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Review

The Promising Role of Amine Transaminase Cascades in the Synthesis of Non-Canonical Amino Acids

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Abstract: Amine transaminases (ATA) are critical players in producing non-canonical amino acids, essential building blocks in pharmaceuticals and fine chemicals. Significant progress has been made in discovering and engineering enzymes in this field, enhancing their use in organic synthesis. However, challenges such as co-factor regeneration, substrate, and product inhibition remain significant limitations to widespread industrial enzyme application. (Chemo-)enzymatic cascades offer efficient and environmentally friendly pathways for synthesizing amino acids, reducing the need for multiple synthesis steps and saving the purification of intermediates. This review focuses specifically on the synthesis of non-canonical amino acids, emphasizing the use of enzymatic and chemoenzymatic cascades involving ATA.

Keywords: amine transaminase; non-canonical amino acids; biocatalysts; high-throughput assays; cascade reaction

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1. Introduction

Amino acids (AAs) are used as building blocks for active ingredients. There is a growing demand for their use in pharmaceuticals, cosmetics, food, animal feed, and agriculture industries [1]. The global market size for AAs reached 11.4 million tons in 2023. According to IMARC Group, the market is expected to reach 16.8 million tons by 2032, with a projected Compound Annual Growth Rate (CAGR) of 4.2% from 2024 to 2032 [2]. Amino acids (AAs) consist of a carboxylic acid and an amino group. They can be linear or cyclic and have diverse structures. The amino group can be located at the alpha, beta, gamma, delta, etc. position concerning the carbonyl group of the acid [3].

The L-isomer of α-AAs plays a crucial role in biosciences as L-isomers are the building blocks of life. Proteins contain 22 genetically encoded amino acids (AAs), including 20 standard AAs, selenocysteine, and pyrrolysine [3]. These AAs, which are named proteinogenic AAs, are incorporated biosynthetically into proteins during translation. They are also known as canonical amino acids, derived from the Greek word kanón, meaning cane or rod. The majority of FDA-approved peptide medicines are composed of canonical amino acids (cAAs). These peptides exhibit low stability in the gastrointestinal system and poor permeability or bioavailability. Therefore, their administration via intravenous or subcutaneous routes is limited [4].

However, the range of natural amino acids from biological sources is much more extensive than the 22 proteinogenic ones. Up to 900 non-canonical amino acids (NcAAs) are known to date, including non-genetically encoded α-L-amino acids, D-amino acids, and β-amino acids (β-AA) [5].

Substituting cAAs with NcAAs enhances the stability of certain medications [6]. For instance, integrating NcAAs into antimicrobial peptides improves their resistance to degradation by microbes and enhances their stability [7]. Similarly, replacing the proline residue

at position 7 of bradykinin (BK) with D-phenylalanine transforms BK agonists into antagonists resistant to degrading enzymes [8].

NcAAs are found in 12% of the 200 top-grossing pharmaceuticals [9]. They can be found in a wide variety of drug classes, such as the HIV protease inhibitor Lopinavir (Abbott), the malaria drug Lariam (Roche), the anti-dementia drug Rivastigmine, the anti-diabetic drug Sitagliptin (Merck), antitumor (Paclitaxel) [10], antiviral agents (CCR-5 receptor antagonist Maraviroc) [11], and pharmaceutical hybrid peptides. They offer greater stability against peptidases while maintaining biological activity and reducing rejection by the human body [12,13]. As plant protection products (or agrochemicals in general), amines are used as herbicides, fungicides, and insecticides [14,15].

Enantiomeric pure NcAAs are highly economically important due to their wide-ranging industrial applications [16,17]. The best-known and most commercially relevant example of the above-mentioned compounds is a type II diabetes drug, namely, sitagliptin [18]. According to the verified market research (VMR) report on 31 July 2024, the global sitagliptin market is projected to exceed $6.6 billion in 2024 and reach USD 60.09 billion by 2031, growing at a CAGR of 34.90% from 2024 to 2031 [19].

Enantiomers are a particular type of stereoisomers. They have the same chemical structure but are non-superimposable mirror images of each other. In chemistry, enantiomers are often represented by classical notations such as (D) or (L) and (*R*) or (*S*) [20]. Enantiomerically pure compounds can be produced using either chemical or biocatalytic methods, or combinations of both. Biocatalysts offer several advantages over chemical synthesis [16]. Enantioselective synthesis is crucial in pharmaceuticals because the different enantiomers of a molecule often display varying biological activities. A classic example is thalidomide, a medication prescribed to pregnant women for morning sickness. The (*S*)-enantiomer of thalidomide exhibited toxicity, while the (*R*)-enantiomer was an effective treatment [21]. Enzymatic processes have been developed to meet strict purity and enantiomeric excess (*ee*) requirements for synthesizing enantiomerically pure amines for pharmaceutical applications [22–25].

Moreover, biocatalysis is key to sustainable chemistry: the need for protection and deprotection steps is usually circumvented leading to less waste and more cost-effective processes [26]. In particular, heavy metals can be eliminated, whereas enzymes are biocompatible, biodegradable, essentially non-toxic, and non-hazardous.

Transaminases (TAs) are enzymes involved in the cell metabolism of all known life forms. However, all industrially applied TAs have been sourced from microorganisms. Transaminases are divided into α-TAs, which transfer the amino group at the α-carbon, and ω-TAs, which transfer the amino group further away from the carboxylic moiety [13].

ω-TAs, commonly known as amine transaminases (ATAs), are crucial in the large-scale production of pharmacophores for various drugs [27]. ATAs (EC 2.6.1) play a key role in the amino acid metabolism of all known life forms and are widely distributed [28]. ATAs catalyze the transfer of an amino group from an amino donor to an amino acceptor. This involves a ping-pong bi-bi redox reaction in which the donor is oxidatively deaminated and followed by reductive amination of the acceptor [29]. The carrier of the amino group is pyridoxal phosphate (PLP), which transforms into pyridoxamine (PMP) in the process (Figure 1). The ATA-catalyzed reductive amination reaction is reversible and prone to substrate and product inhibition [30].

Over the past few years, ATAs have been effectively combined with other enzymes for various synthesis pathways, cofactor/by-product recycling, and removal. They are also applied in multi-enzymatic cascade reactions to produce chiral amino acids [31].

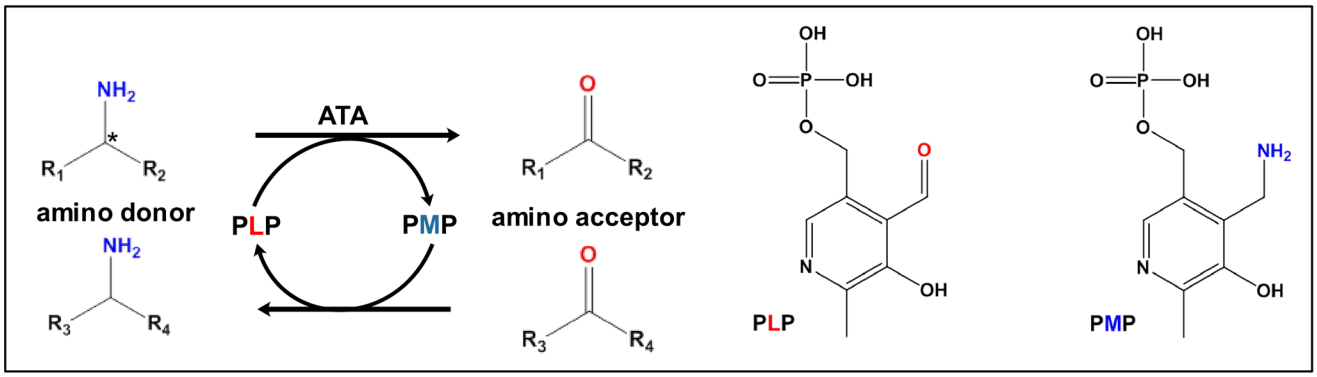


Figure 1. Ping-pong bi-bi mechanism of the transaminase reaction (usually highly enantioselective). The coenzyme is oriented at the ATA via a phosphate-binding pocket; during the reaction, covalent binding occurs via a lysine residue of the enzyme. While a wide variety of organic residues are possible for R_1 and R_3, only carboxyl groups -COOH are accepted as residues R_2 and R_4 of α-TA. For ATA, other (preferably short-chain) alkyl radicals or hydrogen atoms are also possible here; -H, $-CH_3$, $-C_2H_5$, and $-C_3H_7$ (also branched-chain) are preferred. * indicates the potential presence of a chiral center in the amino donor and the emerging product: Most ATAs act strictly stereoselective.

A cascade reaction is a one-pot synthesis in which two or more catalysts are used, and the product of the first catalyst is used as the precursor for the second catalyst [32]. Replacing multiple operations with a single enzymatic cascade has opened up new opportunities, leading to highly efficient and rapid synthesis of complex molecules from readily available starting materials. This concept can be applied in the industry to produce complex chemicals from inexpensive raw materials in a single vessel with the following advantages:

1. Enabling continuous cofactor regeneration: This procedure has enabled many enzymatic processes to operate at a commercial scale [33]. The NAD(P)H regeneration cascade for oxidoreductases is the most well-known co-factor recycling enzymatic system. This system is used to recycle the redox cofactor for many enzymes. Ketoreductases (KREDs) are part of the popular NAD(P)H regeneration cascade systems. KREDs depend on NAD(P)H regeneration to convert ketones and aldehydes into chiral alcohols. The resulting NAD(P) co-product is then used as a substrate for a second enzyme, such as glucose or formate dehydrogenases. These enzymes regenerate NAD(P)H at the expense of glucose or formate [34]. Different variations of this system have been patented for the preparation of *(3R,5S)*-6-chloro-3,5-dihydroxyhexanoic acid tert-butyl ester, a chiral precursor of statins [35,36].

Enzymatic reactions may require ATP as an energy source. It is of interest in in vitro ATP regeneration using newer polyphosphate kinase (PPK) systems, as they use a low-cost and stable phosphate donor. Biocatalytic cascades using PPKs have been created to produce L-theanine, an amino acid analog found in green tea with neurological effects [37]. These processes, however, have remained on a lab scale since then.

2. Eliminating the need to isolate reaction intermediates allows for driving thermodynamically unfavorable reactions to completion by constantly removing intermediates from the equilibrium [34,38]. For example, cathine ((*1S*,*2S*)-norpseudoephedrine) is a psychoactive compound produced in a one-pot reaction. The process combines (*S*)-selective lyase from *Acetobacter pasteurianus* and an (*S*)-ATA from *Chromobacterium violaceum*. In the first step, pyruvate is combined directly with benzaldehyde after decarboxylation to yield enantiomer mixtures of the phenylacetylcarbinol (PAC). In the second step, (*S*)-PAC converts to cathine through amination by (*S*)-ATA in the presence of an amino donor with *ee* > 97%. (*R*)-PAC, an undesirable compound, is converted to benzaldehyde and acetaldehyde by benzaldehyde lyase [39].

This review focuses on synthesizing non-canonical amino acids through transamination using cascade reactions.

