

Review

Recent Advances in Biocatalysis with Threonine Aldolase for the Synthesis of Amino Compounds

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Abstract

Threonine aldolases (TAs) are pyridoxal-5-phosphate (PLP)-dependent enzymes that catalyze the reversible aldol condensation between aldehydes and glycine, enabling the asymmetric synthesis of chiral β -hydroxy- α -amino acids. The discovery of new TAs has greatly enriched the enzyme family and spurred investigations into noncanonical reactions beyond conventional aldol condensation, establishing access to structurally diverse amino compounds. Protein engineering has significantly improved the catalytic performance of this enzyme class, particularly addressing the inherent limitation of low C_β -stereoselectivity. Furthermore, the integration of TAs into multi-enzyme cascade systems has broadened their synthetic applicability. This review systematically presents recent progress in biocatalysis with TAs, including the various reaction types, the engineering of TAs for improved catalytic performance, the application of the enzyme in cascade reactions, and future opportunities to broaden the utility of the enzyme class in amino compound synthesis.

Keywords: threonine aldolase; protein engineering; β -hydroxy- α -amino acids; PLP-dependent enzymes; amino compounds; enzymatic cascades

1. Introduction

Enzymatic aldol condensations, which mediate C–C bond formation, are widespread in the natural metabolic pathways [1]. Enzymes that catalyze this reaction are designated as aldolases and can be classified into distinct classes with different catalytic mechanisms [2]. Among them, pyridoxal-5-phosphate (PLP)-dependent threonine aldolases (TAs) have attracted great interest. The enzymes were initially identified to catalyze the retro-aldol cleavage of threonine, generating glycine and acetaldehyde, as well as the stereoselective reverse aldol condensation (Figure 1) [3]. As the enzymes show good tolerance to varied carbonyl nucleophiles, they can be utilized for the stereoselective synthesis of a broad range of β -hydroxy- α -amino acids. Noteworthy, the synthesis of the class of compounds has aroused great attention, as they serve as important building blocks or even active ingredients in the pharmaceutical and agrochemical industries. For example, *L*-threo- β -4-methylthiophenylserine is used as a key precursor for the synthesis of the broad-spectrum antibiotics florfenicol and thiamphenicol [4]. *L*-threo- β -3,4-(Dihydroxyphenyl)serine, an orally active norepinephrine precursor, is used in the treatment of orthostatic hypotension and Parkinson's disease [5].



Academic Editor: Andres R. Alcantara

Received: 28 April 2026

Revised: 20 May 2026

Accepted: 22 May 2026

Published: 25 May 2026

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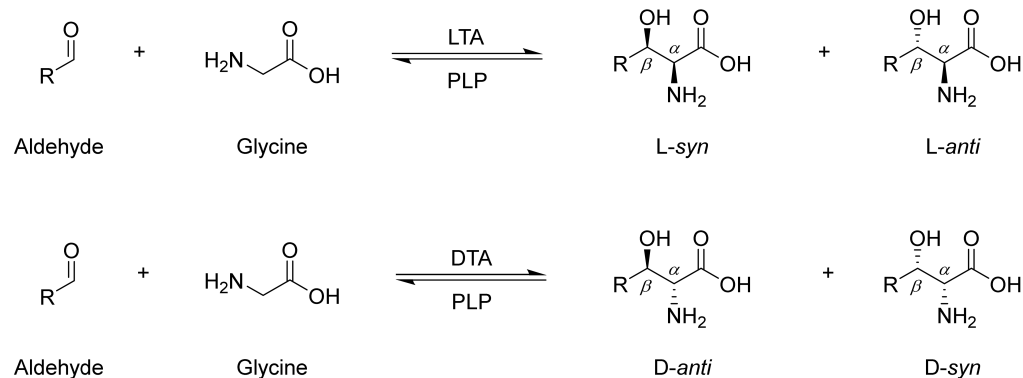


Figure 1. Synthesis of β -hydroxy- α -amino acids via aldol condensation of aldehydes and glycine using L- and D-specific threonine aldolases.

Parallel to enzymatic aldol reactions, non-enzymatic asymmetric aldol methodologies have also seen significant progress. For example, a proline–boronic acid complexation strategy has been developed to enhance both reactivity and enantioselectivity, enabling efficient catalysis for traditionally challenging unbranched linear aliphatic aldehydes [6]. In addition, chiral bidentate halogen bond donors have been introduced as novel organocatalysts for Mukaiyama aldol reactions, achieving up to 98% ee across a broad substrate scope [7]. Transition-metal catalysis continues to advance as well, with chiral nickel(II) complexes enabling direct asymmetric aldol reactions of N-acyl thiazinanethiones with aromatic aldehydes under mild conditions [8]. Despite significant advances in non-enzymatic asymmetric aldol reactions, including organocatalytic and transition-metal-catalyzed systems, these methods often suffer from high catalyst loadings, poor recyclability, and the need for anhydrous conditions [9]. Such limitations have driven the search for efficient biocatalytic alternatives, among which TAs have emerged as promising catalysts for asymmetric aldol condensation [10].

TAs exhibit high stereoselectivity at the α -carbon of β -hydroxy- α -amino acids but relatively poor stereoselectivity at the β -carbon. However, TAs could be afforded higher stereoselectivity under kinetic control (with short reaction time, low reaction temperature, and a low amount of enzyme) compared to the reaction at equilibrium [11,12]. Based on the different stereochemical outcome at the α -carbon, TAs are classified into L- and D-specific TAs (termed LTAs and DTAs, respectively) [13]. L-Specific TAs could be further classified into three sub-groups: L-TAs, L-*allo*-TAs, and low-specificity LTAs, which prefer L-threonine or L-*allo*-threonine, or show low stereoselectivity at the β -carbon, respectively [11,14,15]. Similarly, D-specific TAs could be divided into three types: D-TAs, D-*allo*-TAs, and low-specificity DTAs, owing to their stereoselectivity at the β -carbon [11,12,14,16]. Thus far, TAs have been identified from a wide range of organisms, as exemplified by LTAs identified in *Escherichia coli* [17], *Pseudomonas* sp. [18], *Pseudomonas putida* [19], and *Cellulosilyticum* sp. [4], and DTAs identified in *Arthrobacter* sp. [20], *Alcaligenes xylosoxidans* [15], *Delftia* sp. [12], and *Chlamydomonas reinhardtii* (green alga) [21].

PLP-dependent enzymes can be classified into a number of evolutionarily unrelated families (e.g., transaminases, alanine racemases, amino acid decarboxylases, threonine aldolases, serine hydroxymethyltransferases, tryptophan synthases, etc.) and their structures have five-fold types [22,23]. LTA, serine hydroxymethyltransferase (SHMT), and fugal alanine racemase belong to fold type I PLP-dependent enzymes and have similar active site residues [23,24]. An evolutionary analysis of the fold type I enzymes showed that LTA, SHMT, and fungal alanine racemase might belong to a subgroup composed of closely related proteins [24]. The enzymes all possessed catalytic promiscuity sharing retro-aldol cleavage, transamination, and racemization reactions [24–27]. The catalytic

specificity and substrate preference could be altered via mutagenesis of as few as one single residue site [28]. Kielkopf et al. [29] resolved the first structure of an LTA, which is a homotetramer, and a Lys residue positioned within a domain interface binds the PLP cofactor via a Schiff-base linkage. The key conserved residue positions of LTAs determine the reaction specificity by coordinating the cofactor PLP in the active site, which affects the activation of the cofactor and the tautomeric equilibrium of the reaction intermediates [27]. On the contrary, DTA is dependent on divalent metal ions and belongs to fold type III of PLP-dependent enzymes, which have distinct structures in comparison with LTA [30]. The structure of DTA is a homodimer structure, similar to the structures of bacterial alanine racemases. The metal ion is bound within a metal-binding site adjacent to the PLP cofactor. In comparison to LTA, the crucial active site residues of DTA are situated in an approximately mirror symmetry relative to the cofactor PLP.

The discovery of new TAs and the exploration of the structure–function relationships have laid a solid foundation for applying these enzymes to synthesize structurally diverse amino compounds, beyond the scope of β -hydroxy- α -amino acids. Furthermore, protein engineering of the enzyme class enabled the generation of potent mutants with enhanced catalytic performance and expanded catalytic scope. This review systematically summarizes recent advances in the catalytic scope, engineering, and synthetic applications of TAs.

