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Expanding the threonine aldolase toolbox for the asymmetric synthesis of tertiary α-amino acids

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Abstract The direct biochemical synthesis of tertiary α-amino acids with a wide range of diversity was recently reported using natural threonine aldolases LTA from *Aeromonas jandei* and DTA from *Pseudomonas* sp. Here, we describe the identification of five novel threonine aldolases which accept alanine and serine as amino acid donors. The enzymes were found by sequence database analysis using known aldolases as template. All enzymes were overexpressed in *Escherichia coli* and purified, and their biochemical properties were characterized. The new enantiocomplementary L- and D-threonine aldolases catalyze the asymmetric synthesis of β-hydroxy α-methyl- and α-hydroxymethyl-α-amino acids with good conversion and perfect enantioselectivity at α-carbon of the products (e.e. >99 %). The structural basis for the broad donor specificity of these threonine aldolases is analyzed based on crystal structure alignments and amino acid sequences comparison.

Keywords Threonine aldolase · Aldol reactions · Tertiary amino acids · Enzyme catalysis · Biocatalysis

Introduction

Enzyme-catalyzed approaches using aldolases proved to be an important tool for the asymmetric carbon-carbon bond formation and synthesis of polyfunctional compounds (Clapes and Garrabou 2011; Fesko and Gruber 2013). Aldolases catalyze the reversible aldol addition of a donor component (nucleophile) to an acceptor component (electrophile), with great control of stereochemistry at newly formed stereogenic centers. In general, aldolases can convert a variety of acceptor substrates, whereas the donor components are confined, thus narrowing the possible product range (Fessner 2011). Protein engineering techniques, screening for novel aldolase activities in nature and de novo design of aldolases are currently methods of choice to enable novel synthetic applications of aldolases (Baker and Seah 2012; Wildmann et al. 2012; Windle et al. 2014). Several attempts to change the donor specificity of aldolases have been performed by directed evolution approaches; however, only small structural, isosteric modification of the donor component could be implemented (Müller 2012; Fesko and Gruber 2013). On the other hand, exploring the inherent substrate promiscuity of some enzymes is an efficient method toward discovering catalysts with new properties (Bornscheuer and Kazlauskas 2004; Gatti-Lafranconi and Hollfelder 2013).

Threonine aldolases (TA) are pyridoxal 5′-phosphate (PLP)-dependent enzymes, which catalyze the reversible aldol reaction of glycine as donor with an aldehyde acceptor to form β-hydroxy-α-amino acids (Dückers et al. 2010). These enzymes are highly selective at the α-carbon of the amino acid product. Both L- and D-specific threonine aldolase activities have been found, and various microbial enzymes have been isolated and biochemically characterized so far. Most of the threonine aldolases accept a wide range of aliphatic and aromatic aldehydes as acceptor substrates but only glycine as donor substrate (Steinreiber et al. 2007a, b). Recently, we have reported a natural LTA from *Aeromonas jandei* (LTA_Aj) and a DTA from *Pseudomonas* sp. (DTA_Psp) with hitherto unprecedented donor promiscuity, which accept D-alanine, D-

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serine, and D-cysteine as donor substrates (Scheme 1) (Fesko et al. 2010). Moreover, a serine hydroxymethyl transferase (SHMT) enzyme with LTA activity was rationally designed to increase its promiscuity toward D-alanine and D-serine donor in the aldol condensation with aldehydes. (Hernandez et al. 2015). With that discovery, the application of TAs was expanded toward the asymmetric synthesis of β-hydroxy-α-alkyl-α-amino acids containing a quaternary stereogenic center. Types of these non-natural tertiary amino acids play a special role in the design of short-chain peptides with limited flexibility and as structural units in a number of biologically active molecules (Vogt and Bräse 2007; Zhang et al. 2013). Their direct synthesis by threonine aldolase-catalyzed reactions has become a promising alternative to existing chemical methods due to their sustainability and the high stereospecificity of the biocatalytic route.

To further explore this outstanding activity of TAs, we aimed at expanding the biocatalyst toolbox by a database mining approach to discover more enzymes possessing similar activities. The majority of available sequences annotated as TAs in the databases still has not been expressed and characterized and, thus, new threonine aldolase activities can potentially be found by sequence comparison with previously reported LTA_Aj and DTA_Psp (Davids et al. 2013). Moreover, identification of homologous enzymes with similar activities might help to explain the broader donor specificities compared with TAs that only accept glycine as a donor. In this study, novel L- and D-specific TAs that serve as efficient biocatalysts for the asymmetric synthesis of enantiopure tertiary α-amino acids were identified. Along with a biochemical characterization, the catalytic efficiency and substrate scope of the recombinant enzymes was investigated. In addition, a rationale for the donor specificity based on a thoughtful analysis of the structural and sequence alignment is provided.

Materials and methods

General

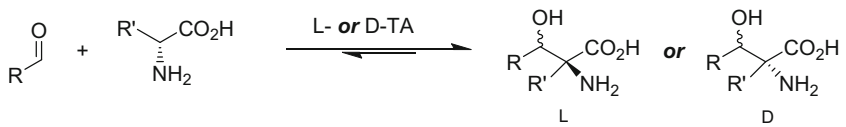
All chemicals were purchased from Sigma-Aldrich, Fluka, Acros Organics, or Alfa Aesar, unless stated otherwise. All enzymes for the genetic work were purchased from Fermentas (now part of Thermo Scientific Molecular Biology). The expression vector pEamTA was produced as stated before (Reisinger et al. 2007), and pET26b(+) was obtained from Novagen. The *Escherichia coli* strains BL21(DE3) and Lemo21(DE3) were obtained from BioLabs (New England). Analytical HPLC was carried out with an Agilent 1100 HPLC system equipped with a G1315A diode array detector. LC-MS analyses were performed on an Agilent 1200 instrument equipped with a 6120 single-quadrupol mass spectrometer configured with an ESI source. A Chromolith Perfomance RP-18e (100×4.6 mm, Merck Millipore) column was used for analysis. Amino acids isomers were determined by HPLC after derivatization with *ortho*-phthaldialdehyde/2-mercaptoethanol (OPA/MCE, achiral derivatization, *d.e.* determination) and *ortho*-phthaldialdehyde/*N*-acetyl cysteine (OPA/NAC, chiral derivatization, *e.e.* determination) (Fesko et al. 2010). The elution order of the different stereoisomers was elucidated by analyzing reference compounds, e.g., commercially available DL-threo-β-phenylserine (Sigma-Aldrich), chemically synthesized DL-*anti*-phenylserine (Steinreiber et al. 2007a) and DL-*anti*-methylthreonine (Dietz and Gröger 2009). ^{1}H-NMR spectra were recorded on a Varian INOVA 500 (^{1}H 499.82 MHz, ^{13}C 125.69 MHz) using the residual peak of D_2O (^{1}H: δ 4.79) as reference. H_2O/D_2O-NMR samples were directly taken from the aqueous solution, diluted with D_2O (1:1) and recorded using H_2O presaturation. HPLC and NMR results were compared to confirm the data obtained with the indirect HPLC analysis methods incorporating literature data (Grandel and Kazmaier 1998; Avenoza et al. 2004).