2. Non-Canonical Amino Acids: The Building Blocks of the Pharmaceutical Industry [40]

2.1. D-*α-Amino Acids*

Besides achiral glycine, proteins exclusively consist of L-configurated amino acids. However, due to the components of the bacterial cell wall, large amounts of D- amino acids (D-AA) are found in all soil samples [41], fermented foods [42], and in animal intestinal and stool samples [43]. D-alanine in particular, as the central component of the peptidoglycan and the teichoic acids of Gram-positive bacteria, is a very frequently occurring D-AA and, not least for this reason, the central target of all penicillins and cephalosporins, and thus around half of the antibiotics on the market. D-phenylglycine (D-PheGly) and *para*-hydroxy-D-phenylglycine (*p*OH-D-PheGly) are required as side chains of the semi-synthetic antibiotics ampicillin and amoxicillin [44]. Moreover, D-phenylalanine is found in the structure of cyclic antibiotics such as gramicidin S and polymyxin B [45]. D-AAs such as D-Tyr are introduced as inhibitors of biofilm formation, reducing cell–cell and cell–membrane attachments [46,47].

In general, D-AAs often have key functions in natural products as a component of non-ribosomal peptides: for example, 13% of these compounds contain D-Ala, 10% each of D-Ser and D-Leu, and 9% D-Glu [48]. A difference in a D-amino acid contents in the molecules often results in different bioactivities [49] as well as significantly increased stability compared to most known proteases, which is particularly important for the oral administration of drugs [50].

Another representative of this drug class is used in reproductive medicine: Cetrorelix (Merck/Darmstadt) prevents a premature increase in luteinizing hormone during in vitro fertilization and thus unwanted early ovulation. This compound is a synthetic decapeptide and contains five D-AA, all of which have a non-proteinogenic residue (Figure 2). Cetrorelix has also been tested as a medication for various types of cancer and is currently being used in research into sex differentiation [51].

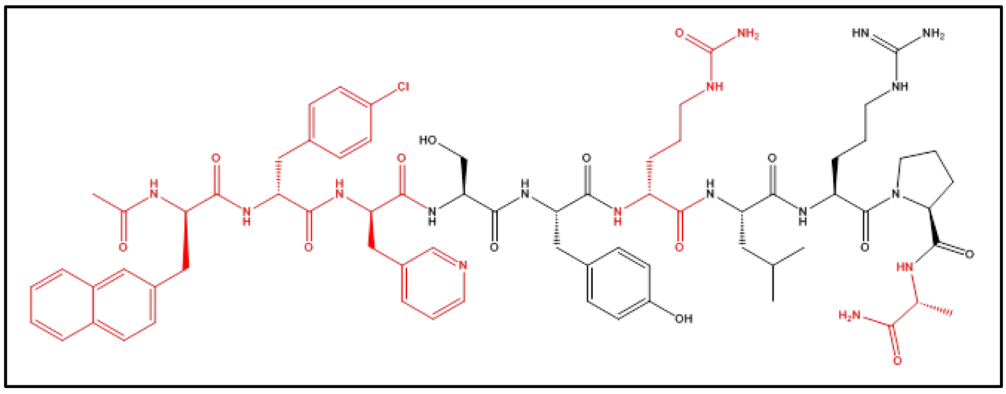


Figure 2. Cetrorelix used in reproductive medicine; D-amino acids in red. IUPAC-conform name according to PubChem: *N*-acetyl-3-(2-naphthyl)-D-alanyl-4-chloro-D-phenylalanyl-3-(3-pyridyl)-D-alanyl-L-seryl-L-tyrosyl-D-citrullyl-L-leucyl-L-arginyl-L-prolyl-D-alaninamide.

2.2. β-Amino Acids

β-Amino acids (β-AAs) are considerably rarer in nature than α-AA. Half of a cell's dry weight consists of proteins made from α-AAs [52,53], which therefore accounts for 10–20% of total biomass, depending on the water content of the cell type. The average occurrence of α-AAs in proteins varies from 1.1 to 9.7% [54]. Thus, according to the amino acid composition of the predominant cell proteins, each of the 22 proteinogenic amino acids averages about 0.1–1% (*w*/*w*) in living cells.

In contrast, the anticancer drug Taxol® (paclitaxel) is isolated from *Taxus baccata* at only 17.5 mg/kg of biomass (before optimizing the production), yielding just a few milligrams of its building block, β-phenylisoserine (a β-phenylalanine derivative) [55]. Thus, a specific β-amino acid (β-AA) is from 1000 to 10,000 times less abundant than its α-configured counterparts.

The difference between α-AA and β-AAs is an additional carbon atom positioned between the carboxylic and amine functionalities [56]. However, like D-AA, they are components of numerous natural products as well as valuable building blocks of pharmacological ingredients, which are often derived from these natural products. Another important field of application is the linkage to β-peptides, which are used as highly stable peptide mimetics for various purposes, as most peptidases do not recognize them [40,57]. The pharmaceutical industry is interested in developing production processes for enantiomerically pure β-amino acids, especially aromatic ones such as β-phenylalanine and β-tyrosine. The production process commonly used to date is based on racemic β-AA esters, which are enzymatically hydrolyzed with high selectivity to the desired β-AA. β-AAs are synthesized from L-AAs in nature through various enzyme-mediated mechanisms [58]. Below is a list of some natural β-AAs:

β-Alanine (3-aminopropanoic acid), a simple β-AA, is formed in the nucleotide metabolism of all living organisms during the degradation of the pyrimidine bases uracil and cytidine. In the human body, β-alanine is found in the brain and muscles, and may prevent muscle loss in old age when used as a dietary supplement [40,59,60].

β-Phenylalanine has a chiral center at the β-C atom, distinguishing D- and L-forms [61]. It is a component of critical chemotherapeutic agents such as paclitaxel and docetaxel [62]. In 2017, researchers accomplished the first purely biocatalytic synthesis of Taxol® analogs by linking enantiomerically pure β-phenylalanine derivative β-phenylisoserine with baccatin III, using a cascade reaction with four enzymes [63]. However, the scalability of this reaction depends on the availability of enantiomerically pure β-phenylalanine and the practicality of the enzymes used, which currently makes it economically unfeasible.

β-Aminoisobutyric acid (BAIBA) is a regulator of carbohydrate and lipid metabolism [64]. It is found widely in various organisms and is typically formed as a byproduct of primary metabolism. There are two enantiomers of BAIBA in biological systems. (*R*)-BAIBA is formed through the degradation of thymine in mammals, while the degradation of L-valine to (*S*)-BAIBA is also recognized in mammals [58].

(*S*)-β-Lysine has been discovered in various *Clostridium* species because it serves as the primary intermediate in the catabolism of L-lysine. Furthermore, (*S*)-β-lysine is a component of myomycin, a glycopeptide antibiotic. Myomycin exhibits moderate but wide-ranging activity against bacteria by restricting bacterial protein synthesis through direct interaction with the 30S subunit of the ribosome [65,66].

Lactams, including β-lactams, are cyclic amides. In the case of β-lactams, the nitrogen is linked to the β-carbon atom, so a cyclic β-AA is formally present (Figure 3). β-lactams, such as penicillins and cephalosporins, are the main class of antibiotics, remaining dominant for more than 80 years since their first use in 1941 [67,68]. Their effect is based on the structural similarity of the β-lactam with a bacterial cell wall component, leading to a halt in cell growth and eventual cell rupture, particularly effective in rapidly growing bacteria and acute infections.

Additional non-α-AAs that are significant for industrial or pharmacological pur-poses are presented in Table 1 [58,69].

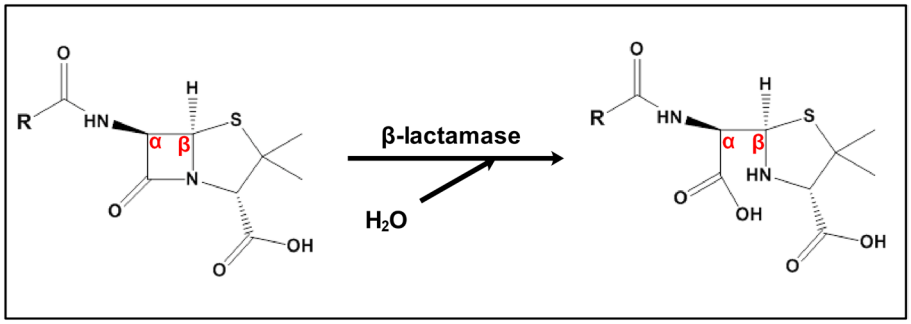
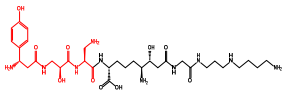
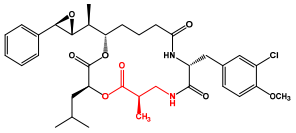
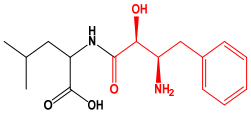
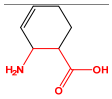
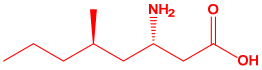
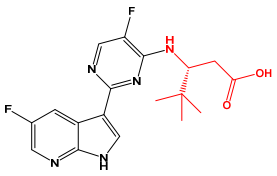


Figure 3. The hydrolytic cleavage of a penicillin by a β-lactamase (or penicillinase, if specifically penicillins are cleaved and no other β-lactams) to the β-AA penicilloic acid (unstable due to decarboxylation, which makes the cleavage irreversible).

Table 1. Additional industrially or pharmacologically important non-α-AA [58,69].

Non-α-Amino Acid	Connection	Function/Application	
β-tyrosine + isoserine + 2,3-diaminopropanoic acid	Edeine 1	Antibiotic	
3-NH_2-isobutyric acid	Cryptophycin 2	Chemotherapeutic agent	
3-NH_2-2-OH-4-phenylbutyric acid	Bestatin 3	Protease inhibitor	
2-NH_2-cyclohex-3-en-1-carboxylic acid	BAY 10-8888 4	Fungicide	
3-NH_2-5-methyloctanoic acid	Imagabalin 5	Candidate for anxiety disorder	
3-NH_2-4,4-dimethylpentanoic acid	PB2 inhibitor 6	Antiviral	

3. Properties and Classification of Amine Transaminases (ATA)

The ATA reaction is the most versatile biocatalytic method for obtaining chiral primary amines [70]. The reasons for this are excellent enantio- and regioselectivity, high reaction rates, and outstanding compatibility with other enzymatic and chemical reaction systems [71]. The broad substrate spectrum of these enzymes also allows access to amino acids, amino alcohols, and other optically pure amino compounds [72], which makes ATAs more practical in the industrial synthesis of medicine [73].

ATA catalyzes the transfer of an amino group from an amino donor to an amino acceptor. The latter is a ketone or aldehyde and becomes an amino donor itself through amination. Meanwhile, the previous amino donor can now in turn act as an amino acceptor. In the transamination reaction, the amino group is transferred from the donor substrate to the ε-amino group of the Lys residue of ATA, covalently bound to the C4′ atom on PLP. This process converts PLP to pyridoxamine-5′-phosphate (PMP), transferring its amino group

to the acceptor substrate to produce a new amine or amino acid [74,75]. The active site of ATAs has two pockets, with a catalytic lysine located between them, the P-pocket and O-pocket, based on their proximity to the phosphate and oxygen groups of PLP. The P-pocket can accommodate the smaller substituents such as methyl or short-chain alkyl group. In comparison, the O-pocket accommodates the bulkier substituents such as hydrophobic and carboxylate groups [74,76,77]. Research showed that mutation in either pocket could lead to accepting non-favorable substrates by ATAs [18,78,79].

PLP-dependent enzymes can be classified into seven different fold types (Table 2) [73], traced back to five evolutionary lineages based on sequence analyses [80]. ATAs are present in two of these fold types (fold type I and IV) [81]. ATAs in fold type I are *(S)*-selective, while those in fold type IV are *(R)*-selective. According to sequence classification, ATA can be categorized into five aminotransferase classes [82] or into six evolutionary protein families (Pfam groups), based on pattern recognition of protein domains in AA sequences (hidden Markov model) [83].