2. Threonine Aldolase-Catalyzed Versatile Reactions for Amino Compound Synthesis

2.1. Threonine Aldolase-Catalyzed Aldol Condensation Between Aldehydes and Glycine

The native reaction of TA is the retro-aldol cleavage of threonine, generating glycine and acetaldehyde, and the reverse aldol condensation [3]. Based on the native reaction, the aldehyde scopes of both LTA and DTA have been expanded to a broad range of aldehydes, including aliphatic and (hetero)aryl-substituted aldehydes, enabling the synthesis of structurally diverse β -hydroxy- α -amino acids [11,14,16]. For instance (Figure 2), LTA has been used to catalyze the aldol condensation between glycine and 4-nitrobenzaldehyde for the stereoselective synthesis of *L*-threo-4-nitrophenylserine, the synthetic intermediate of the antibiotic chloramphenicol [14,31], the aldol condensation between glycine and 4-methylsulphonyl benzaldehyde for the synthesis of *L*-threo-4-methylsulfonylphenylserine (*L*-threo-MTPS), the precursor of the antibiotics florfenicol and thiamphenicol [32], and the aldol condensation between glycine and 3,4-dihydroxybenzaldehyde for the synthesis of droxidopa (*L*-threo-3,4-dihydroxyphenylserine), an effective anti-Parkinson's drug [33–35]. DTAs have been used to catalyze the aldol condensation between glycine and pyridine 4-carboxaldehyde for the synthesis of (2*R*,3*S*)-2-amino-3-hydroxy-3-(pyridin-4-yl)-propanoic acid, the intermediate in the synthesis of an investigational analgesic drug candidate. The reaction was notably scaled up to a 100 L scale, affording up to 74.6% isolated yield and >99.95% de [16]. Representative examples of TA-catalyzed aldol condensations between aldehydes and glycine, including reaction conditions, yields, and stereoselectivities, are summarized in Table 1. Further, as an alternative route, TAs could be harnessed to prepare optically pure β -hydroxy- α -amino acids via the resolution of a diastereomeric mixture by stereoselective retro-aldol cleavage of single isomers to aldehydes and glycine [15,36].

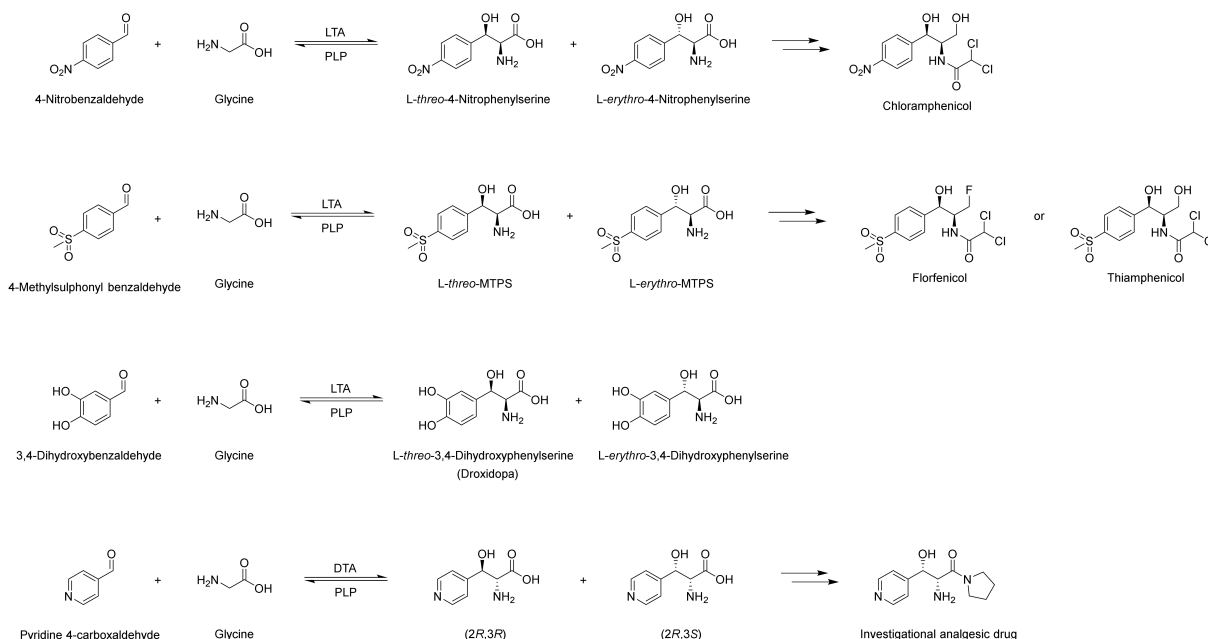


Figure 2. Representative examples of TA-catalyzed synthesis of pharmaceutical intermediates/APIs via aldol condensation of aldehydes and glycine.

Table 1. Representative examples of TA-catalyzed aldol condensation between aldehydes and glycine for the synthesis of β -hydroxy- α -amino acids.

Enzyme (Source)	Aldehyde Substrate	Product	Yield (%)	de (%)	Ref.
LTA (<i>Pseudomonas putida</i>)	4-Nitrobenzaldehyde	<i>L</i> -threo-4-Nitrophenylserine (chloramphenicol intermediate)	79	24	[14,31]
LTA (<i>Actinocorallia herbida</i>)	4-Methylsulphonyl benzaldehyde	<i>L</i> -threo-4-Methylsulfonylphenylserine (florfenicol/thiamphenicol precursor)	77	61	[32]
LTA (<i>Streptomyces avelmitilis</i>)	3,4-Dihydroxybenzaldehyde	<i>L</i> -threo-3,4-Dihydroxyphenylserine (droxidopa, anti-Parkinson drug)	71 (8 g/L)	60	[33–35]
DTA (<i>Alcaligenes xylosoxidans</i>)	Pyridine-4-carboxaldehyde	(2 <i>R</i> ,3 <i>S</i>)-2-Amino-3-hydroxy-3-(pyridin-4-yl)-propanoic acid (analgesic drug intermediate)	74.6	>99.95	[16]

2.2. Threonine Aldolase-Catalyzed Aldol Condensation Between Aldehydes and Non-Glycine Amino Acids as Amino Donors

Other than using glycine as the amino donor, TAs have been demonstrated to catalyze the aldol condensation between aldehydes and other amino acids. Fesko et al. [37] reported the identification of five enantiocomplementary LTAs and DTAs via sequence database mining and their application in catalyzing the aldol condensation between six aliphatic or aromatic aldehydes and D-alanine or D-serine (Figure 3). Thus, the asymmetric synthesis of β -hydroxy α -methyl-/ α -hydroxymethyl- α -amino acids (*syn* or *anti* configuration) were realized with up to 97% conversion and 92% de. Based on this, Blesl et al. [38] further extend the substrate scope of the TA-catalyzed reaction, with 30 aliphatic or aromatic aldehydes and five amino acids (except glycine) displaying apparent reactivity. Hence, β -hydroxy- α,α -dialkyl- α -amino acids (*anti* or *syn* configuration) were produced with up to >80% conversion and >100 (*syn*) diastereomeric ratio. Further, the scalability of the reaction protocol was examined by performing preparative scale synthesis of β -hydroxy- α,α -dialkyl- α -amino acids with an LTA and DTA respectively. The D-threonine aldolase

from *Pseudomonas* sp. could afford >100 (*syn*) diastereomeric ratio for the production of (2*R*,3*S*)-2-amino-3-(2-fluorophenyl)-3-hydroxy-2-methylpropanoic acid with 27% isolated yield. Representative examples of TA-catalyzed aldol condensations using non-glycine amino acids as amino donors, including enzyme sources, substrates, conversions, and stereoselectivities, are summarized in Table 2. Furthermore, the TA-catalyzed transformation could be combined with chemical β -dehydroxylation reactions to synthesize optically pure L- α -methyl-phenylalanine and its benzyl-substituted derivatives.