Plasmids construction

The genomic DNAs from bacteria and yeast were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The threonine aldolase genes were amplified by PCR from the genomic DNA. The primers contained additional 30-bp homologous regions for the plasmids pEamTA (Reisinger et al. 2007) and/or pET26b(+) and $(His)_6$ sequence at C-terminus for LTAs or at N-terminus for DTAs (Table 1). After purification of the PCR product with a commercial kit, it was digested with *Nde*I and *Hin*dIII and gel purified pEamTA (or *Nde*I and EcoRI of pET-26b(+)) plasmid DNA for one-step isothermal Gibson Assembly™. DNA sequencing confirmed the correct DNA assembly.

Threonine aldolase expression with BL21

The expression plasmids (pEamTA-CHis6-LTA-averon, pEamTA-CHis6-LTA-csakaz, pEamTA-CHis6-LTA-gsulfur,



Scheme 1 Biocatalytic synthesis of β-hydroxy-α-quaternary-α-amino acids using threonine aldolases

pEamTA-CHis6-LTA-rornith, and pEamTA-NHis6-DTA-svariic) were transformed into the host strain *Escherichia coli* BL21(DE3). This construct contained the *tac* promoter and the LacI repressor, which control the protein expression level. Three-hundred-milliliter 2xYT medium (in a 2-L flask) containing 50 μg mL^{-1} ampicillin was inoculated with 5 mL of an *E. coli* overnight culture. Cells were grown at 37 °C to an OD_{600} of 0.6–0.8. Protein expression was induced by the addition of 1 mM IPTG, and the culture was allowed to grow overnight at 20 °C.

Threonine aldolases expression with Lemo cells

The expression plasmids (pET26b-CHis6-LTA-sloih, pET26b-NHis6-DTA-psaerug, and pET26b-NHis6-DTA-psprot) were transformed into chemically competent *E. coli* Lemo21(DE3) cells according to the Biolabs transformation protocol (C2528). This construct contained the T7 promoter and terminator. Three-hundred-milliliter LB medium (in a 2-L flask) containing 50 μg mL^{-1} kanamycin, 32 μg mL^{-1} chloramphenicol, and 0.1–1 mM L-rhamnose was inoculated with 5 mL of an *E. coli* overnight culture. Cells were grown at 37 °C to an OD_{600} of 0.6–0.8. Protein expression was induced by the addition of 0.5 mM IPTG, and the culture was allowed to grow overnight at 20 °C.

Preliminary experiments on a 10-mL scale were conducted to determine the optimal expression level for TA production in *E. coli* Lemo21(DE3). Therefore, L-rhamnose was added at different concentrations (0–2000 μM) to the main culture, as described in the Lemo21(DE3) manual.

Protein purification

In all cases, the biomass of the bacterial cultures was harvested by centrifugation at 4500×*g* for 10 min at 4 °C. The cell pellets were resuspended in sodium phosphate (NaPi) buffer (0.1 M, pH 7.4) and disrupted by ultrasonic treatment for 7 min. The crude lysate was cleared by centrifugation at 20,000×*g* for 1 h at 4 °C, and the supernatant (cell-free extract (CFE)) was subjected to further purification.

Recombinant threonine aldolases were expressed as C-terminal $(His)_6$ protein (for LTA) or N-terminal $(His)_6$ protein (for DTA). The CFE was supplied to a Ni-NTA Superflow resin (Qiagen), washed with 50 mM NaPi buffer (pH 7.5) containing 5 mM imidazole and 0.3 M NaCl and subsequently eluted with 0.5 M imidazole in NaPi buffer. To remove the imidazole, the pooled and concentrated protein fractions were loaded on a Sephadex desalting column PD-10 (GE Healthcare) and eluted with 50 mM NaPi buffer (pH 7.4) containing 0.1 mM PLP. The purified proteins were used for the activity measurements and biocatalytic transformations.

Activity measurement toward L- and D-threonine

The assay mixture contained 2–100 mM of L- or D-*syn*-threonine, 50 mM NaPi buffer (pH 8), 50 μM of PLP, 200 μM of NADH, 30 units of YADH in a final volume of 950 μL. The reactions were started by the addition of 50 μL of diluted (1:100) CFE of LTA or DTA. NADH decrease was monitored photometrically at 340 nm ($\varepsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}). Controls without the addition of TAs were carried out under the same conditions. No significant decrease of NADH was observed in these cases.

Activity measurement toward β-phenylserine and a-methyl-β-phenylserine

The activity was verified by monitoring the formation of benzaldehyde upon cleavage of DL-*syn*-β-phenylserine (0.1–5 mM) or L- or D-α-methyl-β-phenylserine spectrophotometrically (0.5–10 mM) at 279 nm ($\varepsilon = 1.4 \times 10^3$ M^{-1} cm^{-1}). The reactions were carried out at 25 °C in 50 mM NaPi buffer (pH 8) containing 50 μM PLP and diluted enzyme.

General procedure for the synthesis of α-tertiary a-amino acids

All enzymatic transformations were performed at 1-mL reaction volume in 50 mM NaH_2PO_4 buffer (pH 8.0), containing 50 μM PLP, 10 % (*v*/*v*) DMSO, 50 mM aldehyde substrate and 0.5 M amino acid donor. Transformations were performed at 30 °C (for LTA) and 18 °C (for DTA) at a total protein concentration of 2 mg mL^{-1}. After 24 h, 50 μL of each reaction mixture was diluted in 950 μL 0.1 M sodium tetraborate buffer (pH 10.5). The diluted reaction mixture was used for the determination of enantiomeric and diastereomeric excesses and conversions by rp-HPLC after a pre-column derivatization as described before (Fesko et al. 2010). Another 50 μL of reaction mixture were diluted in 950 μL acetonitrile and analyzed by LC-MS.

