mg, 2.3 mmol) was added in aliquots under stirring. The mixture was left at 0 °C for 1 h and then at room temperature for 48 h (TLC eluent: BuOH–H_2O–ACOH 4:2:1; R_f: **(*t,e*)-6**=0.48, glycine=0.18, **(*t,e*)-7**=0.60, glycine methyl ester=0.25; detection was carried out with the ninhydrin reagent).

Removal of the liquids under vacuum afforded the methyl esters of **(*t,e*)-6** as a viscous oil, which was subjected to the subsequent reaction.

(b) A mixture of the methyl esters **(*t,e*)-7** (2.32 mmol) dissolved in water (1 ml) and EtOAc (4.7 ml) was cooled to 0 °C followed by the addition of *tert*-butoxycarbonyl anhydride (Boc_2O, 506 mg, 2.32 mmol) under stirring. A saturated solution of $NaHCO_3$ was added dropwise until the pH of the water phase became slightly basic. The reaction mixture was allowed to proceed at 0 °C for 2 h and then at room temperature overnight (TLC eluent: AcOEt–petroleum ether 4:6; R_f: **(*t,e*)-7**=0.10, **(*t,e*)-8**=0.61, *N*-Boc-glycine methyl ester=0.70; detection was carried out with ninhydrin).

The organic fraction was separated and the water phase extracted with EtOAc (3×50 ml). The organic phases were collected together, washed with brine (2×20 ml), and dried over Na_2SO_4. The solvent was removed in vacuo to give the epimeric mixture **(*t,e*)-8** as a viscous colorless oil that was purified by silica gel flash chromatography (eluent: petroleum ether–AcOEt 8:2) to give 80 mg of **(*t,e*)-8** (0.25 mmol, 16% yields calculated on the amount of **5** used

for the biocatalyzed aldolic condensation). The solid was characterized by mono- and bi-dimensional ^{1}H and ^{13}C NMR analyses, which confirmed the structure and the 1:1 ratio of the two diastereomers (*threo*/*erythro*).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.46 (s, 9H, Boc), 3.53–3.61 (m, 2H, H-4$_{erythro,threo}$), 3.68 (s, 3H, OMe), 3.75 (s, 3H, OMe), 4,19 (br m, 1H, H-3$_{threo}$), 4.25–4.42 (br m, 2H, H-2$_{erythro,threo}$), 4.42–4.52 (m, 3H, H-3$_{erythro}$, $ArCH_{2threo}$), 4.54 (s, 2H, $ArCH_{2erythro}$), 5.35 (s, 1H, NH), 5.56 (s, 1H, NH), 7.10–7.40 (m, 5H, H–Ar). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm) 29.0 ($(CH_3)_3C$); 52.3 (OCH_3); 55.6 and 57.2 (C-2$_{erythro,threo}$); 70.5 and 71.2 (C-4); 73.8 ($ArCH_2$); 80.4 and 80.7 (C-3); 128.2, 128.8 and 137.9 (C–Ar); 156.2 and 156.4 ($(CH_3)_3C$–*C*=O); 171.2 and 171.5 (*C*OOMe).

4.6. Synthesis of 2-(*S*)-amino-3-(*R*,*S*)-hydroxy-succinic acid (*t*,*e*)-9

To a solution of glyoxylic acid (**2**, 250 mg, 2.71 mmol), glycine (5 equiv, 1018 mg, 13.60 mmol), pyridoxal phosphate (PLP, 2 mg, 0.125 mM), and dithiothreitol (DTT, 32 mg, 3 mM) in 65 ml H_2O, NaOH (1 M) was added up to pH=7.5. L-TA (60 U) was recovered by centrifugation from the storing suspension, solubilized in 5 ml water, and added to the reaction mixture, which was stirred at 45 °C for 48 h and monitored by TLC (eluent: BuOH–H_2O–AcOH 4:2:1; R_f: **(*t*,*e*)-9**=0.15, glycine=0.18; detection was carried out with the ninhydrin reagent). The mixture was then lyophilized to give a yellow powder that was either purified by ion-exchange chromatography or derivatized as previously described.

The mixture was purified using a strong basic ion-exchange resin (Ionac A-540, Cl^- form) previously conditioned as follows: a column (diameter 2 cm, height 42 cm) was filled with 40 g of resin that was previously washed twice with 1 M NaOH, water up to neutral pH, 2 M AcOH, and water up to neutral pH. The crude products were then dissolved in water at pH 7.5 and loaded onto the column. The resin was initially eluted with water to remove the excess of glycine and then washed with 0.1 M formic acid to elute the product. The fractions containing the product were collected and lyophylized to give the epimeric mixture **(*t*,*e*)-9** as a white powder (272 mg, 1.83 mmol, 67% yields calculated on the amount of **2** initially used for the biotransformation).