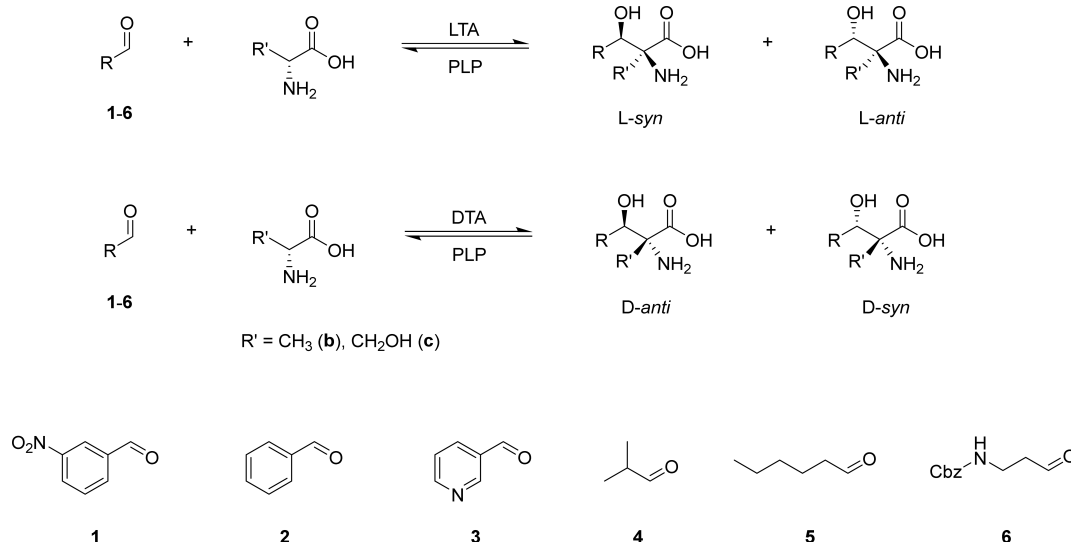


Figure 3. TA-catalyzed synthesis of tertiary α -amino acids via aldol condensation of aldehydes and non-glycine amino donors.

Table 2. Representative examples of TA-catalyzed aldol condensation using non-glycine amino donors for the synthesis of tertiary α -amino acids.

Entry	Enzyme (Source)	Amino Donor	Aldehyde Substrate	Yield (%)	de (%)	Ref.
1	LTA (<i>Aeromonas jandaei</i>)	D-Alanine	3-Nitrobenzaldehyde	92	4 (<i>syn</i>)	[37]
2	LTA (<i>A. jandaei</i>)	D-Serine	3-Nitrobenzaldehyde	39	22 (<i>anti</i>)	[37]
3	DTA (<i>Pseudomonas</i> sp.)	D-Alanine	3-Nitrobenzaldehyde	69	80 (<i>syn</i>)	[37]
4	LTA (<i>Aeromonas veronii</i>)	D-Alanine	Hexanal	48	32 (<i>anti</i>)	[37]
5	DTA (<i>Pseudomonas aeruginosa</i>)	D-Alanine	Hexanal	55	13 (<i>syn</i>)	[37]
6	LTA (<i>A. jandaei</i>)	DL-Alanine	Benzaldehyde	13	2.2 (<i>syn</i>)	[38]
7	DTA (<i>Pseudomonas</i> sp.)	DL-Alanine	3-Fluorobenzaldehyde	27	>100 (<i>syn</i>)	[38]
8	LTA (<i>A. jandaei</i>)	DL-Alanine	3-((Benzyloxy)carbonyl)aminopropanal	21	1.1 (<i>syn</i>)	[38]
9	DTA (<i>Pseudomonas</i> sp.)	DL-Alanine	3-((Benzyloxy)carbonyl)aminopropanal	32	57 (<i>syn</i>)	[38]

2.3. Threonine Aldolase-Catalyzed Aldol Condensation Utilizing Arylmethylamines as Amino Donors

TA-catalyzed aldol condensation reactions are generally restricted to small amino acids as amino donors, which confines the product scope to β -hydroxy- α -amino acids. Recently, Ouyang et al. [39] discovered that TAs were capable of utilizing (heterocyclic)benzyl amines as pro-nucleophiles for the aldol condensation with aldehydes, which provided a new approach for the stereoselective synthesis of a variety of 1,2-amino alcohols (Figure 4) [39]. Using 2-(aminomethyl)pyridine as the model amine donor and 3-phenylpropylaldehyde

as the model acceptor, several LTAs with reactivity were screened, affording 13–20% yield and 7:1 to >20:1 dr. Among the active LTAs, *Tm*LTA from *Thermatoga maritima* was selected for protein engineering due to its relatively high stereoselectivity (>20:1 dr), which enabled the generation of several variants with enhanced activity. Crystallographic studies showed that the key mutations in the active site were beneficial to the binding of sterically more demanding 2-(aminomethyl)pyridine by providing more spatial room. The variants were employed for catalyzing the aldol condensation of a broad range of aldehyde with various (heterocyclic)benzyl amines as pro-nucleophiles, delivering the corresponding 1,2-amino alcohols with up to 80% yield, >99:1 er (>98% ee), and >19:1 dr. At the same time, Smith et al. [40] independently reported that a functionally diverse panel of PLP-dependent enzymes, including threonine aldolases from *Aeromonas jandaei* and *Escherichia coli*, accepted (2-azaaryl)methanamines as pronucleophiles. Through protein engineering, the variant AzAld-V4 was developed, enabling the synthesis of various 1,2-amino alcohols with up to >99:1 er (>98% ee) and 47:1 dr [40]. The utilization of arylmethylanines as amino donors greatly extends the synthetic scope of TA-catalyzed aldol condensation for the synthesis of chiral amines.

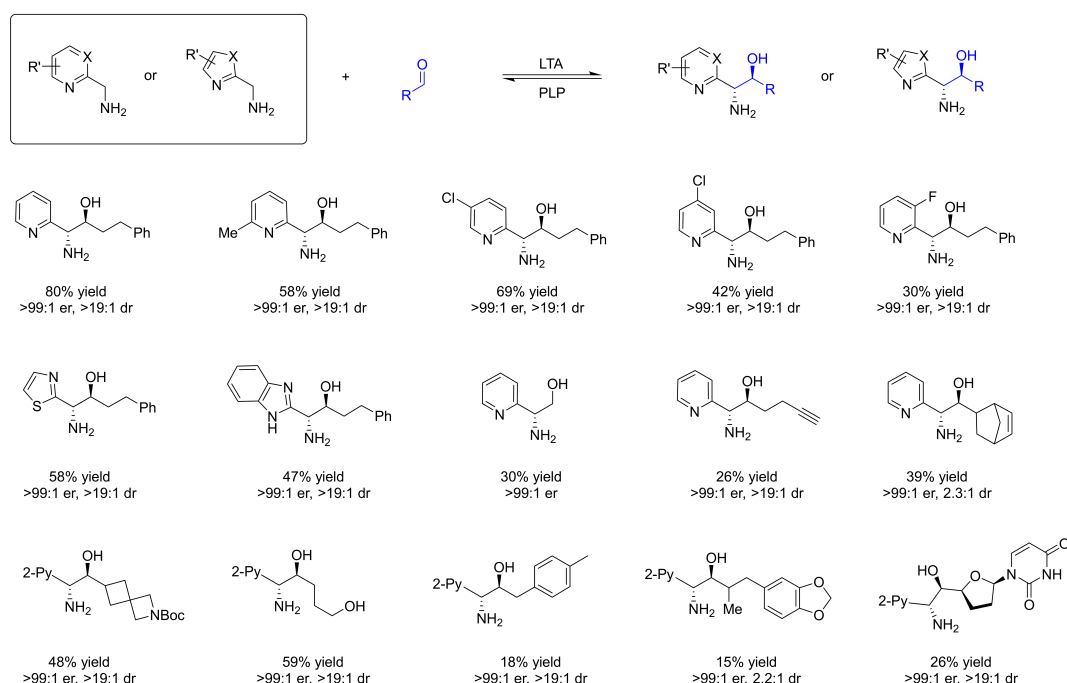


Figure 4. TA-catalyzed aldol condensation utilizing arylmethylanines as amino donors and selected examples.

2.4. Threonine Aldolase-Catalyzed S_N2 Reaction

Nucleophilic substitution (S_N2) reactions in carbon atoms play a pivotal role in organic chemistry and are widely applied in the synthesis of pharmaceuticals and agrochemicals. Natural enzymatic S_N2 reactions are seldomly identified due to the relatively high energy barrier for the trigonal bipyramidal transition state within the reaction process. Recently, Yang et al. [41] unveiled the unprecedented LTA-catalyzed S_N2 reactions between glycine and readily available α -halide carbonyl compounds, enabling the asymmetric synthesis of optically pure γ -oxo- α -amino acids (Figure 5A). A library of LTAs and DTAs was screened for the S_N2 reactions of various benzyl-substituted 2-bromo-acetophenones with glycine, resulting in the identification of several LTAs with up to 99% conversion and >99:1 er (>98% ee). Among the tested LTAs, *Lm*LTA showed the highest conversions and the broadest substrate spectrum, possessing substrate tolerance to 2-bromo-acetophenone

derivatives with various electron-withdrawing groups on the benzene ring. Molecular dynamics (MD) simulations and quantum chemical (QC) calculations were conducted to reveal the precise alignment of the substrate in the active site into a geometry favoring the S_N2 transition state (Figure 5B). The applicability of the biocatalytic protocol was demonstrated by preparative synthesis of a variety of optically pure γ -oxo- α -amino acids with up to 95% isolated yield and noticeable enantioselectivity (from 79:21 er (58% ee) to >99:1 er (>98% ee)).