2-Amino-3-hydroxy-2-methyl-3-(3-nitrophenyl)propanoic acid (**1b**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=85/15, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=5.9 min, $t_{L\text{-}syn}$=6.5 min, $t_{D\text{-}anti}$=13.6 min, $t_{L\text{-}anti}$=13.7 min; ^1H NMR (D_2O): δ 1.54 (s, 3H, $C(CH_3)N$, *anti*), 5.0 (s, 1H, $CHOCH_3$, *anti*); 1.19 (s, 3H, $C(CH_3)N$, *syn*), 5.13 (s, 1H, $CHOCH_3$, *syn*); 7.20–7.40 (m, 4H, Ph).

2-Amino-3-hydroxy-2-methyl-3-phenylpropanoic acid (**2b**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=85/15, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=4.0 min, $t_{L\text{-}syn}$=4.2 min, $t_{D\text{-}anti}$=7.1 min, $t_{L\text{-}anti}$=7.5 min; ^1H NMR (D_2O): δ 1.54 (s, 3H, $C(CH_3)N$, *anti*), 5.0 (s, 1H, $CHOCH_3$, *anti*); 1.19 (s, 3H, $C(CH_3)N$, *syn*), 5.13 (s, 1H, $CHOCH_3$, *syn*); 7.20–7.40 (m, 5H, Ph).

Table 1 Strains, genes NCBI reference sequence, and PCR primers for amplification

	Enzyme	NCBI reference sequence	Forward primers	Reverse primers	Resulting plasmid
1	L-Threonine aldolase *Aeromonas veronii* DSM7386	YP_004392976.1	5′-TTTGTTTAACTTTA AGAAGGAGATGACCAT ATGCGCTATATCGATTTACGA AGTGACACC-3′	5′-GCCAAGCTTGCATG CTTGTTGTCTGACAGTTCAGTGGTGATGG TGATGATGACGCGCTCCGAGATACTCGG-3′	pEamTA-CHis6-LTA-averon
2	L-Threonine aldolase *Cronobacter sakazaki* DSM4485	YP_007441173.1	5′-TTTGTTTAACTTTAA GAAGGAGATGACCAT ATGATCGATTTACGCAGCGATAC CGTC-3′	5′-GCCAAGCTTGCATGC TTGTTGTCTGACAGTTCAGTGGTGATGGTGA TGATGTGCCGTTTGCTGTAAAAATTCCC GC-3′	pEamTA-CHis6-LTA-csakaz
3	L-Threonine aldolase *Shewanella loihica* DSM17748	YP_001093284.1	5′-GTTTAACTTTAAGA AGGAGATATACATATGATTGA TTTTCGCAGTGACACAGTAACTCAG-3′	5′-GTGCGGCCGCAAG CTTGTCGACGGAGCTCGTCAGTGGTGATGGTGAT GATGGAGCAGCGACTTGAGCTTATCGATAAAGA-3′	pET26b-CHis6-LTA-sloih
4	L-*allo*-Threonine aldolase *Geobacter sulfurreducens* DSM12127	WP_010943783.1	5′-TTTGTTTAACTTTA AGAAGGAGATGA CCATATGAACATCATTGACCTGCGCAGC-3′	5′-GCCAAGCTTGCATG CTGTTTGTCTGACAGTTCAGTGGTGATGGTGA TGATGTGACGATGCTCCCGGTTGCAT-3′	pEamTA-CHis6-LTA-gsulfur
5	L-Threonine aldolase *Raoultella ornithinolytica* DSM7464	YP_007874367.1	5′-TTTGTTTAACTTTA AGAAGGAGATGACCATATGATAGAT TTACGCAGTGATACCGTAACCC-3′	5′-GCCAAGCTTGCATG CTTGTTGTCTGACAGTTCAGTGGTGATGG TGATGATGGCGTTGCAAAAATGCCTGCCAGT-3′	pEamTA-CHis6-LTA-rornith
6	Hypothetical protein *Pseudomonas aeruginosa* DSM24068	YP_001351418.1	5′-GTTTAACTTTAAGAA GGAGATATACATATGCATCATCACCA TCACCACAATCGACGCAAGTTCATGTTCGCC-3′	5′-GTGCGGCCGCAAGCT TGTCGACGGAGCTCGCTAGGCGTAAACCGGCCAGC-3′	pET26b-NHis6-DTA-psaerug
7	Alanine racemase domain *Pseudomonas protegens* DSM19095	YP_008001024.1	5′-GTTTAACTTTAAGA AGGAGATATACATATGCATCATCACCAT CACCACAACCGTCGTCATTTCATGGCCG-3′	5′-GTGCGGCCGCAAGCT TGTCGACGGAGCTCGTCAGTACACC GGCCAGCGTT-3′	pET26b-NHis6-DTA-psprot
8	Hypothetical protein *Singularimonas variicoloris DSM15731*	WP_020650309.1	5′-TTTGTTTAACTTTAA GAAGGAGATGACCATATGCATCATCACCATCACC ACAACAGACGCAAGTTCCTGCTCG-3′	5′-GCCAAGCTTGCATGC TGTTTGTCTGACAGTCTACTGG CGGTAGACCGGCC-3′	pEamTA-NHis6-DTA-svariic

2-Amino-3-hydroxy-2-methyl-3-(pyridin-3-yl)propanoic acid (**3b**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=90/10, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=5.61 min, $t_{L\text{-}syn}$=5.66 min, $t_{D\text{-}anti}$=7.08 min, $t_{L\text{-}anti}$=7.12 min.

2-Amino-3-hydroxy-2,4-dimethylpentanoic acid (**4b**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=88/12, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=6.2 min, $t_{L\text{-}syn}$=6.3 min, $t_{D\text{-}anti}$=9.8 min, $t_{L\text{-}anti}$=10.0 min; ^{1}H NMR (D_2O): δ 1.52 (s, 3H, $C(CH_3)N$, *anti*), 4.22 (d, 1H, *J*=6.5 Hz, CHOCH, *anti*); 1.38 (s, 3H, $C(CH_3)N$, *syn*), 4.29 (d, 1H, *J*=6.5 Hz, $CHOCH_3$, *syn*); 1.73 (m, 1H, $CH(CH_3)_2$); 1.0 (d, 6H, *J*=7.5 Hz , $CH(CH_3)_2$).