Table 2. PLP-dependent enzymes based on fold types [73,84].

Fold Type	Example	EC Number
I	Aspartate transaminase	2.6.1.X
II	Tryptophan synthase	4.2.1.20
III	Alanine racemase	5.1.1.1
IV	D-alanine transaminase	2.6.1.21
V	Glycosyl phosphorylase	2.4.1.1
VI	D-Lysine 5,6-aminomutase	5.4.3.4
VII	Lysine 2,3-aminomutase	5.4.3.2

In addition to the structural and evolutionary classification, there is a much older classification [85], which describes the regioselectivity of the enzymes and significantly influences the current functional classification of transaminases. This classification divides transaminase into α-TAs and ω-TAs (well-known ATA) based on the position of the transfer amino group concerning the carboxylic group of the substrate. α-transaminases transfer α-amino groups to the carboxylic acid group in the α-position and allow only the formation of α-AAs, while ω-TAs transfer amine from donor to acceptor, in which at least one of the substrates is a non-α-amine acid or a non-α-keto acid [86].

The substrate binding sites of folding types I and IV have a mirror image structure. The catalytically active lysine, which binds the coenzyme, is located on either the (*si*) or (*re*) side of the PLP (Figure 4). This means that there are complementary enzymes: folding type I ATAs convert (*S*)-amines and L-AAs, while folding type IV ATAs convert (*R*)-amines, D-AAs, and branched-chain L-AAs. The latter enzymes are also known as DATA (D-amino acid transaminase) and BCAT (branched-chain amino acid transferase).

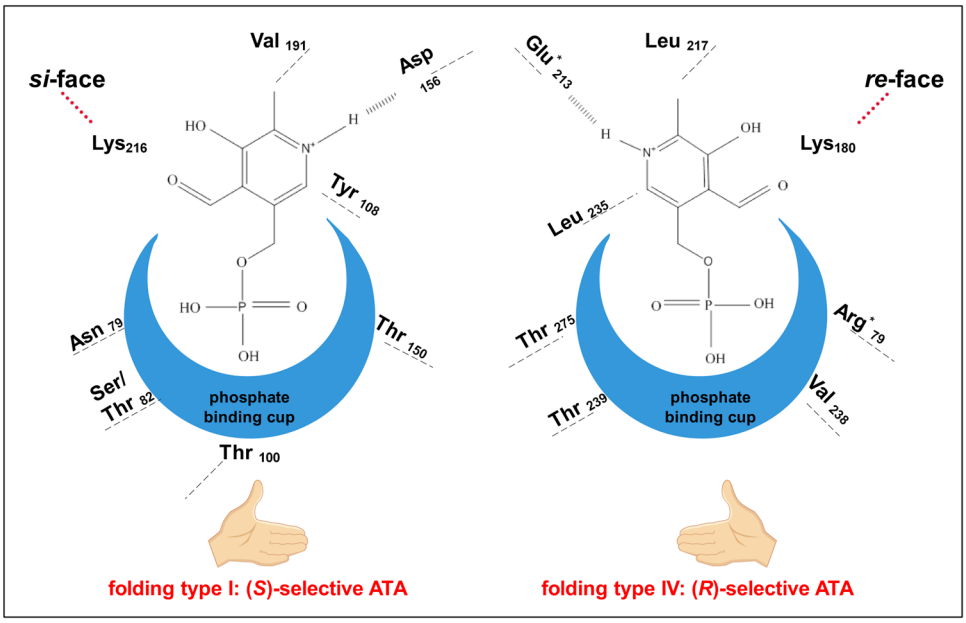


Figure 4. A comparison of the active sites of an *(R)*-selective ATA of folding type IV and an (*S*)-selective ATA of folding type I. The functional AA residues were assigned according to the structurally well-studied (*R*)-ATA from *Aspergillus terreus* (PDB 4CE5) and the *(S)*-ATA from *Mesorhizobium* sp. LUK (PDB 2YKU). The decisive factor is the position of the lysine residue, which is essential for catalysis, in relation to the coenzyme PLP. * indicates the highly conserved position Arg79 is present in all sequences and is part of the conserved PLP-binding cup formed by the likewise conserved Glu213. The Figure is modified after Buß et al. [87].

4. Synthesis Strategies with ATA

There are three strategies for synthesizing chiral amines or AAs by ATAs, which are summarized in Figure 5. In principle, chiral (or at least enantio-enriched) amine functions can be generated by asymmetric synthesis starting from prochiral ketones and by kinetic resolution of racemic amines. By means of deracemization, both the (*R*) and the (*S*)-enantiomer can be generated by using both synthesis strategies with two ATAs of different selectivity.

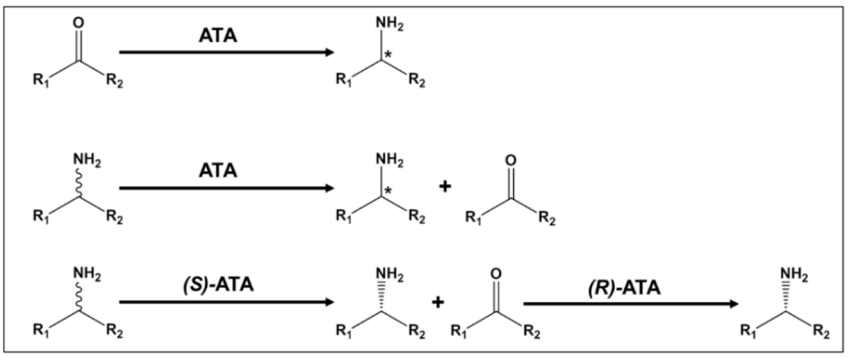


Figure 5. Three methods for synthesizing chiral amines with ATA. For the sake of clarity, the amino donors or acceptors required for the respective reaction (Figure 1). Top: asymmetric synthesis; middle: kinetic resolution; bottom: deracemization (1-pot-2-step, also possible with AA oxidases). * indicates the creation of a new chiral center in the emerging product.

Asymmetric synthesis: Asymmetric synthesis can theoretically yield 100% of the desired enantiomer without unwanted by-products. However, this process faces challenges due to the reaction equilibrium often favoring the formation of the ketone side. For example, the equilibrium constant of the amination of acetophenone with alanine to give α-

methylbenzylamine (MBA) is 8.81×10^{-4} [88]. Numerous approaches have been developed to remove the resulting co-product (then pyruvate) in an enzymatic cascade reaction (lactate dehydrogenase, pyruvate decarboxylase, acetolactate dehydrogenase) or alternatively even to recycle the amino donor (amino acid dehydrogenase) when using other amino donors, especially the universally accepted alanine. With appropriate metabolic turnover, the pyruvate can also be metabolized as a C source if the reaction is carried out as whole-cell catalysis.

Kinetic resolution: The simplest way to kinetically resolve primary amines is to add stoichiometric equivalents of pyruvate (or other α-keto acids suitable as amino acceptors). In the kinetic resolution reaction, the thermodynamic equilibrium is on the product side, where, in addition to the optically enriched amine, the remaining ketone and the corresponding α-AA are present, in the case of pyruvate corresponding to alanine [88]. In contrast to the asymmetric syntheses described above, no enzyme is required apart from the ATA, and there are further limitations in addition to the apparent disadvantage of a theoretical yield limited to a maximum of 50%: First of all, in addition to the pyruvate used, the ketone formed can also have a strong inhibitory effect on the reaction [89]. Furthermore, the co-product formed in stoichiometric amounts (in this case, L-alanine) can interfere with the separation of the product. As a consequence, either the ketone formed can be withdrawn or the concentrations of both the pyruvate used and the alanine formed can be kept low by permanently recycling the amino acceptor [88].

The latter can be achieved with the aid of an amino acid oxidase, which oxidizes alanine to pyruvate by means of molecular oxygen. Although this cancels the advantage described above (only one enzyme is required), the irreversible oxidation (with the formation of H_2O_2 and ammonia) additionally pulls the equilibrium to the desired product side (Figure 6). By using commercially available D- or LAA oxidases, both alanine enantiomers can be recycled to pyruvate, which is the co-product when using an (*R*)- or (*S*)-selective ATA [90].

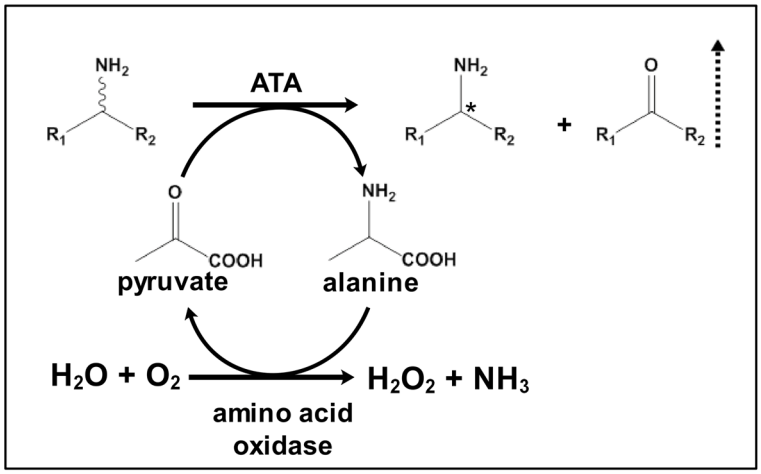


Figure 6. The equilibrium of kinetic resolutions of racemic amines to chiral amines can be shifted to the product side by evaporating the ketone (top; only suitable for small and highly volatile molecules) or by recycling the amino acceptor (bottom). The latter reaction can stereo specifically recycle both D- and L-alanine to pyruvate if a suitable oxidase is used. * indicates the creation of a new chiral center in the emerging product.

The resulting ketone can be removed from the equilibrium in various ways, including evaporation under negative pressure [91], solid-liquid two-phase systems [92], or packed-bed reactors with ATA immobilized in alginate [93].

The separation processes described are also suitable for continuously removing the resulting chiral amine from the reaction in an asymmetric synthesis and thus achieving significantly higher conversions [94].

Deracemization: According to Figure 5, the deracemization of racemic amines requires two ATAs with complementary enantioselectivity. This method is limited by the substrate compatibility of these two enzymes; the cascade reaction must be performed stepwise, with the first enzyme being inactivated [71]. For example, the deracemization of *rac*-2-aminopentane was achieved by a combination of the (*S*)-selective ATA-113 and the (*R*)-selective ATA-117 with enantiomeric excesses of only 75% (*S*) or even only 29% (*R*), depending on the sequence or addition of the enzymes [95]. However, by including an additional heat inactivation step before adding the second enzyme an *ee* > 99% was realized. Further optimization was achieved by using an amino acceptor in the first step, which cannot be used by the ATA in the second step, so that the reaction was pulled toward the product side [96]. This made it possible to avoid the complex inactivation step.

5. Screening of New Microorganisms with ATA Activity

5.1. Discovering Novel ATA

In the last decade, bacterial enrichment cultures have led to the discovery of class III and IV ATAs with previously undescribed activities. This approach, known as culture-based screening, involves screening bacterial isolates expressing genes with the ability to metabolize specific amines as the sole nitrogen source in minimal sugar-based media [13,97]. Several microorganisms from soil samples were identified using this approach by using 1-methylbenzylamine [98–101], 1-cyclopropylethylamine [102], 2-aminobutane, 1-phenylpropylamine, 1-aminoindane, 1-aminotetraline [103] β-phenylalanine [104], 3-amino-3-phenylpropionic [105], or other nitrogen sources.