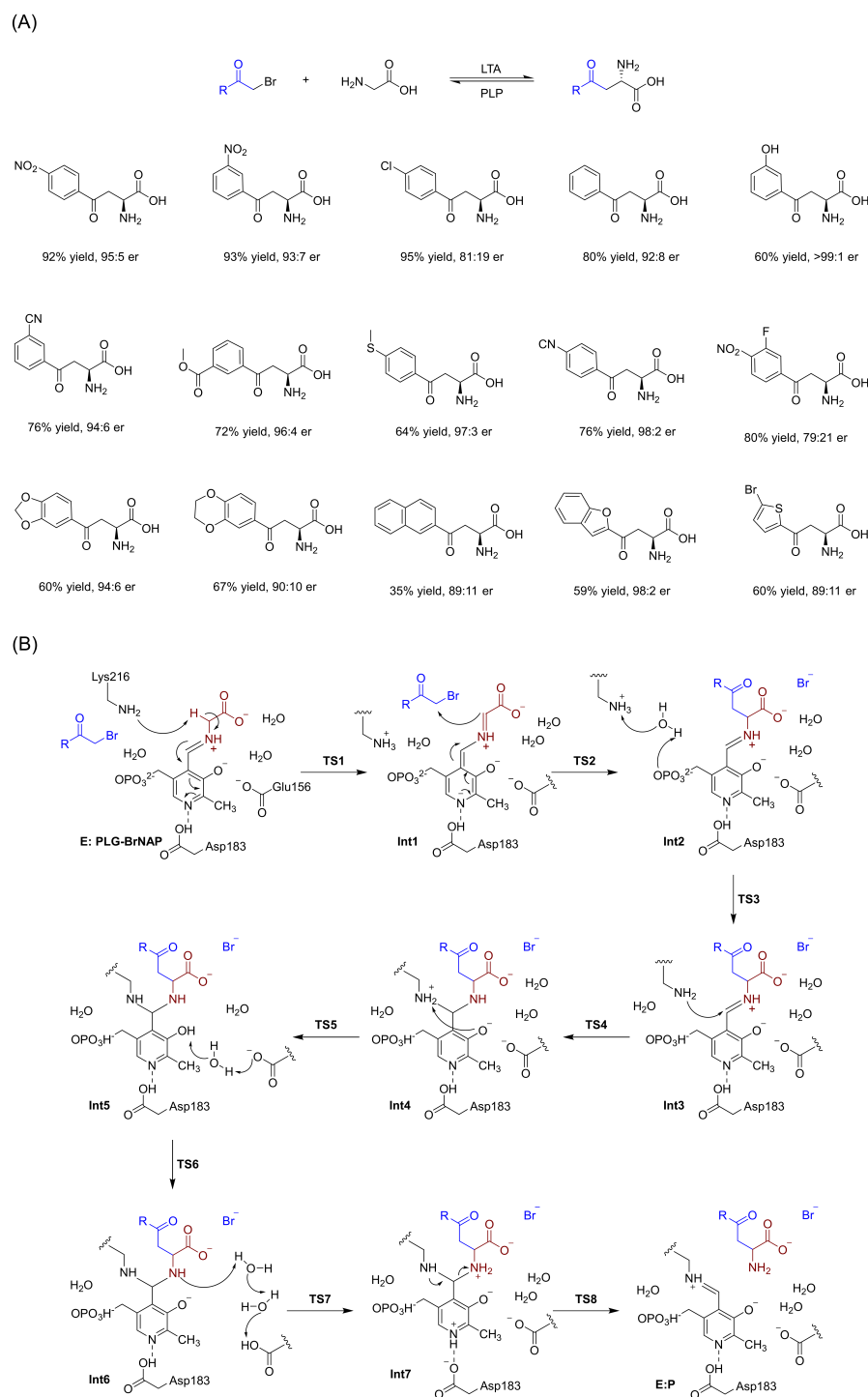


Figure 5. Schematic diagram of the S_N2 reaction catalyzed by threonine aldolase. (A) TA-catalyzed S_N2 reaction for the synthesis of optically pure γ -oxo- α -amino acids. (B) Proposed mechanism of the *LmLTA*-catalyzed S_N2 reaction based on MD simulations and QC calculations (adapted with permission from Ref. [41], copyright 2025, American Chemical Society).

3. Protein Engineering of Threonine Aldolases

3.1. High-Throughput Screening

Development of high-throughput screening is crucial for facilitating the protein engineering of enzymes in a labor-saving and cost-efficient manner. Due to the nature of the TA-catalyzed retro-aldol cleavage of β -hydroxy- α -amino acids (e.g., L-threonine), TA activity could be measured spectrophotometrically at 340 nm (indicating the concentration of NAD(P)H) by quantifying the generated aldehyde via alcohol dehydrogenase-coupled reduction reactions [18,42]. To measure both the activity and stereoselectivity of TAs, Chen et al. [43] developed a stepwise and colorimetric high-throughput screening method (Figure 6). After performing the aldol condensation reaction between benzaldehyde and glycine, the formed L-threo/erythro-phenylserine was quantified by measuring the residual aldehyde by adding 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AMHT, Purpald), which will react with the residual aldehyde to form purple dye with a characteristic absorbance at approximately 640 nm. On the other hand, the formed L-threo-phenylserine was quantified by coupling a stereoselective L-phenylserine dehydratase (L-PSDH), which transformed the formed L-threo-phenylserine to phenylpyruvate. The generated phenylpyruvate was measured by reacting with Fe^{3+} to form a cyan complex with characteristic absorbance at approximately 540 nm. Hence, the colorimetric high-throughput screening method can be utilized to evaluate the activity and stereoselectivity of TAs in a stepwise manner. Zhao et al. [44] developed a spectrophotometric high-throughput screening method for the C_β -stereoselectivity engineering of LTAs. This method employs a stereospecific L-threo-phenylserine dehydrogenase or *Actinomyces* ketone reductase (AcKRED) to specifically oxidize L-threo-4-methylsulfonylphenylserine (L-threo-MTPS) synthesized by LTA, generating NADPH that can be spectrophotometrically detected at 340 nm. The assay allows for rapid quantification of the desired diastereomer and supports high-throughput screening of mutant libraries. When applied to the error-prone PCR libraries of an LTA from *Aquaspirillum* sp., the system successfully identified a variant with markedly enhanced diastereoselectivity, improving from 0.98% de in the wild type to 71.9% de. Gong et al. [45] established a rapid and low-background colorimetric assay based on 2,4-dinitrophenylhydrazine (DNPH) for the high-throughput screening of threonine aldolases (TAs) (Figure 7). The high-throughput detection strategy quantified TA aldol condensation activity by monitoring residual aldehydes: aldehyde substrates react with DNPH to generate hydrazone derivatives, which were further converted into chromogenic 2,4-dinitrobenzoquinone (with characteristic absorbance at approximately 485 nm) under alkaline conditions. The DNPH method was successfully applied to screen the site-directed saturation mutagenesis libraries of *Alcaligenes xylosoxidans* D-threonine aldolase (AxDTA) and to evaluate the substrate specificity of the enzyme and its variants.

TA activity could also be screened using bacterial selection systems. Giger et al. [46] developed a high-throughput screening method for the engineering of TAs, which used an engineered glycine auxotrophic *Escherichia coli* strain, constructed by deleting four glycine biosynthesis-related genes (Figure 8A). This system restores the strain's glycine prototrophy via expressing aldolases that convert medium-supplied β -hydroxy- α -amino acids to glycine and corresponding aldehydes, enabling rapid screening by strain growth. The screening system facilitates the mechanistic studies, directed evolution, and substrate preference investigation of TAs. Bulut et al. [47] developed a growth-dependent high-throughput screening system in *Pseudomonas putida* KT2440, which utilized aromatic aldehydes, the product of TA-mediated retro-aldol cleavage of phenylserine or its derivatives, as the sole carbon source via the β -ketoadipate pathway (Figure 8B). Validation experiments showed that introducing plasmid-encoded LTA genes into the modified strain, whose native TA gene was deleted, restored its growth in minimal medium with DL-threo- β -phenylserine as

the sole carbon source. This system enables the easy identification of new L-specific TAs or benzaldehyde-liberating enzymes from genomic/metagenomic libraries.