2-Amino-3-hydroxy-2-methyloctanoic acid (**5b**). Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=82/18, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=5.9 min, $t_{L\text{-}syn}$=7.2 min, $t_{D\text{-}anti}$=8.9 min, $t_{L\text{-}anti}$=14.7 min; ^{1}H NMR (D_2O): δ 3.62 (m, 1H, CHOH), 1.20–1.40 (m, 7H); 0.91 (t, 3H, *J*=7.4 Hz).

2-Amino-5-(((benzyloxy)carbonyl)amino)-3-hydroxy-2-methylpentanoic acid (**6b**). Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=84/16, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=6.4 min, $t_{L\text{-}syn}$=6.7 min, $t_{D\text{-}anti}$=8.6 min, $t_{L\text{-}anti}$=8.7 min.

2-Amino-3-hydroxy-2-hydroxymethyl-3-(3-nitrophenyl)propanoic acid (**1c**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=86/14, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=4.0 min, $t_{L\text{-}syn}$=6.1 min, $t_{D\text{-}anti}$=11.9 min, $t_{L\text{-}anti}$=12.6 min; ^{1}H NMR (D_2O): δ 4.15 (s, 2H, *J*=8.4 Hz, $C(CH_2)N$, *anti*), 5.71 (s, 1H, CHOH, *anti*); 4.03 (s, 2H, *J*=8.4 Hz, $C(CH_2)N$, *syn*), 6.12 (s, 1H, CHOH, *syn*); 7.20–7.40 (m, 4H, Ph).

2-Amino-3-hydroxy-2-hydroxymethyl-3-phenylpropanoic acid (**2c**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=85/15, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=5.6 min, $t_{L\text{-}syn}$=6.1 min, $t_{D\text{-}anti}$=12.5 min, $t_{L\text{-}anti}$=12.9 min; ^{1}H NMR (D_2O): δ 4.18 (ss, 2H, *J*=8.6 Hz , $C(CH_2)N$, *anti*), 5.85 (s, 1H, CHOH, *anti*); 4.09 (ss, 2H, *J*=8.6 Hz, $C(CH_2)N$, *syn*), 6.05 (s, 1H, CHOH, *syn*); 7.20–7.35 (m, 5H, Ph).

2-Amino-3-hydroxy-2-hydroxymethyl-3-(pyridin-3-yl)propanoic acid (**4c**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=90/10, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=4.4 min, $t_{L\text{-}syn}$=4.6 min, $t_{D\text{-}anti}$=9.4 min, $t_{L\text{-}anti}$=9.67 min.

2-Amino-3-hydroxy-2-hydroxymethyl-octanoic acid (**5c**). Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=83/17, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=4.4 min, $t_{L\text{-}syn}$=5.0 min, $t_{D\text{-}anti}$=7.9 min, $t_{L\text{-}anti}$=8.5 min; ^{1}H NMR (D_2O): δ 0.87 (t, 3H, *J*=7.0 Hz), 1.23–1.65 (m, 8H), 3.90–4.05 (m, 2H, CH_2OH, 1H, CHOH).

2-Amino-5-(((benzyloxy)carbonyl)amino)-3-hydroxy-2-methylpentanoic acid (**6c**). Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=84/16, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=4.8 min, $t_{L\text{-}syn}$=5.0 min, $t_{D\text{-}anti}$=5.7 min, $t_{L\text{-}anti}$=6.0 min.

2-Amino-2-(hydroxyl(3-nitrophenyl)methyl)butanoic acid (**1d**). HPLC: Chromolith® column: OPA/NAC: buffer KH_2PO_4 (20 mM, pH 6.8)/MeCN=84/16, 2.5 mL min^{-1}; $t_{D\text{-}syn}$=7.22 min, $t_{L\text{-}syn}$=7.46 min, $t_{D\text{-}anti}$=12.9 min, $t_{L\text{-}anti}$=13.3 min. LC-MS: Poroshell® 120 SB-C18 column (100× 3.0 mm, 2.7 μm, Agilent), solvent A (0.01 % formic acid in H_2O), solvent B (methanol), gradient elution from 2 to 35 % B over 8 min, 0.7 mL min^{-1}; t_{ret}(prod)=3.8 min; ES^+: $(M+1)^+$ 255.1.

Results

Identification of candidate genes

To expand the scope of threonine aldolases with broad donor specificity, a database mining was performed. The described LTA from *Aeromonas jandei* (LTA_Aj) and DTA from *Pseudomonas* sp. (DTA_Psp) were compared with available protein sequences from GenBank database using the BLASTP program from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). L-Specific TAs are widespread in microorganisms; thus, more than 1000 sequences encoding LTAs were found in the database. The genes were annotated mostly as TA-like proteins of aminotransferase I superfamily. The D-specific TAs belong to the alanine racemase family PLP enzymes; therefore, the genes were mostly annotated as hypothetical alanine racemase. Five sequences encoding LTAs and five sequences encoding DTAs were selected as candidate genes based on 55–85 % sequence similarity to the template genes (Table 2). The exact function of the chosen target enzymes was not characterized before according to our knowledge.

Molecular cloning, heterologous gene expression, and protein purification

The candidate genes were amplified by PCR from the genomic DNA of the corresponding organisms (Table 2). Each of the genes was cloned at first into pEamTA plasmids with C-terminal (for LTA) and N-terminal (for DTA; *C*-His_6 tag results in inactive enzyme) $(His)_6$-tag. Expression of the gene constructs in *E.coli* BL21(DE3) gave the soluble protein after cell lysis except of LTA_Sl, DTA_Pa, and DTA_Pp, where the protein was mainly in the insoluble fraction. Optimization of the expression conditions with respect to temperature and IPTG concentration, as well as application of the self-induction protocol and co-expression with chaperons (according to Takara's protocol), did not lead to significant improvement in protein production. To minimize the inclusion body formation, fine tuning of T7 expression was applied by

Table 2 Enzymes homologues to LTA *Aeromonas jandei* and DTA *Pseudomonas* sp.