In silico-based approaches, ATA activity is quickly discovered by data mining genome, protein, and motif databases. In this regard, searching protein databases by multiple alignments of (*R*)-ATA protein led to identifying putative (*R*)-ATA activity from bacteria and fungi. Among them, 17 (*R*)-selective amine transaminases synthesized several (*R*)-amines with >99% *ee* [106]. Moreover, the motif sequence blast search offers a quick and effective method for in silico identification of new (*R*)-ATA and (*S*)-ATA. In this method, conserved motifs of (*R*)- or (*S*)-ATA were applied as a template sequence for BLASTP in the NCBI [107].

Metagenomics, which is culture-independent, is a promising approach to discovering novel ATAs. DNA is extracted from the environment and analyzed [108,109]. Previously, the extracted DNA was fragmented and inserted into a proper vector. The vector screening went through either a sequence-based approach or a functional-based approach [110,111]. With advancements in sequencing technology, environmental DNA can now undergo whole genome sequencing directly. For example, nine volunteers' whole genome sequencing of oral tongue scrapings identified 11 putative class III transaminases. After cloning in an expression vector, three of them showed transaminase activity [109]. In another study, DNA sequencing of domestic households led to the identification of 36 novel class III transaminases. Twenty-nine genes were successfully cloned and expressed, and their transaminase activity was surveyed against different substrates. Three of them showed promising activity toward heterocyclic ketones [110].

5.2. High-Throughput Assays for the Determination of ATA Activity

Over the past two decades, many rapid high-throughput screening (HTS) methods have been developed to determine the substrate range, suitable amino donors and acceptors, and stability to different reaction conditions such as temperature, pH value, or various (organic) solvents for ATA. These methods were fundamental for the targeted optimization of ATA using valid selection methods. The detection methods described below are summarized in Figure 7.

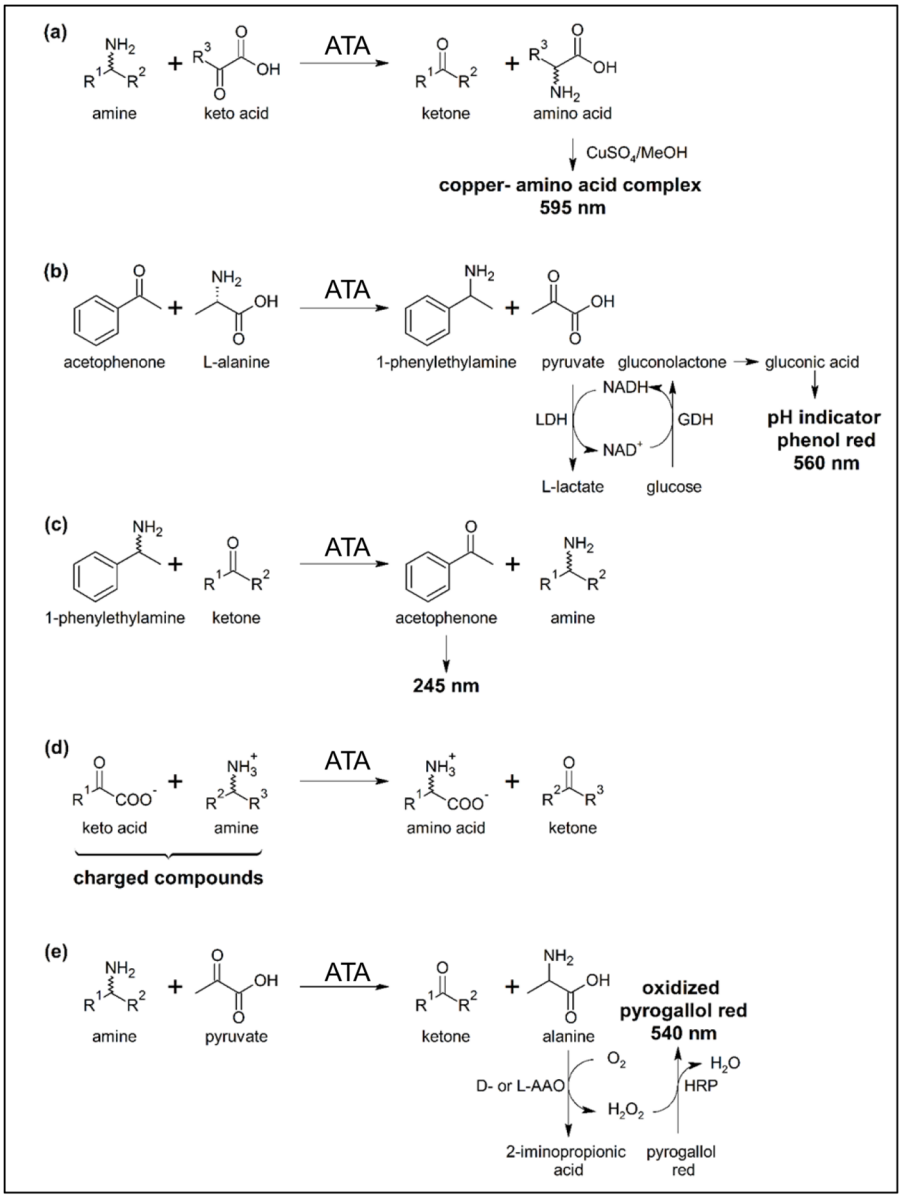


Figure 7. Activity assays for screening and characterization of ATA activity. (**a**) Formation of a blue copper complex; (**b**) pH drop; (**c**) absorption measurement of the resulting acetophenone; (**d**) decrease in conductivity; (**e**) oxidation of pyrogallol red via coupled enzyme reaction. LDH = lactate dehydrogenase, GDH = glucose dehydrogenase, AAO = amino acid oxidase, HRP = horseradish peroxidase. Figure is taken from [40]. Copyright © 2012 Rudat et al.; licensee Springer, Berlin, Germany.

An early assay is based on the formation of a blue amino acid–copper complex using an α-keto acid as amino acceptor [112] and a $CuSO_4$/MeOH staining solution (Figure 7a). This assay can use various amino donors, including aliphatic and aromatic β-AAs. However, the method has limitations, such as potential enzyme inhibition by the test solution, restricting it to endpoint determination. Additionally, it is not compatible with crude extracts due to interference by free α-amino acids, necessitating enzyme purification, which limits its application.

A multi-enzyme system was developed to assess ATA activity efficiently and on a large scale with a shift towards producing the final product [113]. In this system, the pyruvate produced in the transaminase reaction is converted to lactate by lactate dehydrogenase (LDH) (Figure 7b). Glucose dehydrogenase (GDH) regenerates the NADH coenzyme re-

quired for this process. The oxidation of glucose to gluconic acid results in a decrease in pH, which can be precisely and rapidly quantified using a pH indicator (phenol red). However, this approach has some limitations, such as the exclusive use of alanine as an amino donor and the restricted ability to change reaction conditions in a multi-enzyme system, which limits the characterization of the ATA beyond the range of potential amino acceptors.

The assay involves using 1-phenylethylamine to produce acetophenone, which can be detected in the UV range at 245 nm (Figure 7c). This test is sensitive and can be conducted with cell extracts. The use of enantiomerically pure (*R*)- or (*S*)-MBA allows for the determination of the enzyme's enantiopreference [114].

A different method is used to determine amino donor specificity (Figure 7d). This approach measures the change in conductivity of the reaction mixture as the reaction progresses. It offers the advantage of using a wide range of amino donors and acceptors and high sensitivity. However, it is time-consuming to measure many samples simultaneously because each reaction vessel must be equipped with an electrode [115].

Another method involves a multienzyme reaction with an AA oxidase (AAO) that converts D- or L-alanine to the corresponding imine (Figure 7e). The H_2O_2 produced in this process oxidizes the dye pyrogallol red with the help of horseradish peroxidase. The progress of the reaction can be tracked by measuring the decrease in absorbance at 540 nm [116]. This approach offers the flexibility to use various amino donors and determine the enantiopreference of the transaminase. However, free AAs disrupt the reaction, making crude extracts unsuitable. Furthermore, the limitations of a multi-enzyme system restrict the extent to which reaction conditions can be modified.

A breakthrough in the analysis of ATA, especially in high throughput, was the introduction of so-called smart amino donors, whereby a product with easily detectable properties is created without additional reagents or enzymes. The establishment of *ortho*-xylylenediamine (OXD) as a "smart" amino donor is particularly noteworthy [117]: after deamination, the remaining molecule polymerizes spontaneously and forms a black precipitate, which also strongly pulls the equilibrium towards the product side (Figure 8). This method is also suitable for screening whole cells and was modified in the present work so that individual transaminase bands can also be detected in protein gels (SDS-PAGE) of crude extracts from wild-type strains (i.e., without overexpression or further purification) [118].

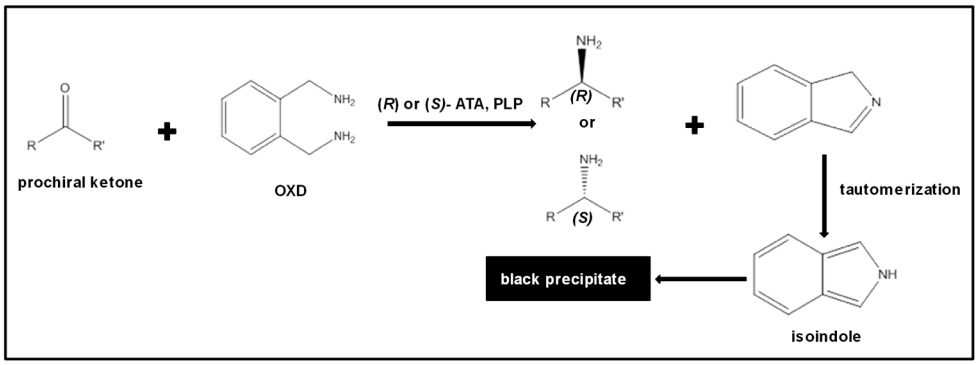


Figure 8. The use of the "smart amino donor" *ortho*-xylylenediamine (OXD): The simple deamination of OXD by ATA leads to spontaneous tautomerization of the remaining molecule, which polymerizes and forms the black precipitates. Figure taken from © 2021 Gord Noshahri et al., licensee Springer, Berlin, Germany [118].

Tetrazolium red (TTC)-based colorimetric assay developed for high-throughput screening ATAs in a microtiter plate. As TTC is reduced, α-hydroxy ketones undergo oxidation to form diketones or aldehyde ketones, forming an intense red formazane precipitate. The absorbance of the precipitate is measured using a microplate reader at 510 nm [119].

A recent high-throughput assay using fluorescence has been developed to improve the efficiency of smart amino donors. This assay was utilized for ATA and expands its substrate spectrum [120]. The measurement of enzyme activity is based on the blue light fluorescence (460 nm) of 2-acetyl-6-aminonaphthalene, formed from the amino donor 6-amino-α-methyl-2-naphthalene methanamine during the reaction. Fluorescence measurement can be performed with 310 nm excitation in micro-titer plates, allowing for the screening of protein engineering success on a large scale. Figure 9 provides an example of the feedback from the assay and protein engineering approaches for ATA optimization, which were generally pursued using high-throughput screening methods similar to those mentioned above.