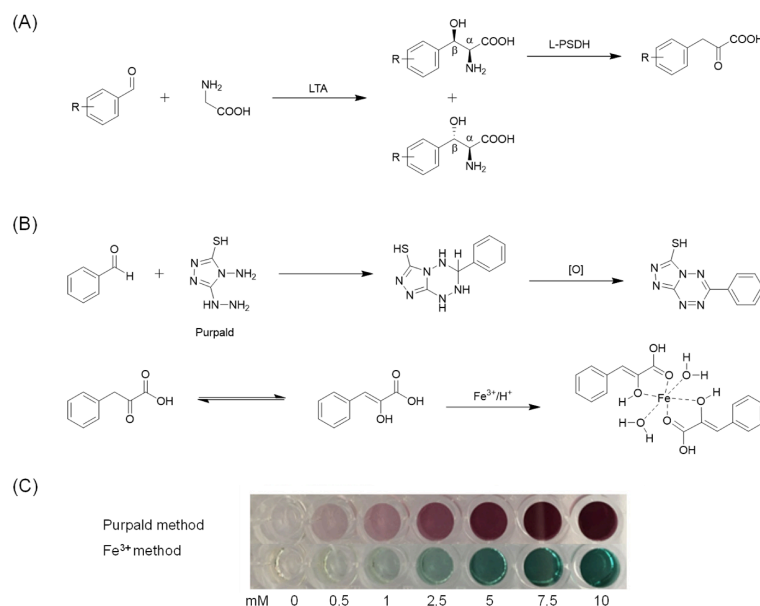


Figure 6. A stepwise and colorimetric high-throughput screening method for evaluating both the activity and stereoselectivity of TAs. **(A)** LTA-catalyzed reaction coupled with an L-PSDH for stereoselective transformation of L-threo- β -hydroxy- α -amino acids to 3-arylpyruvates. **(B)** Colorimetric assay reactions for benzaldehyde and phenylpyruvic acid. **(C)** Colorimetric assay results at varied concentrations of the analytes, adapted with permission from Ref. [43], copyright 2019, American Chemical Society. Quantitative absorbance measurements at 540 nm (provided in the original reference) confirmed the assay reliability and concentration dependence.

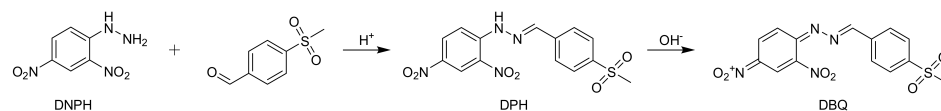


Figure 7. The DNPH method for screening the aldol activity of threonine aldolase via reaction between the residual aldehyde and DNPH.

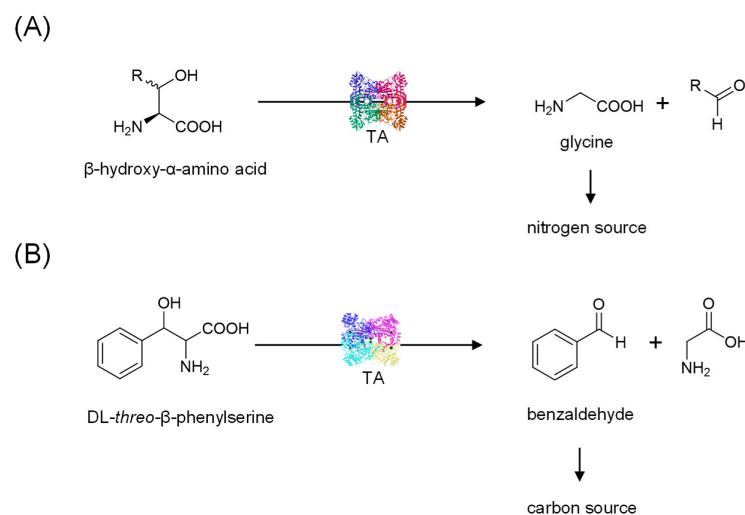


Figure 8. High-throughput screening of TA activity using bacterial growth-dependent selection systems. **(A)** Screening of LTA activity using the glycine auxotroph of *Escherichia coli* with tetracycline-inducible P_{tet} system. **(B)** Screening of LTA activity in *Pseudomonas putida* KT2440 using minimal medium M9 with DL-threo- β -phenylserine as sole carbon source.

3.2. Engineering the Stereoselectivity

TAs, catalyzing the reversible aldol condensation between aldehydes and amino acids, generally have high stereoselectivity in the α -amino group but suffer from limited stereoselectivity in the β -hydroxy group. Hence, great efforts have been made in engineering the class of enzymes for enhanced stereoselectivity. Compared to directed evolution, a semi-rational design enables the improvement of TA stereoselectivity with significantly smaller mutant libraries focused on key functional sites and has been widely employed in recent studies.

For instance, Chen et al. [43] engineered L-PsTA from *Pseudomonas* sp. to modulate its C_β -stereoselectivity toward aromatic aldehydes through rational multiple-site mutagenesis. With the colorimetric high-throughput screening method, the simultaneous saturation mutagenesis of key residues interacting with the amino and hydroxyl moieties of the substrate generated L-PsTA mutants (e.g., D93H/E147D and D93N/E147D) with enhanced or fully inverted stereoselectivity. Molecular docking analysis suggested that these mutations altered the spatial distances between the substrate and the critical histidine residues within the active site. He et al. [48] reported the discovery and rational engineering of an L-threonine aldolase from *Neptunomonas marine* (NmLTA) for the asymmetric synthesis of β -hydroxy- α -amino acids. Rational engineering yielded a triple mutant N18S/Q39R/Y319L (SRL), which achieved >99% diastereomeric excess and ~1.5-fold higher activity than the wild type. The variant displayed an expanded substrate scope toward aromatic aldehydes and showed enhanced stereoselectivity for para-substituted derivatives. Structural analysis revealed a dual-conformation mechanism governing diastereoselectivity, where 4-methylsulfonylbenzaldehyde could bind in the active cavity in two different orientations, leading to the formation of *threo*- and *erythro*-diastereoisomers, respectively. Zheng et al. [4,49] engineered the C_β -stereoselectivity of an LTA from *Cellulosilyticum* sp. (CpLTA) via a mutability landscape constructed by the saturation mutagenesis of residues lining the substrate access tunnel, combined with the combinatorial active-site saturation test/iterative saturation mutagenesis (CAST/ISM). The obtained triple variant H305L/Y8H/V143R showed drastically enhanced *syn*-selectivity, with its *syn* diastereoselectivity rising from 37.2% to 99.4%, while variant H305Y/Y8I/W307E displayed fully reversed stereopreference with 97.2% *anti*-diastereoselectivity. Molecular dynamics simulations and coevolution analysis uncovered two distinct substrate access tunnels responsible for *syn* and *anti*-product generation. The Prelog rule was further adopted to clarify the stereocontrol mechanism, in which *re*- or *si*-face nucleophilic attacks toward the carbonyl of the aldehyde due to its two distinct access tunnels determines the product configuration (Figure 9). Key mutations reshaped the two substrate access tunnels, thereby modulating the nucleophilic attack mode and altering the enzyme stereoselectivity.

A growing number of recent studies have described tuning the C_β -stereoselectivity of TAs via semi-rational design for the asymmetric synthesis of β -hydroxy- α -amino acids, particularly active pharmaceutical ingredients (APIs) and pharmaceutical intermediates. For instance, LTAs derived from *Pseudomonas putida* [50,51] and *Staphylococcus epidermidis* [52] were engineered for enhanced diastereoselectivity (up to 92.7% de) for the synthesis of L-*threo*-4-nitrophenylserine, the precursor of the broad-spectrum antibiotic chloramphenicol. LTAs derived from *Pseudomonas* sp. [53], *Bacillus nealsonii* [54], *Actinocorallia herbida* [55], *Leishmania major* [49], *Pseudomonas putida* [56], *Neptunomonas marine* [48], and *Cellulosilyticum* sp. [4] were engineered for improved diastereoselectivity (up to >99% de) for the synthesis of L-*threo*-4-(methylsulfonyl)phenylserine, the intermediate in the synthesis of the antibiotics florfenicol and thiamphenicol. LTAs derived from *Firmicutes bacterium* [5] and the gut microbiota of a black bear [57,58] were engineered for enhanced diastere-

oselectivity (up to 92.9% de) for the synthesis of L-threo-3,4-dihydroxyphenylserine, an anti-Parkinson's drug.