Enzyme	Amino acids	Identity (aa) to LTA_Aj	Enzyme	Amino acids	Identity (aa) to DTA_Psp
LTA *Aeromonas jandei*[a] (LTA_Aj)	337	100	DTA *pseudomonas*[a] sp.(DTA_Psp)	426	100
LTA *Aeromonas veronii* (LTA_Av)	339	85	Hypothetical protein *Pseudomonas aeruginosa* (DTA_Pa)	425	81
LTA *Cronobacter sakazaki* (LTA_Cs)	335	55	alanine racemase domain protein *Pseudomon. protegens* (DTA_Pp)	424	80
LTA *Shewanella loihica* (LTA_Sh)	335	55	Hypothetical protein *Singularimonas variicoloris* (DTA_Sv)	425	64
L-*allo*-TA *Geobacter sulfurreducens* (LTA_Gs)	348	60	Alanine racemase domain protein *Variovorax paradoxus* (DTA_Vp)	405	53
LTA *Raoultella ornithinolytica* (LTA_Ro)	333	55			
LTA *Pseudomonas mendocina* (LTA_Pm)	334	61			

[a] Template sequences

varying the level of lysozyme, the natural inhibitor of T7 RNA polymerase. In order to do this, LTA_Sl, DTA_Pa, and DTA_Pp genes were cloned into pET-26b(+) vectors as *C*- and *N*-$(His)_6$-tagged enzymes, and then, *E. coli* Lemo21(DE3) expression host strain was used. The addition of L-rhamnose to the expression culture tuned the expression of the protein of interest. Five hundred micromolars of L-rhamnose was found as optimal concentration to produce soluble proteins. After purification by affinity chromatography using Ni-NTA beads, 15–25 mg of pure protein was obtained from 1-L expression culture.

Donor specificity of novel threonine aldolases

The donor specificity of the expressed TAs was tested in vitro in the aldol condensation of 3-nitro-benzaldehyde and a range of amino acid donors, e.g., glycine (physiological donor) (**a**), D-alanine (**b**), D-serine (**c**), or D-2-aminobutanoic acid (**d**) (Fig. 1). The product formation was monitored by TLC, HPLC, and ^{1}H-NMR. Among the ten expressed TAs, LTA_Av, LTA_Sl, LTA_Ro, DTA_Pa, and DTA_Pp were able to accept D-alanine and D-serine as donor, whereas other tested TAs only catalyzed reactions with glycine as a donor (Fig. 1). Good to excellent substrate conversion was observed in the aldol reactions with D-alanine, catalyzed by the novel TAs (conversions up to more than 95 % for LTA_Av, respectively, up to more than 60 % for DTA_Pa and LTA_Ro): in the case of D-serine, the maximum conversions after 24 h were close to 40 % with LTA_Av but lower than 10 % with all DTAs tested (Table 4 and Fig. 1). With increasing donor size, the conversion drops, leading to less than 2 % conversion in the reaction with D-2-aminobutanoic acid for LTA_Av and DTA_Pa and no detectable amount of product for other TAs (Fig. 1). Other natural amino acids were not accepted by any of the tested enzymes. The biotransformations proceeded with perfect enantioselectivity at the quaternary α-carbon with both types of enzymes. Thus, enantiocomplementary L- or D-amino acid products can be obtained (e.e.$_L$ >99 %; e.e.$_D$ >99 %). The selected enzymes represent valuable complements to the previously described enzymes for the synthesis of optically pure α-branched amino acids.

Kinetic parameters of the new threonine aldolases

To explore the biocatalytic properties of the newly discovered threonine aldolases with broad donor specificity and to compare them with the known LTA_Aj and DTA_Ps, kinetic parameters were determined by spectrophotometric assays. Enzyme activities were measured for the retro-aldol reaction of α-methyl-β-phenylserine and compared with those obtained for β-phenylserine and the natural substrate threonine (Table 3).

The catalytic efficiency of the new enzymes similarly to the previously described threonine aldolases significantly decreases when having an α-quaternary center in the substrate, which results in a drop of the k_{cat} values (Table 3). Interestingly, the kinetic parameters for the cleavage of non-natural α-methyl-β-phenylserine lie in a similar range or are even higher as for the cleavage of natural L-threonine in the case of L-specific TAs. This can be explained by the high stereospecificity of these enzymes toward the L-*allo*-threonine isomer compared to L-threonine, as was shown before (Kataoka et al. 1997). In contrast, the catalytic efficiency k_{cat}/K_m for the D-specific enzymes was lower for the retro-aldol cleavage of α-methyl-β-phenylserine (e.g., k_{cat}/K_m 0.19 min^{-1} mM^{-1}