The product was characterized by mono- and bi-dimensional ^{1}H and ^{13}C NMR, which also allowed the evaluation of the diastereoisomeric *threo*/*erythro* ratio (1:1) of **(*t*,*e*)-9**.

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): **(*e*)-9**: 3.78 (d, 1H, *J*=8.0 Hz, H-2), 4.06 (d, 1H, *J*=8.0 Hz, H-3); **(*t*)-9**: 3.98 (d, 1H, *J*=1.2 Hz, H-2), 4.26 (d, 1H, *J*=1.2 Hz, H-3). ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): **(*e*)-9**: 55.16 (C-2); 70.71 (C-3); 168.08 and 171.77 (C-1 and C-4). **(*t*)-9**: 54.19 (C-2); 67.06 (C-3); 168.63 and 172.67 (C-1 and C-4).

4.7. Synthesis of dimethyl 2-(*S*)-*tert*-butoxycarbonylamino-3-(*R*,*S*)-hydroxy-succinate (*t*,*e*)-11

A solution of thionyl chloride (1.45 ml, 19.8 mmol) in MeOH (3.14 ml) was cooled to 0 °C and the crude residue of the biocatalyzed condensation (**(*t*,*e*)-9**, 500 mg, 6.6 mmol) was added in aliquots under stirring. The reaction was allowed to proceed at 0 °C for 1 h and then at room temperature for 48 h (TLC eluent: BuOH–H_2O–AcOH 4:2:1; R_f: **(*t*,*e*)-9**=0.15, dimethyl esters of **(*t*,*e*)-9**=0.20; detection was carried out with ninhydrin). Removal of the liquids in vacuo afforded the dimethyl esters **(*t*,*e*)-10** as a viscous oil, which was immediately used for the subsequent Boc protection reaction.

The previously obtained mixture of the dimethyl esters of **(*t*,*e*)-10** (6.6 mmol) was dissolved in water (1.5 ml) and AcOEt (10 ml). The biphasic solution was cooled to 0 °C and *tert*-butoxycarbonyl anhydride (Boc_2O, 1.4 g, 6.6 mmol) was added under stirring. A saturated solution of $NaHCO_3$ was added dropwise until the pH of the water phase became slightly basic. The reaction was allowed to proceed at 0 °C for 2 h and then at room temperature overnight, following the conversion by TLC (eluent: AcOEt–petrolem ether 4:6; R_f: **(*t*)-11**=0.35, **(*e*)-11**=0.44; detection was carried out with ninhydrin). The organic phase was separated and the water phase extracted with AcOEt (3×50 ml); the organic phases were collected, washed with brine (2×20 ml), and dried over Na_2SO_4. The solvent was removed in vacuo to give the epimeric *threo*/*erythro* mixture **(*t*,*e*)-11** that could be purified by silica gel flash chromatography (eluent: petroleum ether–AcOEt 85:15, then petroleum ether–AcOEt 70:30) affording the two pure epimers **(*t*)-11** (55 mg, 0.20 mmol) and **(*e*)-11** (60 mg, 0.22 mmol) as viscous colorless oils.

The two epimers were characterized by mono- and bi-dimensional ^{1}H NMR, ^{13}C NMR, and mass analyses, which confirmed the proposed structures. The relative configuration (*threo*/*erythro*) of the two products could be assigned by comparison with the literature values.[8]

Compound ***(e)-11***. ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.47 (s, 9H, Boc), 3.82 (s, 3H, OMe), 3.84 (s, 3H, OMe), 4.71 (br s, 1H, H-3), 4.80 (d, 1H, *J*=9.2 Hz, H-2), 5.31 (br d, 1H, N*H*). ^{13}C NMR (300 MHz, $CDCl_3$), δ (ppm,): 28.50 ($(CH_3)_3C$); 53.23 (OCH_3); 56.51 (OCH_3); 71.34 (C-3); 56.44 (C-2); 155.0, 170, 173.0 (3C=O). FAB$^+$-MS: 278 $(M+H)^+$.

Compound ***(t)-11***. ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 1.44 (s, 9H, Boc), 3.73 (s, 3H, OMe), 3.83 (s, 3H, OMe), 4.52 (br s, 1H, H-3), 4.84 (d, 1H, *J*=7.6 Hz, H-2), 5.51 (br d, 1H, N*H*). ^{13}C NMR (300 MHz, $CDCl_3$), δ (ppm): 28.58 ($(CH_3)_3C$); 53.05 (OCH_3); 53.25 (OCH_3); 57.37 (C-2); 72.42 (C-3); 155.68, 169.18, 172.05 (3C=O). FAB$^+$-MS: 278 $(M+H)^+$.