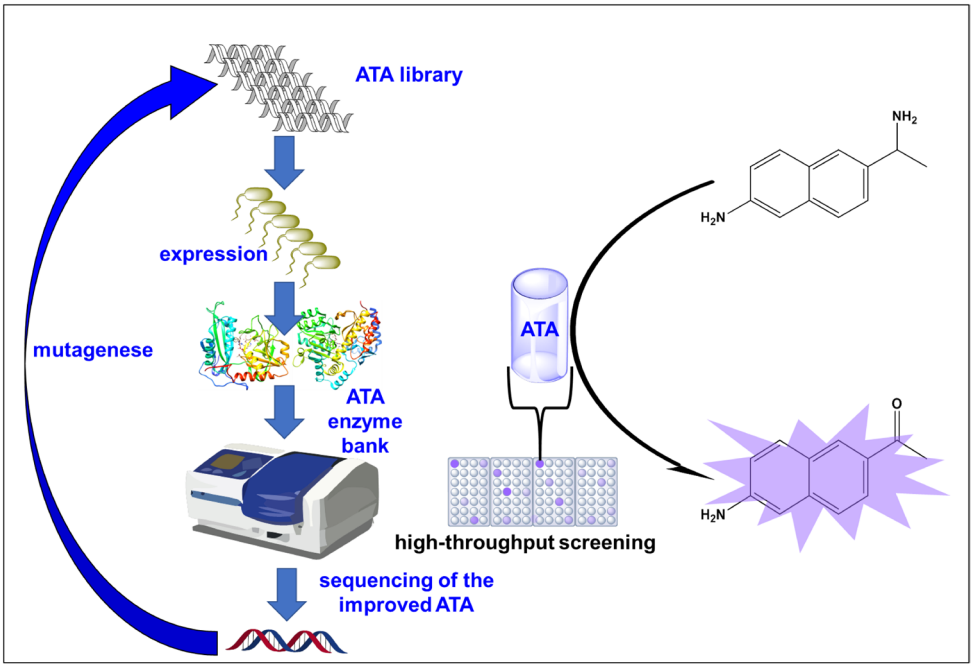


Figure 9. High-throughput screenings for the rapid optimization of ATA: Fast quantitative detection methods are crucial; shown here on the "smart amino donor" 6-amino-α-methyl-2-naphthalene methanamine [modified after Cheng et al., 2020 [120].

The main limitations of ATA-catalyzed reactions are the inhibitory effects of the co-products (resulting in low yield and purity) and the enzymes' limited pH and temperature stability [121]. Furthermore, an expansion of the substrate spectrum is desirable. All this is the subject of protein engineering approaches to adapt ATA to industrial needs for process suitability [122]. In principle, there are two options: targeted protein modification by directed evolution and rational design.

Since predictions are only possible to a limited extent, especially for the stabilization of proteins, even with good structural knowledge (in contrast to the estimation of substrate and stereoselectivity, for example), a fast and robust assay system is still desirable in most cases today, hybrid approaches of directed evolution and rational design are chosen. The number of suitable exchanges is minimized with the help of the available information. This is often referred to as a "semi-rational approach", with which, for example, the enantiopreference of ATA from *Chromatium violaceum* has recently been changed from (*S*) to (*R*) [123]. A frequently cited prime example of the successful targeted optimization of an ATA for the industrial production of the diabetes drug sitagliptin is described in the Section 6.1.

6. Cascade Reaction

Cascade reactions involve the sequential combination of different enzyme activities. Multi-enzyme cascades can be categorized into five main types: linear, parallel, orthogonal, cyclic, and triangular. In the linear cascade, one substrate is transformed into one or more intermediates and then into a product through sequential enzymatic steps. Parallel cascades form when two enzymatic reactions are coupled and share a reciprocal demand for the cofactor [124]. In orthogonal cascades, the conversion of substrate to product is coupled with a second enzyme that regenerates a cofactor or removes one or more by-products [124,125]. Cyclic cascades occur when the product of a multistep reaction is converted back to one of the starting materials. Therefore, the by-products are to be recycled directly to form the co-substrate. In triangular cascades, a series of catalytic reactions occur simultaneously, starting from the substrate and then converging into the same product (Figure 10) [124].

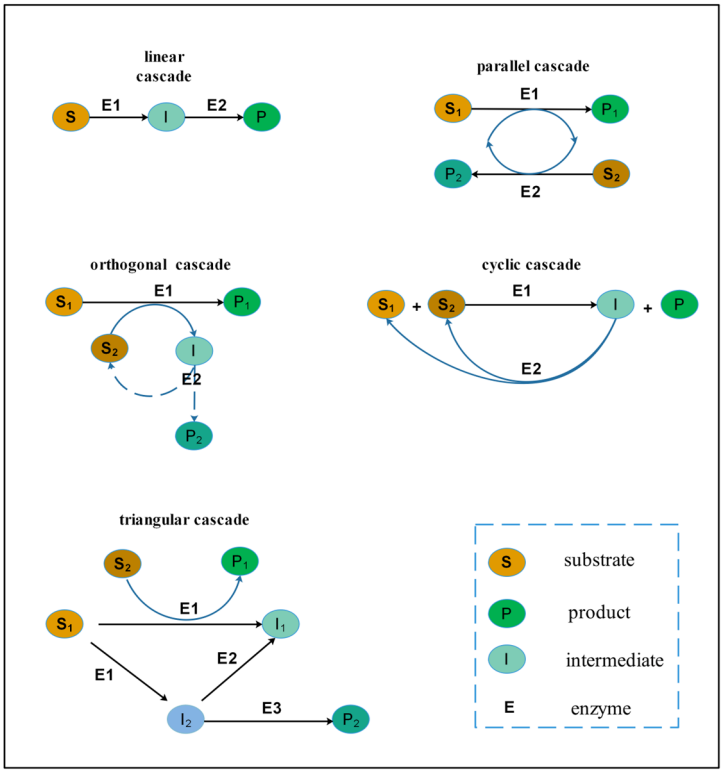


Figure 10. Enzymatic cascade classification. Linear cascade: One product is synthesized via multiple enzymatic steps in a linear cascade. Parallel cascade: A parallel cascade contains two enzymatic steps where the cofactor is consumed and regenerated. Orthogonal cascade: Product formation is coupled with two enzymatic reactions for cofactor regeneration and removal of a by-product. Cyclic cascade: a combination of substrates produces a product, and the product then converts back to one of the substrates again. Triangular cascade starts from the substrate during a multi-step enzymatic reaction when a combination between the substrate and an intermediate occurs. Originally based on a simple diagram by [126], this concept has been refined by [127] and further developed by [124]. The figure depicts a simplified compilation of all three references.

These cascades can occur within a living (usually bacterial) cell or in vitro, in one-pot systems where the desired enzymes are mixed to carry out the multi-enzyme reaction [128]. Using a living cell eliminates the need for costly cofactors, providing self-replicating biotransformation catalysts, producing a wide range of high-value (chiral) chemicals and protecting enzymes from harsh reaction conditions [129]. However, it can harm the cell with reaction intermediates and make retrieving desired products more difficult. Unwanted side reactions reduce yield, and managing metabolic flux adds another challenge [128,130].

The challenges in the wide application of ATA for chiral amine production include inefficient equilibrium displacement and high by-product removal costs. ATAs can also be integrated into one-pot cascade reactions, either in linear sequences [131] or for by-product removal or recycling (orthogonal or parallel cascades) [124].

Not least, cascades mimic the living cell where usually no high concentrations of substrate, intermediate, or product are present; everything is imported, converted, synthesized and degraded on demand. In this regard, the establishment of cascade reactions in bioreactors resemble a natural environment and avoid inhibition by high concentrations of substrates, intermediates, and/or products, which often inhibits enzymes and the progress of the desired reaction direction. Thus, one-pot reactions offer multiple advantages, such as shifting reversible reaction equilibrium, increasing yields and reaction rates, overcoming inhibition effects, and addressing solubility issues, resulting in a significant improvement of process efficiency. All these developments are important steps towards a "greener" chemical future [126].

6.1. β-s Keto Esters as Promising Substrates to Synthesis β-AAs

Numerous enzymatic cascade reactions ATA based for the synthesis of optically pure non-canonical AAs have been reported. Biotransformation of β-keto acids is a promising approach in the synthesis of β-AAs but faces the challenge that the substrates tend to spontaneous decarboxylation in aqueous solutions [132]. To address this issue, stable β-keto esters can be used as the initial substrate [40]. The linear cascade of lipase and ATA to produce β-AA was introduced in Mathew's et al. studies.

They overcame the decomposition problem of β-keto acids by hydrolyzing β-keto ester during a cascade reaction system by using recombinant purified lipase and ATA from *Candida rugosa* and *Sphaerobacter thermophilus*, respectively [133]. In a separate study by this group, they optimized lipase concentration; β-keto esters were hydrolyzed by lipase from *Candida rugosa* to produce their keto forms. Subsequently, β-keto acids were aminated during the asymmetric reaction to generate their corresponding amine forms by purified (*S*)-ATA from *Polaromonas sp. JS666*, which is heterologously expressed in *Escherichia coli* (Figure 11) [134].

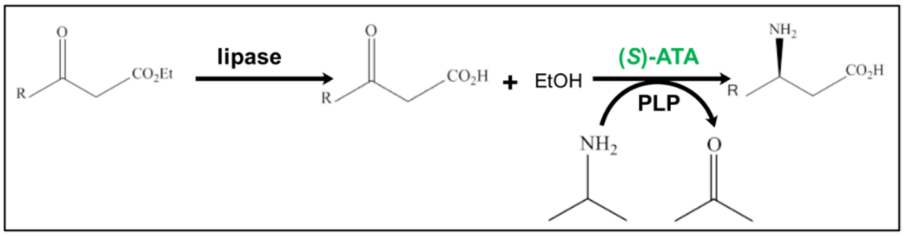


Figure 11. Asymmetric synthesis of aromatic β-AAs from β-keto esters. The process involves hydrolyzing the β-keto ester to β-keto acids using lipase, then aminating the β-keto acids using the (S)-specific ATA from *Polaromonas* sp. JS666 (ω-TAPO). R represents differently substituted phenyl residues. The figure is adapted from Mathew et al. [134].

In 2017, Mathew et al. developed an alternative enzyme cascade system to convert β-keto nitriles to β-AAs (Figure 12). This was achieved by hydrolyzing β-keto nitriles to β-keto acids by nitrilase, followed by the conversion of the β-keto acids to β-amino acids using ATA from *Bradyrhizobium japonicum*. (*S*)-α-MBA was used as an amino donor [135].

A recyclable glutamate (Glu) amine donor system was developed by Kim et al. to synthesize β-AAs. This orthogonal cascade system contains amine transaminase (ATA), glutamate dehydrogenase (GLDH), and mutant formate dehydrogenase (FDH), in which the expensive amino donor glutamate was replaced with α-MBA. β-keto esters were used as starting materials due to the instability of β-keto acids. *Candida rugosa* lipase was employed to hydrolyze β-keto esters to their corresponding β-keto acids. In this cascade, Glu was recycled using GLDH, which helped overcome the inhibition of ATA by α-ketoglutarate.

NADH was regenerated by FDH in the presence of formate (Figure 13). This system also successfully synthesized various γ-AAs and amines with 70% conversions and excellent *ee* > 99% [136].