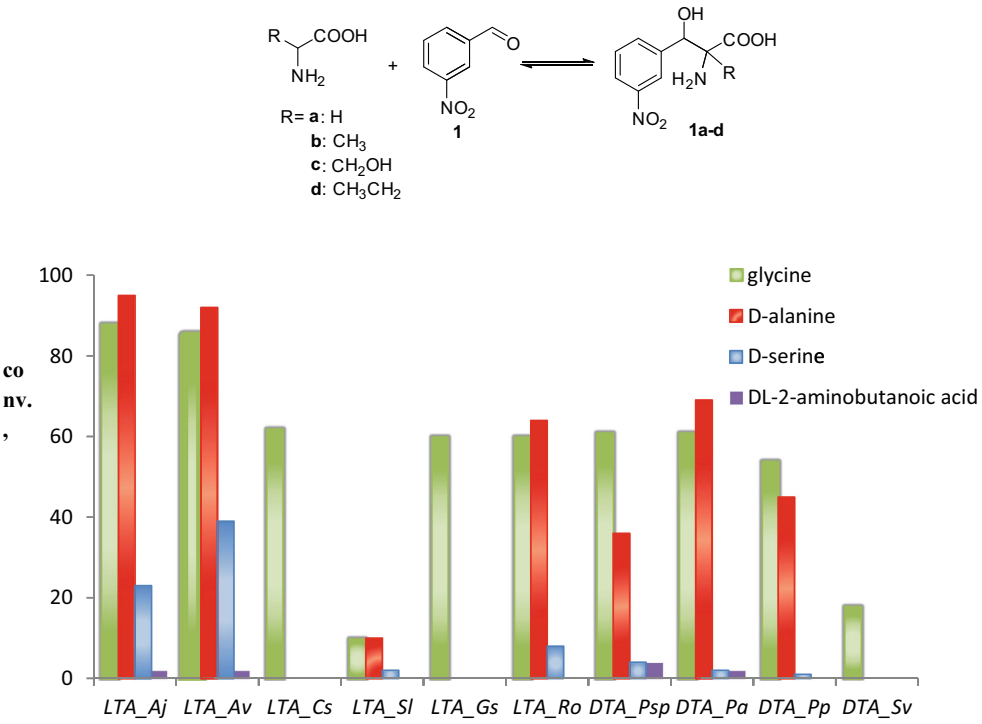


Fig. 1 Formation of β-(3-nitrophenyl)-serine (**1a**), β-(3-nitrophenyl)-α-methyl-serine (**1b**), β-(3-nitrophenyl)-α-hydroxymethyl-serine (**1c**), and 2-amino-2-(hydroxyl(3-nitrophenyl)methyl)butanoic acid (**1d**) with new threonine aldolases. Conditions: 1-mL reaction volume, 50 mM 3-nitrobenzaldehyde, 0.5 M glycine (*green*), D-alanine (*red*), D-serine (*blue*), or DL-2-aminobutanoic acid (*violet*), 50 μM PLP, 10 %*v*/*v* DMSO, 25 °C, 2 mg mL^{-1} TAs; conversions were determined after 24 h by rp-HPLC (color figure online)

for DTA_Pa) compared to the reaction with natural substrate D-threonine (k_{cat}/K_m 1.91 min^{-1} mM^{-1} for DTA_Pa) and especially to the cleavage of D-β-phenylserine (k_{cat}/K_m 47.2 min^{-1} mM^{-1} for DTA_Pa). The highest catalytic efficiency for the cleavage of quaternary α-methyl-β-phenylserine was obtained for LTA_Av with a k_{cat} of 16.7 min^{-1}, a K_m of 1.0 mM and a k_{cat}/K_m of 16.7 min^{-1} mM^{-1}. The D-specific TAs are almost 50 times less active with α-quaternary substrate compared to L-specific TAs. Hence, the catalytic efficiency of the novel TAs is up to the same order of magnitude as for the previously described LTA_Aj and DTA_Psp.

Aldol synthesis of α-tertiary α-amino acids with the new threonine aldolases

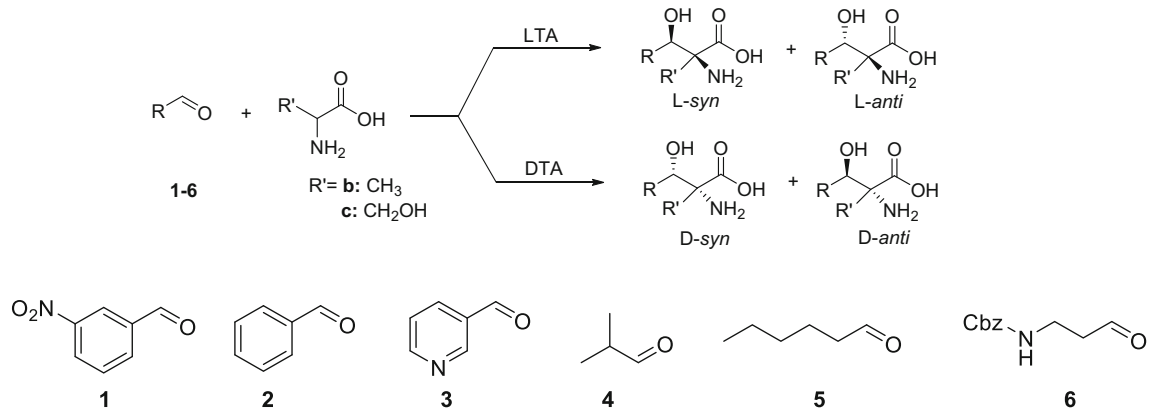
Having identified five novel TAs with broad donor specificity, these enzymes were tested in the aldol condensation of a set of aldehydes **1**–**6** and D-alanine (**b**), respectively, D-serine (**c**) as donor to produce β-hydroxy-α-alkyl-α-amino acids (Scheme 2, R'=CH_3; CH_2OH).

The investigation of reaction parameters revealed similar conditions to the previously reported ones as optimal for the TA-catalyzed reactions: pH 8.0 and 30 °C for LTAs, respectively, 18 °C for DTAs (Fesko et al. 2010). Moreover, the enzymes can tolerate up to 20 % of water-soluble organic

Table 3 Kinetic parameters of purified TAs with threonine, β-phenylserine, and α-methyl-β-phenylserine as substrates

TA variant	*syn*-Threonine			*syn*-β-Phenylserine			α-Methyl-β-phenylserine		
	k_{cat} [min^{-1}]	K_m [mM]	k_{cat}/K_m [mM^{-1} min^{-1}]	k_{cat} [min^{-1}]	K_m [mM]	k_{cat}/K_m [min^{-1} mM^{-1}]	k_{cat} [min^{-1}]	K_m [mM]	k_{cat}/K_m [min^{-1} mM^{-1}]
LTA-A*j**	17.4	10.9	1.6	1.1×10^3	2.0	550	32.3	2.76	11.7
LTA-*Av*	18.5	11.8	1.6	1.9×10^3	1.28	1055	16.7	1.0	16.7
LTA-*Sl*	28.8	14.9	1.93	13.45	0.15	90	3.3	0.46	7.17
LTA-*Ro*	9.3	7	1.33	29.1	0.19	153	0.73	0.67	1.1
DTA-*Ps**	24.0	15.9	1.5	82.1	1.5	54.7	1.97	6.2	0.32
DTA-*Pa*	26.6	13.9	1.91	84.5	1.79	47.2	1.71	8.98	0.19
DTA-*Pp*	18.9	15.6	1.21	70.0	4.45	15.7	1.44	11.2	0.12

*Template enzymes



Scheme 2 Aldol synthesis of β-hydroxy-α-alkyl-α-amino acids using L- or D-threonine aldolases

solvents like dimethylsulfoxide, acetonitrile, or ethanol. Threonine aldolase catalyzed reactions are known often to suffer from an unfavorable thermodynamic equilibrium. To partially overcome this limitation, an excess of amino acid donor was applied.