4.8. Synthesis of 2-(*S*)-amino-3-(*R*,*S*)-hydroxy-6-carboxy-hexanoic acid (*t*,*e*)-12

To a solution of hemisuccinic aldehyde (**4**, 1.76 ml, 2.71 mmol), glycine (1.02 g, 13.57 mmol), pyridoxal phosphate (PLP, 4.25 mg, 0.125 mM), and dithiothreitol (DTT, 40 mg, 3 mM) in 13.6 ml of H_2O, NaOH (1 M) was added up to pH=7.5. L-TA (40 U) was recovered by centrifugation from the storing suspension, solubilized in 4 ml water, and added to the reaction mixture, which was stirred for 48 h at 45 °C and monitored by reverse phase TLC (eluent: MeOH–H_2O–AcOH 95:5:0.5; R_f: **(*t*,*e*)-12**=0.73, glycine=0.50; detection was carried out with ninhydrin).

The product mixture was purified using a strong basic ion-exchange resin (Ionac A-540, Cl^- form) previously conditioned with the following procedure: a column (diameter 2 cm, height 42 cm) was filled with 40 g of resin that was previously washed twice with 1 M NaOH, water up to neutral pH, 2 M AcOH, and water up neutral pH. The product mixture was dissolved in water at pH 7.5 and loaded onto the column; the resin was initially eluted with water to remove the excess of glycine and then washed with 0.1 M formic acid to elute the product. The recovered solution was lyophilized to give the epimeric mixture of the desired products **(*t*,*e*)-12** (163 mg, 0.92 mmol, 34% yields). The material was redissolved in water, the pH was adjusted to 11 using 1 M NaOH, and then the solution was lyophilized again to give a white solid that was analyzed by mono- and bi-dimensional ^{1}H and ^{13}C NMR.[19]

Compounds ***(t,e)-12***. ^{1}H NMR (400 MHz, D_2O), δ (ppm): 1.60–1.80 (m, 4H, CH_2-4$_{threo,erythro}$), 2.20–2.40 (m, 4H, CH_2-5$_{threo,erythro}$), 3.16 (d, 1H, *J*=6.5 Hz, H-1$_{threo}$), 3.29 (d, 1H, *J*=6.5 Hz, H-1$_{erythro}$), 3.75 (m, 1H, H-2$_{erythro}$), 3.79 (m, 1H, H-2$_{threo}$). ^{13}C NMR (400 MHz, D_2O), δ (ppm): *threo*: 29.71 (C-4); 33.82 (C-5); 59.81 (C-2); 72.69 (C-3); 180.47 (C-1); 182.48 (C-6); *erythro*: 27.58 (C-4); 33.68 (C-5); 60.11 (C-2); 72.92 (C-3); 179.55 (C-1); 182.58 (C-6).

The two epimeric lactams (**(*a*)-14**, **(*e*)-14**), spontaneously formed in acidic solution, were also characterized by mono- and

bi-dimensional ^{1}H and ^{13}C NMR. ^{1}H NMR (400 MHz, D_2O), δ (ppm): **(*a*)-14**: 1.51–1.59 (m, 1H, H-4_{eq}), 1.82–1.87 (m, 1H, H-4_{ax}), 2.21–2.26 (m, 2H, H-$5_{ax,eq}$), 3.12 (d, 1H, J=8.9 Hz, H-2), 3.49 (ddd, 1H, J_1=4.0 Hz, J_2=10.5 Hz, J_3=9.0 Hz, H-3), 6.83 (br s, 1H, NH); **(*e*)-14**: 1.61–1.71 (m, 2H, H-$4_{eq,ax}$), 1.94–2.07 (m, 1H, H-5_{eq}), 2.12–2.19 (m, 1H, H-5_{ax}), 3.41 (q, 1H, J=1.5 Hz, H-2), 4.05 (q, 1H, J=4.0 Hz, H-3), 6.63 (br s, 1H, NH). ^{13}C NMR (400 MHz, D_2O), δ (ppm): **(*a*)-14** and **(*e*)-14**: 27.13, 27.10 (C-4); 28.56, 29.20 (C-5); 58.82, 59.13 (C-2); 63.62, 67.31 (C-3); 168.82, 169.59 (C-1); 172.14, 172.60 (C-6).

Acknowledgements

We thank Dr. Martino Luigi di Salvo (Rome University), Dr. Daniela Monti, Dr. Lara Baratto, and Mrs. Federica Loiacono (ICRM-CNR) for their valuable technical assistance in the production of L-TA and in the work-up of the biocatalyzed reactions. Thanks are also due to Mr. Walter Panzeri (ICRM-CNR) for recording the NMR spectra.