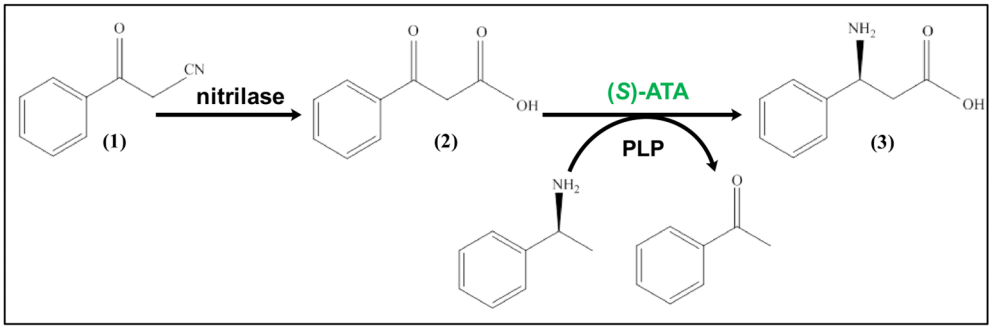


Figure 12. The synthesis of various chiral β-AAs (3) using β-keto nitriles (1) as the initial substrates. β-keto nitriles (1) were initially hydrolyzed by nitrilase, and five different (S)- specific ATAaminated the produced β-keto acids (2). (*S*)- MBA was used as an amino donor. The figure is adapted from Mathew et al. [135].

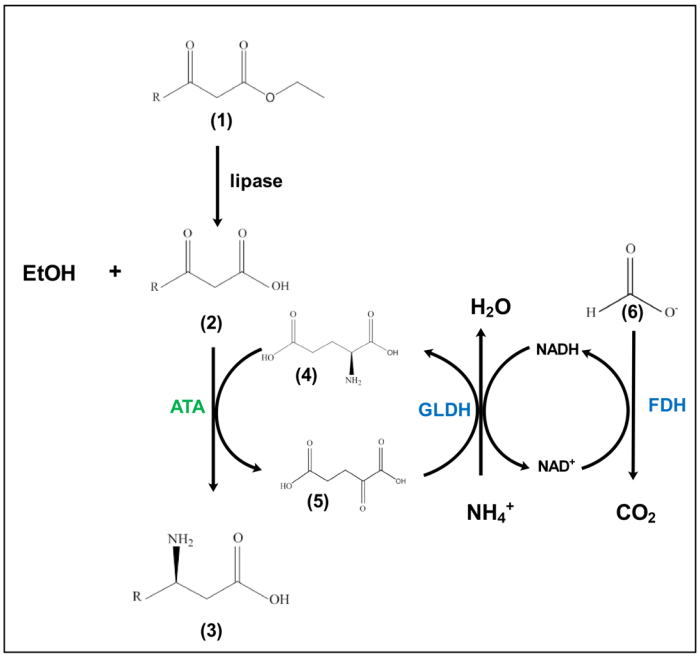


Figure 13. The synthesis of β-AA (3) using Glu (4) as an amino donor is carried out by the catalytic system ATA/GlDH/FDHm. First, the β-keto ester (1) is hydrolyzed by lipase to form β-keto acid (2). Then, (2) is converted to (3) by ATA. The α-ketoglutarate (5) produced in this process is recycled to (4) by glutamate dehydrogenase (GLDH). NADH was regenerated by formate dehydrogenase (FDH) in the presence of formate (6). R represents an aromatic compound. The figure has been adapted from Kim et al. [136].

Several enzymatic cascades reactions have been developed for synthesis of sitagliptin from β-keto esters.

Synthesis of the precursor of sitagliptin from β-keto esters was introduced in 2016 (Figure 14). A linear cascade system contains an amination reaction of hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl)butanoate (1) to 3-amino-4-(2,4,5-trifluorophenyl)butyric ester (2) by a crude extract of ATA variant (ATA117) and an autohydrolysis to produce Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl) butyric acid (3) [137].

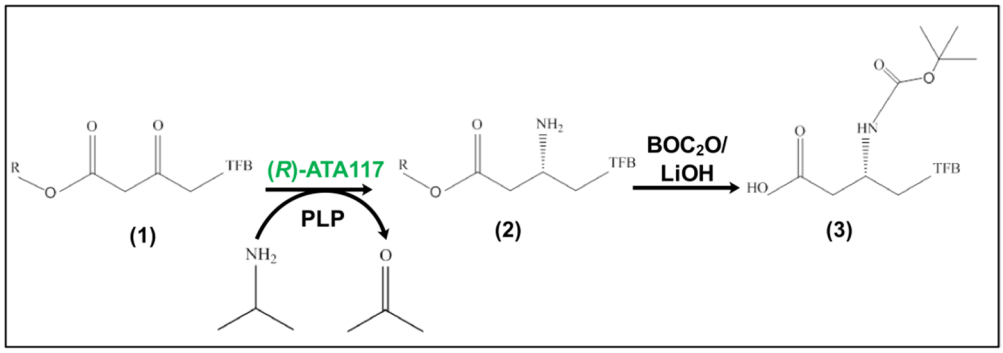


Figure 14. The enzymatic cascade synthesis of Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl) butyric acid (3). Hydroxyethyl-3-oxo-4-(2,4,5-trifluorophenyl) butanoate (1) was used as a substrate aminated to amino acid ester (2) by ATA117. TFB denotes trifluorbenzyl residue and R is hydroxyl ethyl ester. The $(BOC)_2O$, chemically a di-tert-butyl decarbonate, is an amine-protecting group during synthesis. The BOC group can later be removed from the amine using moderately strong acids. The figure has been adapted from Hou et al. [137].

Khobragade et al. (2021) asymmetrically synthesized a sitagliptin intermediate on a gram scale using a multi-enzyme cascade from corresponding β-keto ester (Figure 15). This single whole-cell system was co-expressed ATA, esterase, aldehyde reductase (AHR), and formate dehydrogenase (FDH). In this cascade, benzylamine served as the amino donor. The ATA from *Roseomonas deserti* demonstrated the highest activity towards benzylamine, approximately 70%. Khobragade et al. set a parallel reaction, including aldehyde reductase (AHR) from *Synechocystis* sp. and formate dehydrogenase (FDH) from *Pseudomonas* sp. to counter the inhibitory effect of benzaldehyde. These enzymes are essential for converting benzaldehyde to benzyl alcohol by utilizing NAD(P)H, a significant step in the enzymatic cascade. The reported conversion rate was between 72% and 91% to produce sitagliptin intermediate [138].

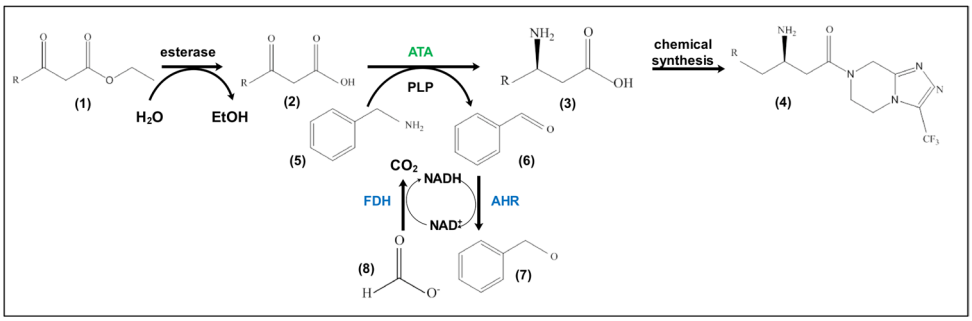


Figure 15. Multi-enzymatic synthesis of sitagliptin intermediate using benzylamine as an amino donor. The β-keto ester (1) is hydrolyzed to β-keto acid (2) by esterase and then aminated to sitagliptin intermediate (3) by ATA. Compound (3) undergoes chemical synthesis to convert to sitagliptin (4). Benzylamine (5) is used as the amino donor and deaminated to benzaldehyde (6). (6) is converted to benzyl alcohol (7) by aldehyde reductase (AHR). Formate (8) is used as substrate for formate dehydrogenase (FDH) to regenerate NADH and produce CO_2. R represents a fluorinated ring. The figure from Khobragade et al. has been modified [138].

However, removing deaminated products to avoid product inhibition remains a challenge during sitagliptin synthesis. Recently, Khobragade et al. developed a cyclic cascade reaction using three enzymes. The construct involves co-expressing three plasmids containing two ATA and one esterase in a single cell. In this cascade reaction (Figure 16), an esterase converts the β-keto ester to a β-keto acid, followed by the formation of the sitagliptin intermediate using the first ATA-1 from *Ilumatobacter coccineus*. During the reaction, (*S*)-MBA is deaminated, and acetophenone is produced, which is then utilized as a substrate for syn-

thesizing (*S*)-α-MBA using the second ATA-2 from *Silicibacter pomeroyi*. A preparative scale reaction (gram scale) achieved 98% conversion of the sitagliptin intermediate in 24 h [139].

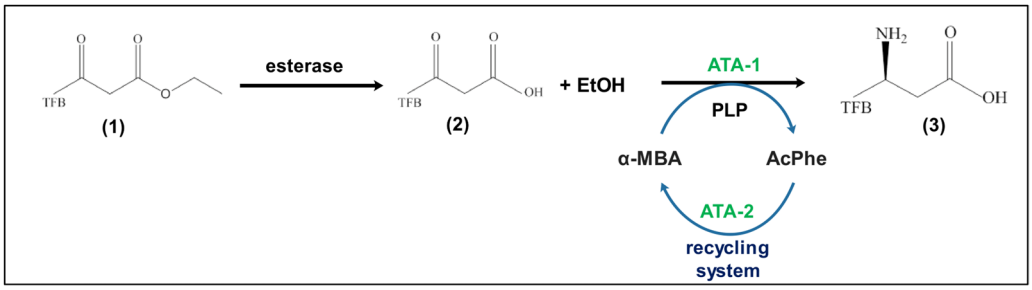


Figure 16. Sitagliptin intermediate synthesis utilizes a cascade reaction with two ATAs. ATA-1 is derived from *Ilumatobacter coccineus*, while ATA-2 is obtained from *Silicibacter pomeroyi*. Firstly, β-keto ester (1) is hydrolyzed to β-keto acid (2) by esterase and then aminated to sitagliptin intermediate (3) by ATA. TFB denotes trifluorbenzyl residue. AcPhe is acetophenone. The figure from Khobragade et al. has been modified [139].

The combination of biotic ATA and esterase activities, known as PluriZyme, has been developed for the one-pot cascade synthesis of β-AAs (Figure 17). In this process, ATA from the *Acidihalobacter* genus acts as a scaffold for an artificial esterase. This bienzymatic cascade reaction synthesizes *(R)*-3-amino-4-(2,4,5-trifluorophenyl)-butanoic acid (3-ATfBA) from β-keto ester with a conversion rate of over 99% and enantioselectivity (*ee* > 99%). 3-ATfBA is a critical precursor for synthesizing sitagliptins. Using one enzyme with two biotic sites to catalyze different chemistries can reduce development costs associated with producing multiple enzymes. However, the advantages and potential variations in different enzyme functionalities are yet to be fully defined [140].