In aldol reactions catalyzed by TAs, two new stereocenters are formed. All tested aldolases showed excellent enantioselectivity at the α-carbon of the amino acid products which in all cases was found with an e.e. >99 % and enantiopure L- or D-α-alkyl-serine derivatives were produced depending on type of enzyme applied (Table 4). The stereochemistry at C_α is controlled by the enzymes; however, the selectivity at C_β also depends on the acceptor aldehyde used. A mixture of *syn*- and *anti*-diastereomers was typically observed. The isomer ratio can be manipulated by the type of enzyme used or by performing the reaction under kinetically controlled conditions. Unfortunately, using the latter conditions would lead to a decline in yields and therefore make the protocol less useful on a practical point of view (Fesko et al. 2008). In general, higher diastereoselectivities were obtained with D-specific TAs and/or at low conversions (d.e.'s up to 95 %). Frequently, the inversion from *syn* to *anti* isomer was obtained when long chain aliphatic aldehydes were used as acceptors or D-serine was used as donor (Table 4).

The novel threonine aldolases, similar to previously published TAs from *Aeromonas jandaei* and *Pseudomonas* sp., accept a wide range of acceptor substrates. The aromatic, linear, and branched aliphatic, as well as heterocyclic aldehydes, were converted with reasonable conversions using D-alanine (**b**) and D-serine (**c**) as donors (Table 4). As previously described (Steinreiber et al. 2007a, b; Fesko et al. 2010), introducing electron-withdrawing groups to the aromatic aldehydes increases the electrophilic reactivity and the transformation proceeds with higher yields. In the aldol reaction catalyzed by the novel TAs, 3-nitro-benzaldehyde (**1**) was efficiently converted to **1b** with up to 95 % analytical yield, whereas unsubstituted benzaldehyde (**2**) was converted with maximum yield of 30 % (Table 4). *N*-Cbz-protected 3-aminopropanal **6** was converted with an analytical yield of 10–30 % by the novel TAs leading to a polyfunctional β-hydroxy-α-alkyl-α,ω-diamino acid derivative, a non-fluorinated analog to β-hydroxy-eflornithine. The best conversions were obtained in reactions with LTA-Av, which showed the highest sequence similarity to the previously published LTA-Aj. LTA-Sl proved to be unstable under the reaction conditions and showed fast denaturation, which resulted in generally low conversions. Among all D-specific enzymes, DTA-Pa gave the best conversions with long-chain aliphatic substrate hexanal (**5**) (conversions up to 55 %). With increasing donor size, the yields decrease significantly. Thus, the enzymatic transformations where D-serine (**c**) was used as donor gave lower conversions compared to those reactions with D-alanine (**b**).

Discussion

By means of homology search using the amino acid sequence of LTA_Aj and DTA_Psp as templates, the scope of biocatalysts for the asymmetric synthesis of tertiary α-amino acids has been expanded. Five new previously not characterized enantiocomplementary L- and D-specific TAs were found to possess broad donor specificity. The enzymes were heterologously produced in *E. coli*, and their expression was improved by the application of a tunable expression using the Lemo System™. The kinetic parameters of the new enzymes were in the same range as the previously reported TAs (Fesko et al. 2010). The herein described new TAs were applied for the simple one-step synthesis of α-quaternary α-amino acids. In all cases, enantiopure L- or D-products were obtained depending on the type of enzyme used. The conversions and diastereoselectivities depend largely on the substrates used. Superior results were obtained with LTA-*Av* and nitro-substituted benzaldehyde. The D-specific enzymes give high conversions with long-chain aliphatic aldehydes. In order to improve the outcome of reactions, further reaction

Table 4 Conversions and diastereomeric excesses in the aldol reactions catalyzed by novel L- and D-TAs

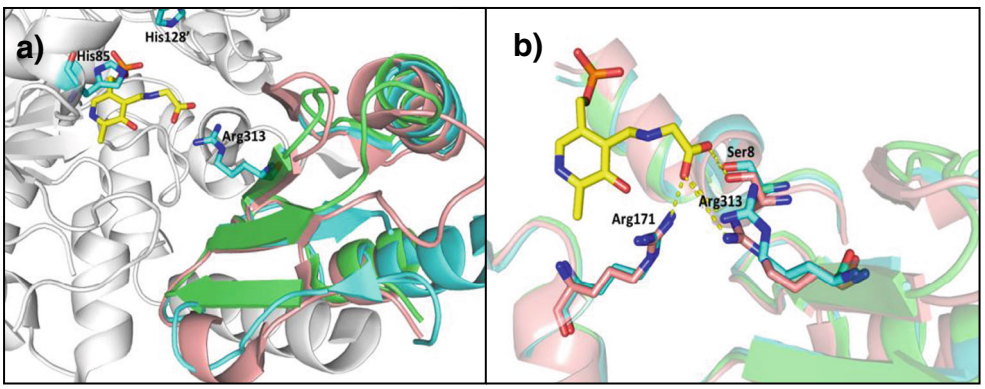
Acceptor	Donor	LTA-Av		LTA-Sl		LTA-Ro		DTA-Pa		DTA-pp	
		conv., %	*de*, %	*conv.*, %	*de*, %	*conv.*, %	*de*, %	*conv.*, %	*de*, %	*conv.*, %	*de*, %
1	**b**	92	4 (*syn*)	23	16 (*anti*)	64	30 (*syn*)	69	80 (*syn*)	45	92 (*syn*)
	c	39	22 (*anti*)	2	0	8	0	4	23 (*anti*)	1	4 (*syn*)
2	**b**	30	0	10	74 (*syn*)	21	0	11	65 (*syn*)	9	55 (*syn*)
	c	15	55 (*syn*)	4	62 (*syn*)	6	54 (*syn*)	n.d.		n.d.	
3	**b**	97	20 (*syn*)	18	73 (*syn*)	52	12 (*syn*)	56	88 (*syn*)	38	80 (*syn*)
	c	25	44 (*anti*)	5	80 (*anti*)	4	95 (*anti*)	5	79 (*anti*)	n.d.	
4	**b**	45	21 (*syn*)	6	11 (*anti*)	8	5 (*anti*)	29	60 (*syn*)	21	17 (*syn*)
5	**b**	48	32 (*anti*)	4	18 (*anti*)	18	21 (*anti*)	55	13 (*syn*)	30	16 (*syn*)
	c	23	41 (*anti*)	<1	95 (*anti*)	6	53 (*anti*)	11	2 (*anti*)	8	9 (*anti*)
6	**b**	29	28 (*anti*)	10	36 (*anti*)	27	31 (*anti*)	27	17 (*syn*)	16	49 (*syn*)
	c	17	60 (*anti*)	n.d.		4	95 (*anti*)	6	35 (*anti*)	n.d.	