References and notes

1. Danbolt, N. C. *Prog. Neurobiol.* **2001**, *65*, 1–105.
2. Pellicciari, R.; Costantino, G.; Macchiarulo, A. *Pharm. Acta Helv.* **2000**, *74*, 231–237.
3. Rao, V. R.; Finkbeiner, S. *Trends Neurosci.* **2007**, *30*, 284–291.
4. Schkeryantz, J. M.; Kingston, A. E.; Johnson, M. P. *J. Med. Chem.* **2007**, *50*, 2563–2568.
5. Kanai, Y.; Hediger, M. A. *Eur. J. Pharmacol.* **2003**, *479*, 237–247.
6. Beart, P. M.; O'Shea, R. D. *Br. J. Pharmacol.* **2007**, *150*, 5–17.
7. Levi, G.; Raiteri, M. *Trends Neurosci.* **1993**, *16*, 415–419.
8. (a) Shimamoto, K.; Sakai, R.; Takaoka, K.; Yumoto, N.; Nakajima, T.; Amara, S. G.; Shigeri, Y. *Mol. Pharmacol.* **2004**, *65*, 1008–1015; (b) Shimamoto, K.; Shigeri, Y.; Yasuda-Kamatani, Y.; Lebrun, B.; Yumoto, N.; Nakajima, T. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2407–2410.
9. Saeed, A.; Young, D. W. *Tetrahedron* **1992**, *48*, 2507–2514 and references therein.
10. Vassilev, V. P.; Uchiyama, T.; Kajimoto, T.; Wong, C. H. *Tetrahedron Lett.* **1995**, *36*, 5063–5064.
11. (a) Cardillo, G.; Gentilucci, L.; Tolomelli, A.; Tomasini, C. *Synlett* **1999**, 1727–1730; (b) Hanessian, S.; Vanasse, B. *Can. J. Chem.* **1993**, *71*, 1401–1406; (c) De Angelis, M.; Campiani, G. *Tetrahedron Lett.* **2004**, *45*, 2355–2357.
12. See, for instance: (a) Riva, S. *Trends Biotechnol.* **2006**, *24*, 219–226; (b) Monti, D.; Candido, A.; Cruz Silva, M. M.; Kren, V.; Riva, S.; Danieli, B. *Adv. Synth. Catal.* **2005**, *347*, 1168–1174; (c) Riva, S. *J. Mol. Catal. B: Enzym.* **2002**, *19 and 20*, 43–54; (d) Carrea, G.; Riva, S. *Angew. Chem., Int. Ed.* **2000**, *39*, 2226–2254 and references therein.
13. (a) Steinreiber, J.; Fesko, K.; Reisinger, C.; Schurmann, M.; van Assema, F.; Wolberg, M.; Mink, D.; Griengl, H. *Tetrahedron* **2007**, *63*, 918–926; (b) Nishide, K.; Shibata, K.; Fujita, T.; Kajimoto, T.; Wong, C. H.; Node, M. *Heterocycles* **2000**, *52*, 1191; (c) Vassilev, V. P.; Uchiyama, T.; Kajimoto, T.; Wong, C. H. *Tetrahedron Lett.* **1995**, *36*, 4081–4084.
14. Contestabile, R.; Paiardini, A.; Pascarella, S.; Di Salvo, M. L.; D'Aguanno, S.; Bossa, F. *FEBS J.* **2001**, *268*, 6508–6525.
15. Percudani, R.; Peracchi, A. *Embo Rep.* **2003**, *4*, 850–854.
16. Liu, J. Q.; Dairi, T.; Itoh, N.; Kataoka, M.; Shimizu, S.; Yamada, H. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 107–115.
17. (a) Kimura, T.; Vassilev, V. P.; Shen, G. J.; Wong, C. H. *J. Am. Chem. Soc.* **1997**, *119*, 11734–11742; (b) Shibata, K.; Shingu, K.; Vassilev, V. P.; Nishide, K.; Fujita, T.; Node, M.; Kajimoto, T.; Wong, C. H. *Tetrahedron Lett.* **1996**, *37*, 2791–2794.
18. (a) Steinreiber, J.; Schurmann, M.; Wolberg, M.; van Assema, F.; Reisinger, C.; Tesko, K.; Mink, D.; Griengl, H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1624–1626; (b) Steinreiber, J.; Schuermann, M.; van Assema, F.; Wolberg, M.; Fesko, K.; Reisinger, C.; Mink, D.; Griengl, H. *Adv. Synth. Catal.* **2007**, *349*, 1379–1386.
19. Jackson, B. G.; Pedersen, S. W.; Fisher, J. W.; Misner, J. W.; Gardner, J. P.; Staszak, M. A.; Doecke, C.; Rizzo, J.; Aikins, J.; Farkas, E.; Trinkle, K. L.; Vicenzi, J.; Reinhard, M.; Kroeff, E. P.; Higginbotham, C. A.; Gazak, R. J.; Zhang, T. Y. *Tetrahedron* **2000**, *56*, 5667–5677.