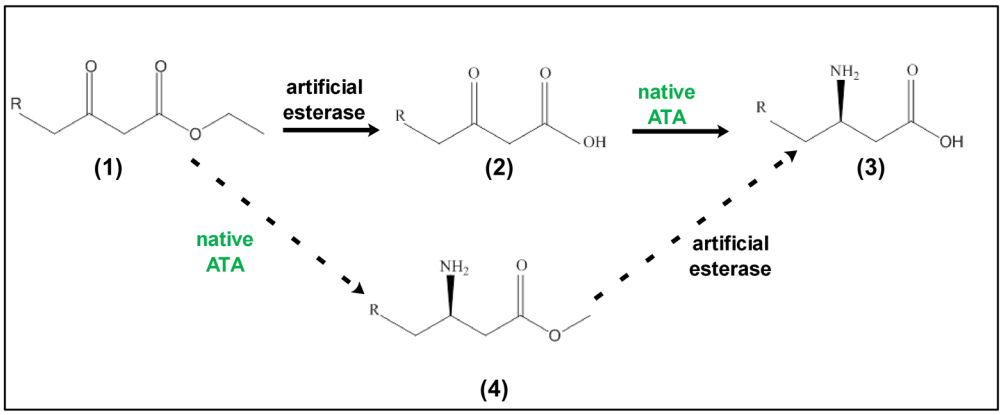


Figure 17. A one-pot reaction can convert β-keto ester (1) to 3-ATfBA (3) using PluriZyme. There are two approaches for producing 3-ATfBA (3). In the first approach, β-keto ester (1) is hydrolyzed to β-keto acid (2) by esterase and then aminated to (3) by ATA. In the second approach, the reaction can occur by aminating (1) to (4) using ATA and then esterifying (4) to (3). In this context, R represents a fluorinated ring. The figure modification is sourced from Roda et al. [140].

β-phenylalanine, a β-AA found in the natural compound paclitaxel (Taxol®), was synthesized through a cascade reaction from β-keto ester.

Buß et al. transaminated (ethyl benzoylacetate) ethyl ester to β-phenylalanine ethyl ester using ATA from *Vibrio fluvialis* (Figure 18). It was then hydrolyzed to form either *(R)*-β-phenylalanine or *(S)*-β-phenylalanine by lipase [132].

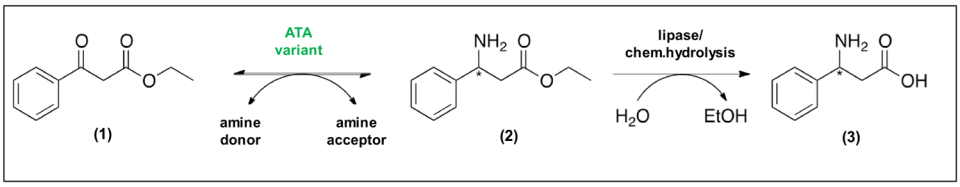


Figure 18. Asymmetric synthesis of β-phenylalanine (3) from ethyl benzoylacetate (1). ATA activity from *V. fluvialis* on (3) in the presence of an amino donor leads to the production of β-phenylalanine ethyl ester (2), which can be converted to (3) by chemical hydrolysis or via lipase activity, the latter probably leading to additional enantiomeric excess. Alternatively, (2) can be isolated for transesterification purposes. Figure modified from [132], where steps (1) to (2) was realized with both stereoselectivities by two different ATA using the smart amino donor OXD-assay shown in Figure 8 for screening an ATA library in high throughput. (2) was subsequently isolated by performing a semi-preparative synthesis. * indicates the creation of a new chiral center in the emerging product. Figure taken from © 2018 by Buß et al. Licensee MDPI, Basel, Switzerland [132].

Feng et al. developed a method for producing pure *(S)-β*-phenylalanine using the recombinant ATA from *Enhydrobacter aerosaccus* (Figure 19). They utilized β-phenylpyruvic acid, produced by lipase through the hydrolysis of ethyl phenylpyruvate, as the amino acceptor. This compound was then used in an asymmetric reaction with 3-aminobutyric acid as the amino donor and ATA. The result was the formation of *(S)-β*-phenylalanine with a yield of 82% and an *ee* > 99% [141].

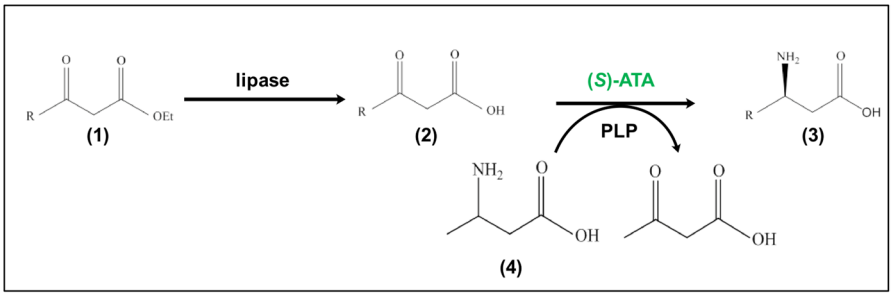


Figure 19. Synthesis of optically pure (*S*)-β-phenylalanine (3): First, ethyl phenylpyruvate (1) was hydrolyzed to form β-phenylpyruvic acid (2). Then, in the presence of 3-aminobutyric acid (4) as the amino donor, it was aminated to (*S*)-β-phenylalanine (3) using ATA from *Enhydrobacter aerosaccus*. R represents an aromatic ring. The figure has been adapted from Feng et al. [141].

6.2. Recycling Enzymatic Cascade System

In the typical asymmetric synthesis, L-alanine is used as an amino donor, which transfers its amino group to the amino acceptor and produces pyruvate. Therefore, removing pyruvate and even recycling it back into alanine is of great interest in the process engineering of these asymmetric syntheses and makes it more economical. For this purpose, the application of several cascade systems along with ATAs has been studied. Lactate dehydrogenase (LDH) was used to reduce pyruvate which is dependent on cofactor regeneration. Therefore, glucose dehydrogenase (GDH) accompanies systems (ATA/LDH/GDH) to recycle the cofactor NADH [142,143]. In the four enzyme cascade system (ATA/TdcE/FDH/LDH), formate acetyltransferase (TdcE) was enhanced to system to covert pyruvate to formate; then, formate dehydrogenase (FDH) was introduced to degrade formate and produce NADH, used in conversion of the pyruvate to lactate by LDH [143]. Conversion of pyruvate to (*R*)-3-OH-PAC by pyruvate decarboxylase (PDC) and transformation of PAC to metaraminol using ATA was introduced in enzymatic cascade [144].

Richter et al. described coupling system with (*R*)-ATA containing alanine racemase (AlaR) to provide D-alanine from L-alanine. This system was accompanied by AlaDH (alanine dehydrogenase) for recycling amino donor [145].

6.3. Immobilized Multi-Enzymatic Systems

Enzyme stability can be improved through co-immobilization. Co-localizing cascade enzymes offers the advantage of promptly transporting the product of one enzyme reaction to the next biocatalyst, thus improving overall performance [146,147].

It was reported that co-immobilization of a multi-enzyme cascade, which means immobilizing the enzymes together rather than immobilizing them on separate beads, showed higher reaction rates due to fast co-substrate channeling.

Heinks et al. introduced a co-immobilization cascade system on glutaraldehyde-functionalized amine beads in which L-norleucine was produced after transamination of 2-oxohexanoic acid by (*S*)-selective ATA (Figure 20). The co-substrate L-norleucine was oxidized by L-amino acid oxidase from *Hebeloma cylindrosporum* and continuously recycled to the corresponding α-keto acid 2-oxohexanoic acid, while hydrogen peroxide (H_2O_2) was removed by human catalase. After reusing three cycles, this system showed high enantiomeric purity (97.3%) [148].

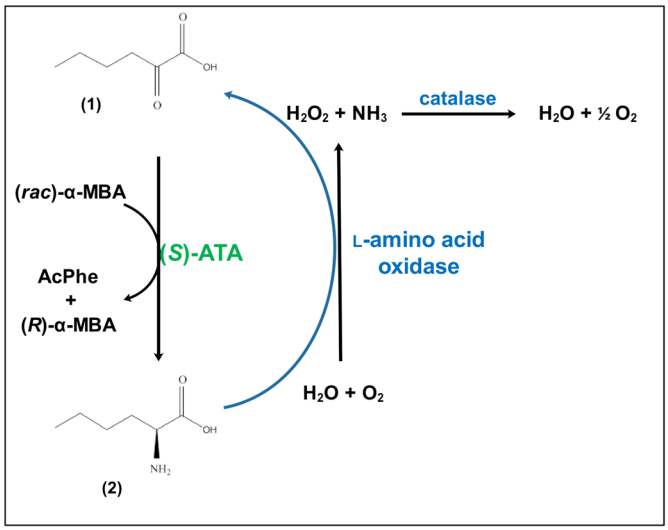


Figure 20. L-norleucine (2) synthesis by (*S*)-ATA via kinetic resolution. *rac*-MBA and 2-oxohexanoic acid (1) were used as amino donors and acceptors, respectively. The recycling of (1) was conducted by oxidizing (2) with L-amino acid oxidase. Catalase removed the produced hydrogen peroxide. AcPhe is acetophenone. The picture has been adapted from Heinks et al. [148].

A model D-AA *(R)*-2-amino-3-(2-bromophenyl) propanoic acid was synthesized through cascade reaction. Three enzymes, namely, D-amino acid ATA from *Bacillus cereus*, a D-lactate dehydrogenase (*lh*D-LDH) from *Lactobacillus helveticus*, and a formate dehydrogenase (*cb*FDH) from *Candida boidinii*, were immobilized on glutaraldehyde-activated amino polymer beads. The immobilized multi-enzymes exhibited excellent stability, resulting in an overall isolated yield of 65% of *(R)*-2-amino-3-(2-bromophenyl) propanoic acid. The immobilized enzyme was also evaluated in a continuous-flow reaction, achieving a stable conversion of >95% for 36 h. This approach could be a potential manufacturing platform for D-AAs [149].

6.4. Multi-Enzymatic Synthesis of Non-α- AAs

Recombinant *E. coli* cells containing both transaminase and aldolase enzymes were used to produce L-homoserine. L-homoserine is a non-standard AA, and it is a critical component in the synthesis of 3-hydroxypropionaldehyde and 1,3-propanediol. In this cascade system, aldolase catalyzed the addition of pyruvate to formaldehyde, followed by the reductive amination of the product by transaminase using an amino donor [150].

Walton et al. set up an *E. coli* whole-cell system for one-pot enantioselective synthesis of D-phenylalanine (Figure 21). D-phenylalanine is an essential building block in

synthesizing antibiotics, antidiabetics, and chemotherapeutic agents for cancer treatment. A single-pot cascade reaction involves oxidative deamination by *Proteus mirabilis* L-amino acid deaminase and reductive amination of L-AAs into D-AAs using an engineering D-phenylalanine-specific aminotransferase from *Bacillus* sp. YM-1. The cascade reaction showed excellent enantiomeric excesses (90% to >99%) in 4 h [151].

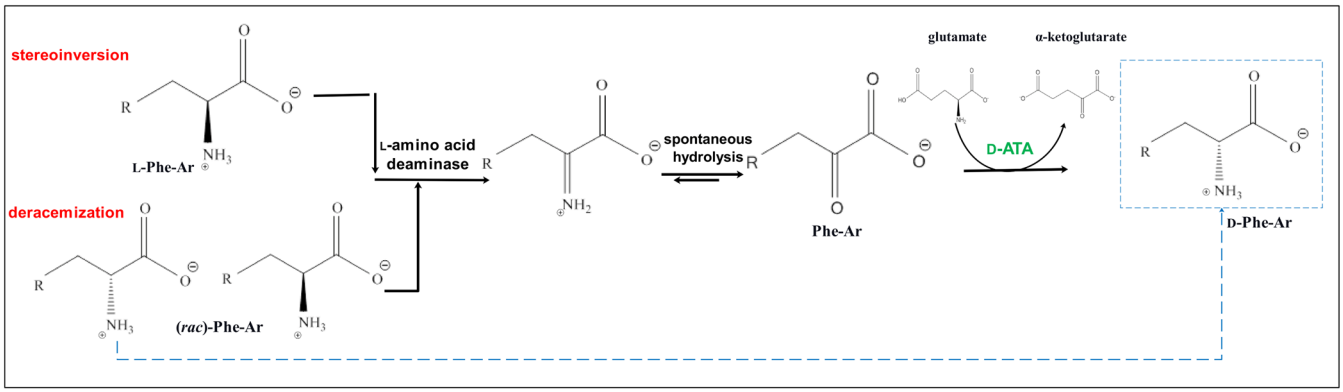


Figure 21. An asymmetric synthesis of D-phenylalanine aromatic derivatives (Phe-Ar) is achieved through a cascade reaction. The reaction involves the oxidative deamination by L-amino acid deaminase and the reductive amination of L-AAs into D-AAs using an engineered ATA. Glutamate is utilized as an amino donor and converted to α-ketoglutarate. In this context, R represents an aromatic ring with alkyl or nitrite group. The figure modification is sourced from Walton et al. [151].