Conditions: 1-mL reaction volume, 50 mM aldehyde (**1–6**), 0.5 M D-alanine (**b**) or D-serine (**c**), 50 μM PLP, 10 %*v*/*v* DMSO, 30 °C (for LTA) or 18 °C (for DTA), 2 mg mL^{-1} TA; conversions, *d.e.* and *e.e.* were determined after 24 h by rp-HPLC after pre-column derivatization

n.d. not detected due to low conversion

engineering is envisaged in order to shift the equilibrium to the product side.

To get an understanding how a certain donor specificity is regulated in TAs, a sequence and crystal structure comparison between LTAs with broad donor specificities and those which strictly only accept glycine was performed. In the LTAs, the active-site residues that recognize the substrate/product amino acid are conserved, including Ser8, His85, Arg171, Lys199, and Arg313 (position numbers are given for LTA_Aj). The major sequence dissimilarities were found within 270–315 amino acid positions (Fig. 2c). As shown recently, the carboxylate group of an amino acid donor interacts with Arg171, Arg313, and Ser8 side chains that anchor the donor to the active site (Qin et al. 2014). The donor ligand should be bound in the active site in the orientation that the scissile C_α-H bond is oriented perpendicular to the plane of the PLP-donor ring (Toney 2005). This orientation is essential for the catalysis. It was postulated that the activated water molecule in the close position to C_α acts as a base to abstract the C_α proton and initiates the aldol reaction. The other residues, which participate in the protonation of the hydroxyl



c)

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LTA_Ro    ADVTRHDTNMLFVR-VGEE-HAEAL 290   GAFMQQRGVLINAS--PIVRLVTH 312
LTA_Ec    ADVMRQDTNMLFVR-VGEE-NAAAL 290   GEYMKARNVLINAS--PIVRLVTH 312
LTA_Cz    AEVRRHDTNMLFVS-VPQA-QVAAL 290   GAFMKSRNVLISAA--PVTRLVTH 314
LTA_Aj    LDLAQVQTNMVFLQ-LTSGESAPLL 296   AF-MKARGILFSGY--GELRLVTH 317
LTA_Av    LDLSLVQSNMVFLR-LREGESAPLL 296   AF-MKERGILFSGY--GELRLVTH 317
LTA_Sl    VDLGEVQTNMLFAR-VVEGVAIDKL 294   ATSLKASGILISPG--KTLRMVTH 314
LTA_Gs    LDPSQVQTNMVFLC-LPLR-TADRL 295   ASFLNEQGILISGR--ESIRLVTH 317
LTA_Tm    VNPEDVKTNMVILRTDNLKVNAHGF 296   IEALRNSGVLANAVSDTEIRLVTH 320
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Fig. 2 Structural and sequence alignment of L-specific TAs. **a** Structural superposition of region 268–333 (*blue*, LTA_Aj; *red*, LTA_Tm; *green*, LTA_Ec). **b** Residues alignment in the active site responsible for carboxylate stabilization (*blue*, LTA_Aj; *red*, LTA_Tm). **c** Sequence alignment of the expressed LTAs in region 273–317 for LTA-Aj (*gray*, LTAs with broad donor specificity) (color figure online)

group of the product, were postulated to be His128 and His85 and are conserved among LTAs (Qin et al. 2014; di Salvo et al. 2014).

A structural alignment using the Pymol program showed that the overall structure of LTA_Aj (accept glycine, alanine, and serine as donor) is highly similar to the previously reported LTA_Tm (Kielkopf and Burley 2002) with 49 % sequence identity and LTA_Ec (di Salvo et al. 2014) with 54 % sequence identity (LTA_Tm and LTA_Ec accept only glycine as donor). Their superposed structures implied that the conformational changes were only found in the region Met281-Arg313, which might be responsible for the substrate recognition as well as the flexibility of the active site (Fig. 2a). Moreover, the spatial orientation of Arg313, which participates in the stabilization of the carboxylate group of a donor, was slightly different in all structures (Fig. 2b). These differences may interfere the hydrogen-bonding network which is necessary for the stabilization of a donor substrate in a catalytically active orientation and the transition state during the enzymatic reaction.

Based on the sequence and structural comparison, we assume that the extended donor specificity of LTAs described herein results from the productive stabilization of the donor in the active site of enzyme, which is determined by the overall microenvironment that surrounds the donor substrate rather than by one or more specific residues. As was shown before, the binding of glycine in the active site and its deprotonation is also the rate-limiting step in the aldol reactions catalyzed by L-specific TAs (Fesko et al. 2008). To optimize the extended donor specificity, a crystal structure of LTA_Aj in a complex with D-alanine would help to disclose the conformational changes happening during binding of sterically hindered substrates.

Unfortunately, no crystal structure is currently available for D-specific TAs, and therefore, it was not possible to make similar reflections on their broad donor specificity.

In summary, we have demonstrated that novel L- and D-TAs with donor specificities beyond glycine could be found by database screening. For a long time, it was believed that glycine is the only accepted donor in TA-catalyzed reactions. Nowadays, with an increasing amount of gene sequences, enzyme crystal structures and information on the catalytic mechanism available, chemists have got a powerful tool at hand to reveal novel biocatalysts with desired properties by simple database search. The herein shown biocatalytic approach using new TAs opens up new opportunities for enzymatic carboligation reactions via enabling the direct formation of quaternary stereocenters. The reported enzymes convert a representative subset of aromatic and aliphatic aldehyde substrates, which provide a smart synthesis of a range of tertiary α-amino acids, which can be further used in peptide syntheses and as building block in multifunctional pharmacologically active molecules. Moreover, the availability of enantiocomplementary L- and D-specific TAs makes it possible to obtain both enantiomeric forms. The potential of our new enzymes is highlighted by the asymmetric biotransformations that led to high conversions and excellent e.e.s. Additionally, we have proposed structural prerequisites for the broad donor specificity of the tested TAs. However, deeper knowledge on the structure-function relationship is still required to optimize the enzyme activities and to understand their naturally evolved properties.

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Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no competing interests.

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