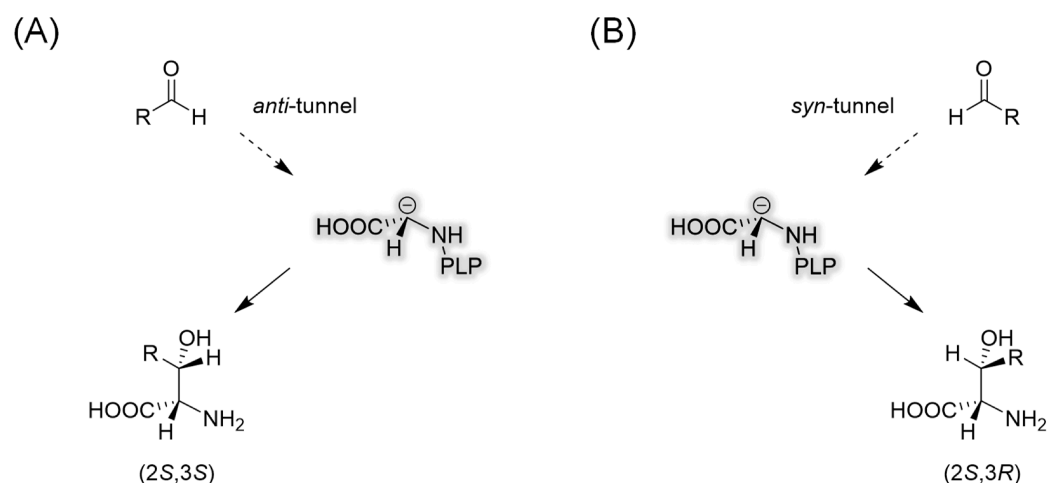


Figure 9. Illustration of the stereocontrol of LTA by aldehyde substrate access through distinct tunnels (*anti*-tunnel (A) and *syn*-tunnel (B)).

3.3. Engineering the Stability

The stability of the enzyme played a pivotal role in the synthetic efficiency of a biocatalytic system. Although great efforts have been made to improve the stereoselectivity of TAs, acquiring robust TAs with high thermostability is also critical, especially for transformations with high process intensity. Cai et al. [51] adopted computer-aided directed evolution to engineer an L-threonine aldolase (LTA) from *Pseudomonas putida* for the industrial asymmetric synthesis of L-threo-4-nitrophenylserine. With the aid of molecular docking and molecular dynamics simulations, key regions affecting stereoselectivity and cosolvent tolerance (e.g., unstable loop regions close to the tetramer interface potentially affecting cosolvent resistance) were identified to guide the design of site-saturation mutagenesis (SSM) libraries and combinatorial libraries. Through three rounds of iterative evolution and screening under stringent conditions (up to 40% ethanol and 45 °C), the optimized 18-site variant EM-ALDO031 exhibited drastically enhanced organic solvent stability, alongside greatly improved diastereoselectivity from 31.5% to 85% de. This semi-rational engineering approach greatly reduced the screening workload. The engineered variant achieved over 96% conversion at a high substrate loading of 150 g/L in 40% ethanol, well meeting industrial demands.

Improving the thermostability of enzymes with interfacial active centers while avoiding the undesirable stability–activity trade-off remains challenging. Fang et al. [59] reported a rational engineering strategy for LTA by modulating the rigidity of the V-shaped subunit through modification of its distal opening and hinge regions, rather than residues around the active center. A total of 25 key residues within these regions were subjected to SSM and Iterative Saturation Mutagenesis (ISM). A triple mutant G85A/M207L/A12C was ultimately identified, exhibiting substantially elevated thermostability with a 16 °C increase in T_m and a 34% improvement in specific activity at an optimal temperature, along with maintained diastereoselectivity. Molecular dynamics simulations further indicated that enhanced hydrophobic interactions and newly formed hydrogen bonds reinforced subunit rigidity, constrained domain fluctuation, and reshaped the active pocket. These structural changes facilitated proton transfer and NAC formation, thereby leading to simultaneous enhanced catalytic performance. Ruan et al. [60] reported the structure-guided interfacial engineering of an LTA from *Faecalimicrobium dakarensense* to improve stability. Key residues situated at homotetramer interfaces potentially posing a negative effect on the stability

of the complex were identified by calculating solvation free energy changes (Δ^iG) using the PDBePISA server. After subjecting the residues to virtual saturation mutagenesis and calculation of the folding free energy change ($\Delta\Delta G$), beneficial mutations predicted to have improved stability were constructed and examined. Thus, the optimal triple mutant M3 (L17F/V129R/L206W) was acquired, with prominently improved specific activity and thermal parameters (T_{50}^{60} increased by 20.5 °C; T_m increased by 13.1 °C). Mechanistically, molecular simulations confirmed that interfacial mutations strengthened subunit packing, expanded hydrogen bond networks and elevated structural rigidity, thereby resulting in the enhanced stability of the tetrameric complex.

4. Synthetic Applications of TAs in Cascade Reactions

The main applications of TA-involved cascade reactions in the synthesis of amino compounds are summarized in Table 3, including enzyme components, substrate scope, target products, and key performance data.

Table 3. Summary of representative cascade applications of threonine aldolases (TAs) in the synthesis of amino compounds.

Cascade System	Enzyme Components	Substrate Scope	Target Products	Key Results	Ref.
TA-AADC	LTA + L-TyrDC	Benzaldehyde + glycine	(R)-2-Amino-1-phenylethanol	91% yield, 78% ee	[61]
	LTA + DTA + L-TyrDC	DL-Phenylserine	(R)-2-Amino-1-phenylethanol	67% yield, >99% ee	[61]
LTA-TD-DH/TA	BM: LTA + TD; EM1: L-/D-HicDH + FDH; EM2: L-AADH + FDH; EM3: D-AATA + GluDH + GLR + FDH	9 Aldehydes (aromatic, heteroaromatic, heterocyclic, aliphatic)	α -Keto acids, α -Hydroxy acids, (S)- and (R)- α -Amino acids	α -Keto acids: 68–91% conv.; α -Hydroxy acids: 55–92% conv., 96–99% ee; (S)- α -Amino acids: 65–93% conv., >89% ee; (R)- α -Amino acids: 32–89% conv., >98% ee	[62]
AOX-LTA	AOX + Catalase + LTA	2-Fluoroethanol + glycine	4-Fluoro-L-threonine	91.7% yield, 21.3% de (syn)	[63]
TA-PSDH-PPDC-LTTA	PsTA + PxPSDH + PfPPDC + PfTTA + PtALDH + MgFR	Aromatic aldehydes + glycine + L-Thr	Aryl β -hydroxy- α -aminobutyric acids (β -HABAs)	For <i>p</i> -F, <i>p</i> -Cl, <i>p</i> -Br, <i>p</i> -NO ₂ , <i>p</i> -CF ₃ : >99% yield, >99% de	[64]

4.1. Cascades Combining L-Threonine Aldolase and L-Tyrosine Decarboxylase for the Synthesis of Vicinal Amino Alcohols

Griengl et al. [61] reported an elegant one-pot two-enzyme dynamic kinetic asymmetric transformation (DYKAT) that overcame both the thermodynamic and kinetic limitations inherent in aldolase-catalyzed reactions (Figure 10A). This cascade combines L-threonine aldolase (LTA) and L-tyrosine decarboxylase (L-TyrDC). LTA catalyzes the reversible aldol condensation of glycine with an aldehyde to form a β -hydroxy- α -amino acid intermediate (a mixture of L-*syn* and L-*anti* diastereomers); subsequently, L-TyrDC performs an irreversible decarboxylation, shifting the equilibrium toward the product side. Importantly, the intrinsic *syn*/*anti*-selectivity of L-TyrDC (10:1) dramatically improved the overall stereocontrol at the C $_{\beta}$ position, which is inherently poor when LTA is used alone. Using benzaldehyde as the model substrate, the two-enzyme system (LTA from *Pseudomonas putida* and L-TyrDC from *Enterococcus faecalis*) achieved >99% conversion of benzaldehyde after 58 h at pH 5.5 and 25 °C, affording a yield of 91% (R)-2-amino-1-phenylethanol. The C $_{\beta}$ stereoselectivity was enhanced from 60:40 (R/S, LTA alone) to 89:11 (R/S), corresponding to 78% ee for the R enantiomer. The further addition of D-threonine aldolase enabled a three-enzyme system for the dynamic kinetic resolution of DL-phenylserine to synthesize (R)-2-amino-1-phenylethanol, with up to 67% analytic yield (58% isolated yield) and >99% ee (Figure 10B).