Malik et al. eliminated the need to use an expensive keto acid as an amino acceptor by coupling two reactions (Figure 22). In a sequential process, a threonine deaminase from *E. coli* initiated the oxidative deamination of L-threonine, generating 2-oxobutyrate as an amine acceptor. This compound was then utilized by the (*S*)-selective ATA from *Ochrobacterium anthropi* to produce L-homoalanine, deaminated ketone, and enriched (*R*)-amine [152].

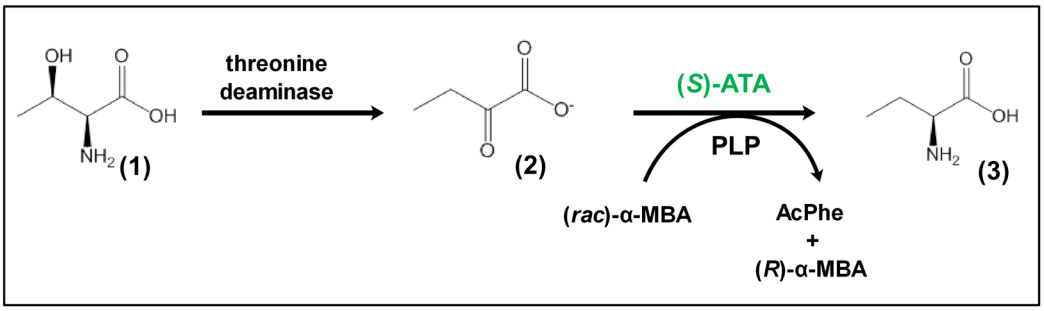


Figure 22. Enzymatic cascade reaction using *(S)*-selective ATA and threonine deaminase converts racemic α-MBA and L-threonine (1) to *(R)*-α-MBA and L-homoalanine (3). (2) and (AcPhe) are 2-oxobutyrate and acetophenone, respectively. The figure has been adapted from Malik et al. [152].

Deracemization cascade with ATA activity has been introduced in the synthesis of *(S)* and *(R)* AAs. The enzymatic cascade synthesis of two pharmaceutical compounds is described below.

(*S*)-2-Amino-3-(6-o-tolylpyridin-3-yl)propanoic acid (ATPA) is a vital intermediate required for the synthesis of glucagon-like peptide-1 (GLP-1) mimics that could be beneficial for the treatment of type II diabetes. Chen et al. developed a linear enzymatic cascade system to synthesize this (*S*)-ATPA. First, the (*rac*)-ATPA was deracemized to a mixture of (*S*)-AA and keto acid using (*R*)-amino acid oxidase from *Trigonopsis variabilis* expressed in *E. coli*. Subsequently, the keto acid was converted to (*S*)-ATPA by (*S*)-ATA from *Burkholderia* sp. expressed in *E. coli*, using (*S*)-aspartate as an amino donor. The yield at the end of the

biotransformation process was 85%. This process was scaled up to 78 L reaction containing substrate, PLP cofactor, and phosphate buffer in a 100 L vessel (Figure 23) [16,153].

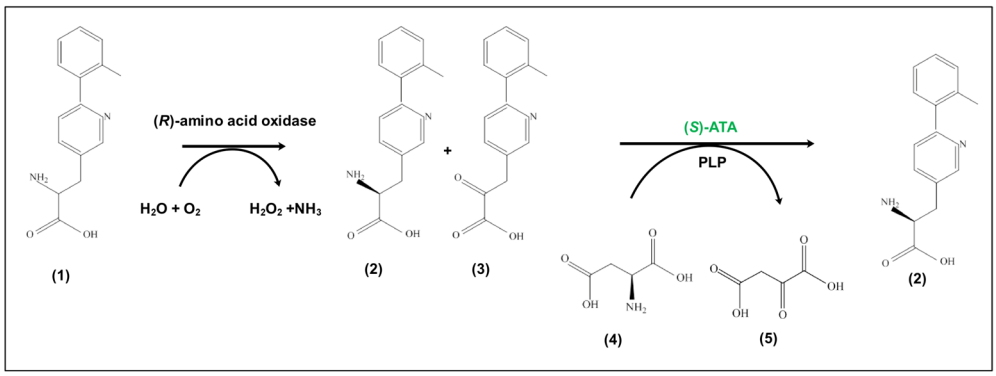


Figure 23. Cascade reaction in the synthesis of (*S*)-2-amino-3-(6-*o*-tolylpyridin-3-yl) propanoic acid (ATPA) (2) by (*R*)-amino acid oxidase and (*S*)-ATA. Firstly, (*rac*)-ATPA is (1) was oxidized to (2) and keto acid (3). Then, keto acid (3) is aminated to (2) by (*S*)-ATA. L-glutamate (4) serves as amino donor and is converted to α-keto glutarate (5). The figure has been adapted from Patel et al. [16].

The (*R*)-AA, (*R*)-2-amino-3-(7-methyl-1H-indazol-5-yl) propanoic acid (AMHP), plays a pivotal role as an important intermediate in the creation of antagonists of calcitonin gene-related peptide receptors. These antagonists are used in the treatment of migraine and other medical conditions [16].

Hanson et al. described the enzymatic synthesis of (R)-2-amino-3-(7-methyl-1H-indazol-5-yl) propanoic acid ((*R*)-AMHP) through a combination of (*S*)-amino acid deaminaseand (*R*)-ATA activity [102]. *(R)*-AMHP is an intermediate used to synthesize an antagonist of the calcitonin gene-related peptide receptor employed in migraine treatment [154]. The process begins with the deracemization of (*rac*)-AMHP to (*R*)-AMHP and the corresponding keto acid using (*S*)-amino acid deaminase from *Proteus mirabilis* expressed in *E. coli*. In the next step, the keto acid is converted to (R)-AMHP using (*R*)-ATA from *Bacillus thuringiensis* expressed in *E. coli*. D-alanine serves as the amino donor. To improve the reaction rate and yield, lactate dehydrogenase (along with NAD+, formate, and formate dehydrogenase to regenerate NADH) is added to eliminate the inhibitory pyruvate produced during the reaction (Figure 24) [102].

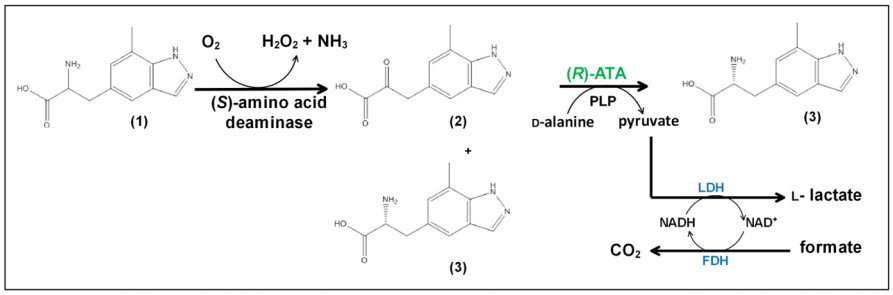


Figure 24. The enzymatic preparation of (*R*)-2-amino-3-(7-methyl-1H-indazol-5-yl) propanoic acid (AMHP) involves using a combination of (*S*)-amino acid oxidase and (*R*)-ATA activity. First, (*rac*)-AMHP (1) is converted to (*R*)-AMHP (3) and the corresponding keto acid (2) by (*S*)-amino acid deaminase. Then, keto acid (2) is converted to (*R*)-AMHP (3) by (*R*)-ATA. Lactate dehydrogenase (LDH) and formate dehydrogenase (FDH) were included in the reaction to prevent the inhibition of pyruvate.The figure has been adapted from Hanson et al. [102].

7. Conclusions and Outlook

This review has discussed amine transaminase (ATA) and high-throughput screening approaches for introducing new ATAs. Additionally, it has focused on applying ATAs in cascade reactions to synthesize NcAAs and amines.

The examples presented in this review underscore the immense potential of enzymatic cascade systems. This potential is particularly evident in overcoming ATA challenges such as cofactor regeneration and product inhibition.

However, there is still a need to optimize cascade reactions to meet the requirements of industrial applications in terms of productivity, space–time yield, and long-term stability up to continuous processes. To the best of our knowledge, all current transaminase applications on industrial scale are batch processes due to the relatively low stability and the complex co-factor recycling (in contrast to hydrolyses and many oxidoreductases).

Merging different catalysts can be challenging due to their incompatibility in reaction media. One effective way to address this issue is by applying sequential cascade reactions. In these systems, not all reagents are initially present in the reaction; they are added sequentially after each prior step is completed. This approach enables the utilization of cost-effective starting materials, such as fatty acids and alkynes, in one-pot cascade ATA reactions [31].

Another approach involves using nanostructures to create substrate channeling inspired by natural biological systems. This design facilitates the movement of intermediates from one active site to another, reducing their diffusion during the reaction. As a result, this method increases the yield of the desired product [155,156]. Compartmentalization is necessary in cascade reactions to prevent unwanted interactions between multiple reactions and to reduce the unfavorable effect of nonconventional media or solvents on enzymes. Enzymes immobilized by self-assembling different enzymes in hydrogels could provide compartmental flow channels to produce valuable chemicals. These so-called all-enzyme hydrogels are covalently crosslinked upon mixing via the genetically encoded SpyCatcher/SpyTag system [157]. Attachment of one or multiple binding sites allow the use of mono- and multimeric enzyme building blocks. The design has already been applied successfully for a broad range of enzymes such as ketoreductases [157,158], imine reductases [159], or phenolic acid decarboxylases [160]. An application of this system for a transaminase-catalyzed reaction is still pending (as of July 2024), but appears promising especially for cascaded ATA reactions. The same applies for the more recent technology of foaming of the forming hydrogels via microfluidic crossing [161]: this development allows the formation of full enzyme foams, circumventing diffusion limitations due to the large pore size after a drying step.

Fusion biocatalyst technology has been introduced to develop cascade reactions. These artificial biocatalysts are composed of a fusion of different domains: a catalytic unit, a co-factor recycling unit, and an immobilization unit [124,162]. Fusion protein and co-immobilizing approaches could be used to optimize enzymatic cascade reactions by decreasing the transport distance of reaction intermediates between enzyme active sites (also known as a substrate channeling) [163,164].

In conclusion, procedural progress such as the development of immobilization methods and continuous flow techniques will contribute as much to providing a robust toolbox for developing enzymatic cascade systems as to the discovery of novel enzymes and the engineering of established biocatalysts. These advances are certain to broaden the application of transaminases in the near future and may allow for the development of more sustainable continuous processes.

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