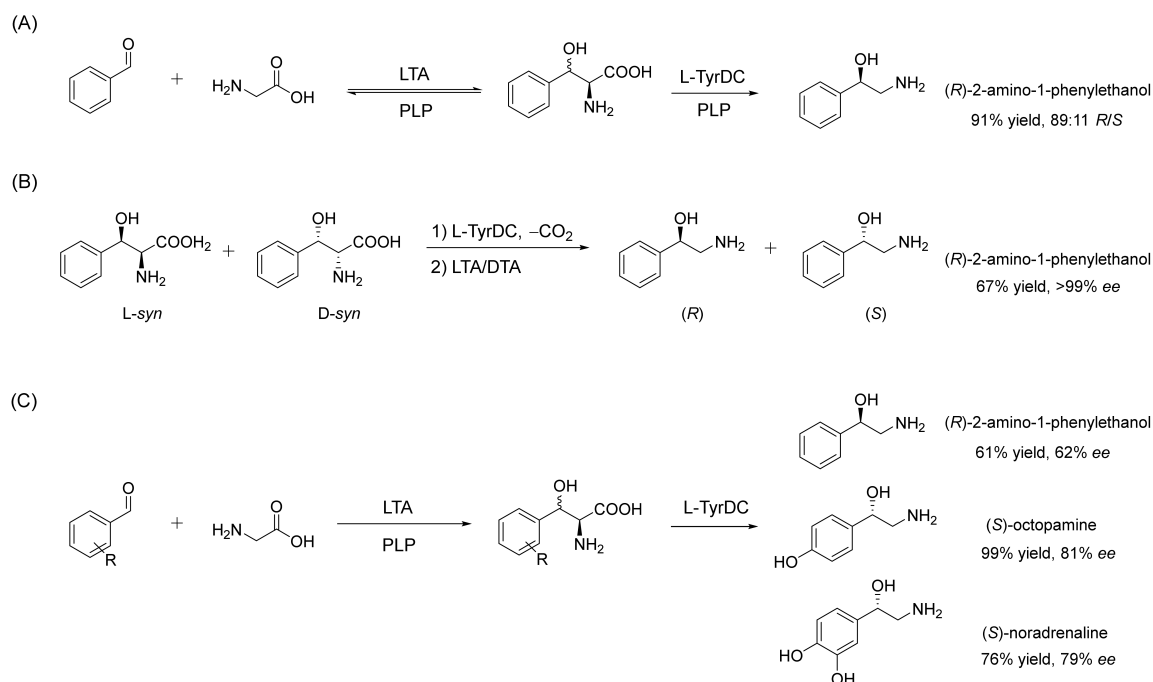


Figure 10. (A) A cascade combining L-threonine aldolase (LTA) and L-tyrosine decarboxylase (L-TyrDC) for the synthesis of (R)-2-amino-1-phenylethanol. (B) A cascade comprising L-TyrDC, LTA, and DTA for dynamic kinetic asymmetric transformation of DL-*syn*-3-phenylserine to (R)-2-amino-1-phenylethanol. (C) This work extends the application of the LTA–L-TyrDC cascade to synthesize chiral phenyl-substituted 2-amino-1-phenylethanols.

In a follow-up study, Steinreiber et al. [65] applied the LTA–L-TyrDC cascade to the transformation of 21 substituted benzaldehydes and evaluated three different L-TyrDCs (Efa, Efi1, and Efi2) (Figure 10C). Using LTA from *Pseudomonas putida* and L-TyrDC from *Enterococcus faecium* (Efi1) for the cascade reaction, TLC screening revealed that 3-hydroxy-, 4-hydroxy-, 2-fluoro-, and 3-fluoro-benzaldehydes were well tolerated, while bulky bromo- or nitro-substituted substrates afforded low conversion. For example, the cascade enabled the synthesis of (S)-octopamine from 4-hydroxybenzaldehyde with 99% yield and 81% ee, (S)-noradrenaline from 3,4-dihydroxybenzaldehyde with 76% yield and 79% ee, and (R)-2-amino-1-phenylethanol from benzaldehyde with 61% yield and 62% ee. The stereochemical outcome of *R* or *S* can be attributed to the changed diastereoselectivities of Efi1. The cascade was scaled-up to 160 mL for the synthesis of (S)-octopamine, affording 47% yield. This work demonstrated the substrate generality and preparative feasibility of the cascade strategy and revealed that the absolute configuration of the product (*R* vs. *S*) could be switched by changing the aldehyde substituent.

4.2. A Modular Enzymatic Cascade System for the Synthesis of α -Functionalized Organic Acids

Song et al. [62] developed an artificial biocatalytic cascade that integrates LTA, threonine deaminase (TD), and L- or D-hydroxyisocaproate dehydrogenase (HicDH)/L-amino acid dehydrogenase (L-AADH) to enable the chiral-group-resetting asymmetric synthesis of high-value α -functionalized organic acids from achiral glycine and aldehydes (Figure 11). The cascade operates in a modular assembly manner, comprising a basic module (BM) that harnesses LTA and TD to convert glycine and aldehydes into prochiral α -keto acids, followed by three extender modules (EM1–EM3) that transform these intermediates into the desired products. In the BM, LTA (*PaTA* from *Pseudomonas aeruginosa*) catalyzes the aldol condensation of glycine with an aldehyde to form a β -hydroxy- α -amino acid, which is then subjected to an irreversible α,β -elimination catalyzed by TD (*CgTD* from *Corynebacterium*

terium glutamicum), generating a prochiral α -keto acid. This two-step process effectively overcomes the thermodynamic equilibrium and low diastereoselectivity inherent in TA-catalyzed reactions. Subsequently, the α -keto acid is converted into the desired product by one of three extender modules: EM1 (L- or D-hydroxyisocaproate dehydrogenase (HicDH)) produces chiral α -hydroxy acids; EM2 (L-AADH) produces (S)- α -amino acids; EM3 (D-amino acid transaminase (D-AATA), coupled with glutamate dehydrogenase (GluDH), formate dehydrogenase (FDH), and glutamate racemase (GLR) for amino donor recycling), produces (R)- α -amino acids. Using this platform with nine structurally diverse aldehydes (aromatic, heteroaromatic, heterocyclic, and aliphatic), nine α -keto acids, 18 chiral α -hydroxy acids, and 18 chiral α -amino acids were produced. The conversions for the α -keto acids ranged from 68 to 91% (e.g., phenylpyruvic acid: 91%). For α -hydroxy acids, (S)- and (R)-products were obtained in 55–92% conversion with 96–99% ee. For (S)- α -amino acids and (R)- α -amino acids, 65–93% conversion with >89% ee and 32–89% conversion with >98% ee were achieved, respectively. The utility of the cascade system was demonstrated by scaling-up to 100 mL, giving isolated yields of 45–78% for the representative products (e.g., (R)-phenylalanine: 11.9 g/L, 81% conversion, 99% ee).

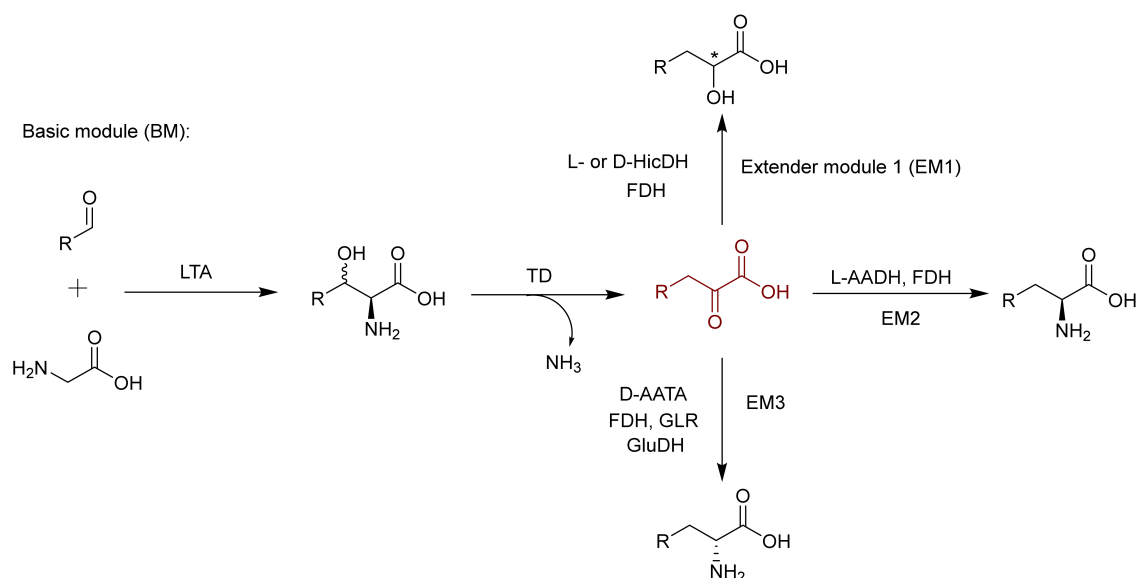


Figure 11. Synthesis of α -functionalized organic acids using the modular enzymatic cascade system. The asterisk (*) in the figure denotes (S)-/(R)-hydroxy acids.

4.3. A Two-Step Cascade Combining Alcohol Oxidase and L-Threonine Aldolase

De Maria et al. [63] described a two-step enzymatic cascade consisting of alcohol oxidase (AOX) and LTA for the efficient transformation of 2-fluoroethanol (F-EtOH) and glycine to 4-fluoro-L-threonine (F-Thr) (Figure 12). In this cascade, AOX first oxidizes the stable F-EtOH in situ to 2-fluoroacetaldehyde (F-MeCHO), which is then condensed with glycine by LTA to generate F-Thr. The strong electron-withdrawing effect of the fluorine atom shifts the reaction equilibrium toward the side of product formation, thereby overcoming the reversibility limitation inherent in conventional LTA-catalyzed reactions. Under optimized conditions (pH 8.0, 100 mM glycine, 10 mM F-EtOH, and 5 μ M LTA), the yield of F-Thr reached 91.7% after 24 h with 21.3% de (L-*syn* form). Comparison of the two LTAs showed that the LTA from *Pseudomonas putida* exhibited significantly higher catalytic efficiency than the LTA from *Escherichia coli* (39% vs. 13% conversion at 12 h). The fluorinated substrate gave approximately 2-fold higher conversion in comparison with the reaction using the non-fluorinated substrate (2-fluoroacetaldehyde vs. acetaldehyde, 39% vs. 21% conversion), suggesting that fluorine substitution efficiently drives the equilibrium

toward aldol addition. This two-enzyme cascade provides a concise and efficient route for the green synthesis of fluorinated non-canonical amino acids.

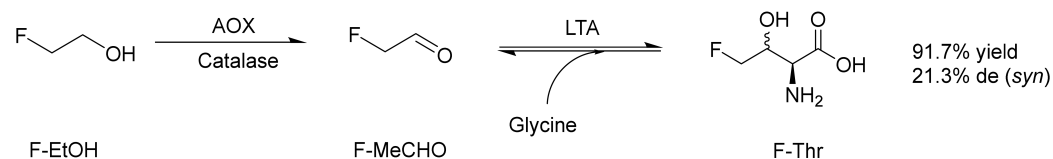


Figure 12. A two-step enzymatic cascade combining alcohol oxidase (AOX) and L-threonine aldolase (LTA) for the transformation of 2-fluoroethanol (F-EtOH) and glycine to 4-fluoro-L-threonine (F-Thr).

4.4. A Biocatalytic Cascade for the Synthesis of Aryl L-Threo- β -hydroxy- α -aminobutyric Acids via Carbon-Chain Extension

Chen et al. [64] developed an artificial biocatalytic cascade for the synthesis of aryl L-threo- β -hydroxy- α -aminobutyric acids (aryl L-threo- β -HABA) from achiral aromatic aldehydes, glycine, and L-threonine via a carbon-chain extension strategy (Figure 13). The cascade comprises LTA, phenylserine dehydratase (PSDH), phenylpyruvate decarboxylase (PPDC), and L-threonine transaldolase (LTTA), and operates as a one-pot, two-stage whole-cell catalysis system with two modules. Module 1 (TA and PSDH) catalyzes the aldol condensation of an aldehyde with glycine followed by dehydration to afford the corresponding α -keto acid; Module 2 (PPDC, LTTA, ALDH, and FR) catalyzes the decarboxylation of the α -keto acid to an aryl acetaldehyde, which then undergoes transaldolation with L-threonine catalyzed by LTTA. The system is further coupled with an acetaldehyde dehydrogenase (ALDH) and a cofactor regeneration module to remove the byproduct acetaldehyde, thereby shifting the equilibrium toward product formation. This strategy can circumvent the inherent instability and polymerization of aryl acetaldehyde intermediates. Under the optimized conditions using benzaldehyde as the model substrate, the two-step cascade employing *E. coli* (AD 4) and *E. coli* (DTOR 11) transformed 20 mM of the starting material to β -hydroxy-homophenylalanine (β -OH-homoPhe), with >99% yield and >99% de. Substrate scope investigation showed that for para-substituted benzaldehyde derivatives (*p*-F, *p*-Cl, *p*-Br, *p*-NO₂, and *p*-CF₃), the cascade could afford the target products in >99% yield and >99% de. For ortho- and meta-halogenated substrates, the conversion decreased in the order of F > Cl > Br. This work provides a versatile biocatalytic cascade approach for the in situ transformation of unstable aryl acetaldehydes and the efficient synthesis of chiral β -hydroxy- α -aminobutyric acids.

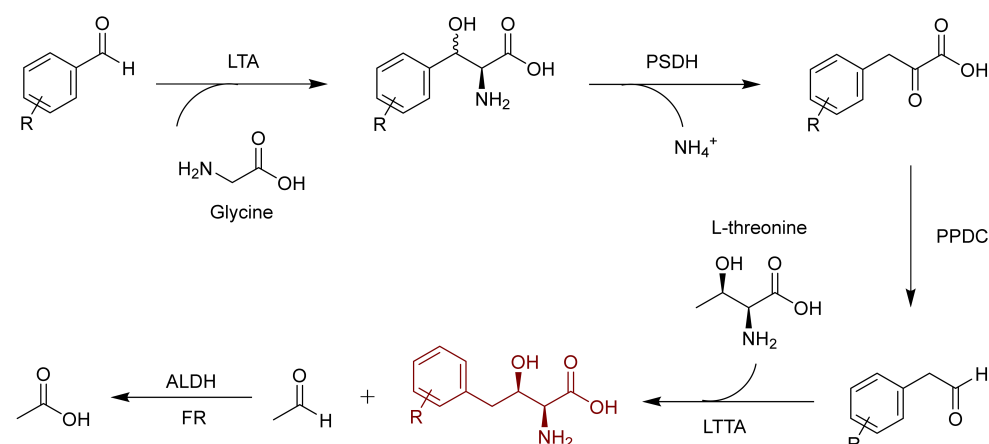


Figure 13. A multi-enzymatic cascade for the transformation of aromatic aldehydes to aryl L-threo- β -HABA with LTA, phenylserine dehydratase (PSDH), phenylpyruvate decarboxylase (PPDC), L-threonine transaldolase (LTTA), acetaldehyde dehydrogenase (ALDH), and flavin reductase (FR).

5. Conclusions and Outlook

In conclusion, TAs (LTAs and DTAs) are an important class for catalyzing the reversible aldol condensation between glycine and a broad range of aldehydes for the asymmetric synthesis of various β -hydroxy- α -amino acids. Other than the conventional reactions, a series of new reaction types have been unveiled with TAs, including aldol reactions between ketones and glycine, aldol reactions using non-glycine amino acids or arylmethanamines, and S_N2 reactions, which significantly extend the synthetic scope of the enzyme class. Furthermore, protein engineering has been widely utilized to evolve TAs, leading to the remarked improvement in C_β -stereoselectivity, activity, and thermostability. Moreover, the application of TAs in cascade reactions has greatly broadened the synthetic application of the enzymes in the production of structurally diverse amino compounds. Compared to non-enzymatic asymmetric aldol methods, TA-based biocatalysis offers distinct advantages, including mild aqueous conditions [66], perfect α -carbon stereocontrol [67], and the ability to operate in one-pot cascade systems without protecting groups [68]. With the rapid progress of AI-based enzyme mining and engineering technologies [69,70], the discovery and engineering of TAs will be achieved in a more timely and efficient fashion for certain reactions, thus greatly enriching the biocatalyst arsenal. Moreover, other PLP-dependent enzymes (e.g., LTAA [71,72] and UstD [73]) have been identified to catalyze different types of aldol reactions (e.g., decarboxylative aldol and transaldol reactions) compared to TA. Investigations of their differences in structure and catalytic mechanism may provide rational guidance for engineering TAs to expand substrate scope or develop new catalytic reactions.

Author Contributions: Conceptualization, F.-F.C.; writing—original draft preparation, X.-T.L. and F.-F.C.; writing—review and editing, A.-L.Z., F.-F.C., and K.-Q.C.; visualization, X.-T.L., R.-H.Z., Q.-W.W., and F.-F.C.; funding acquisition, F.-F.C. and K.-Q.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key R&D Program of China (2024YFA0917800) and the Basic Research Program of Jiangsu (No. BK20233003).

Data Availability Statement: No new data were created or analyzed in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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