Review article

Extremophilic and modified aminotransferases as a versatile tool for the synthesis of optically pure building blocks for pharmaceutical industry

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Abstract: *Considerable progress has been made in the past few years with industrial use of essential key intermediates for chemical and pharmaceutical industry. The increasing demand for obtaining chiral drugs in enantiomerically pure form makes it necessary to search for novel biocatalysts useful in the synthesis of amino acids, chiral amines, amino sugars and alcohols. According to the reaction mechanism, aminotransferases (ATs) have useful applications because of their capability of transfer of an amino group from a donor substrate to an acceptor, thus resulting in the synthesis of a wide variety of building blocks. This article reviews current biocatalytic approaches using microbial ATs in the synthesis of optically active products. Focus is also put on the engineering of ATs and their limitations in the industrial applications. Moreover this review covers biocatalytic approaches using ATs isolated from extreme environments.*

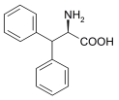
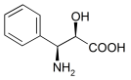
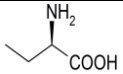
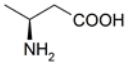
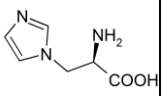
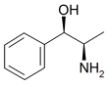
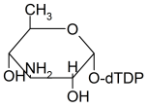
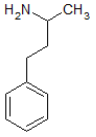
Keywords: *aminotransferases, biotransformations, unnatural amino acids.*

Introduction

Since the discovery of the chirality phenomenon and its effect on differentiation in the biological functioning of structurally similar molecules, the importance of chirality in drugs production has been the subject of scientific research. The synthesis of single stereoisomers of chiral drugs is still a major challenge and is being investigated for the development of new chirotechnology to produce optically pure isomers and/or racemic mixtures formulation [1]. Biotransformations have become key technologies to produce chiral substances and have a big potential for the future large-scale synthesis of the desired and structurally diverse products. Among the many biocatalysts used in industry, the ones that play a significant role in the synthesis of valuable key intermediates are aminotransferases (ATs). They are useful in the synthesis of unnatural

substances, because of their capability of reversible transfer of amino groups from a donor substrate to an acceptor. Moreover they are characterized by high chemo-, enantio- and regioselectivity, high reaction rate and no requirement for additional cofactor regeneration. Furthermore, combining the transaminase reaction in enzymatic cascades allows for the efficient synthesis of a wide range of chiral building blocks [2]. The examples of valuable intermediates and products, such as unnatural amino acids, amino sugars and amino alcohols obtained by ATs are shown in Table 1.

Table 1. Diversity of potential products obtained by transamination reaction

Structure of selected example	Supply in therapeutic area	Ref.
aromatic amino acid (L-diphenylalanine) 	synthesis of the pseudopeptide pharmaceuticals and the preparation of the peptide nanotubes	[3]
aromatic β-amino acid (L-phenylisoserine) 	key component of a variety of bioactive molecules, such as taxol, one of the most active antitumor agents	[4]
aliphatic amino acid (L-2-aminobutyric acid) 	the pharmaceutical intermediate widely used in synthetic antiepileptic drugs, such as: Levetiracetam and Ethambutol	[5]
aliphatic β-amino acid (L-3- aminobutyric acid) 	one of the major inhibitory neurotransmitters in the central nervous system and the ligand of GABA receptor	[6]
β-heterocyclic amino acid (L-thienylalanine) 	an important as L-dopa against Parkinson's disease, alpha-methyldopa against hypertension or L-phosphinothricin as active component of a herbicidal substance	[7]
amino alcohol (L-norephedrine) 	an agent used to relieve allergic reactions or respiratory infection	[8]
aminosugar (TDP-3-amino-4,6-dideoxy-D-glucose) 	a key intermediate in the biosynthesis of TDP-D-desosamine which exists in macrolide antibacterial antibiotics	[9]
α-chiral primary amine ((R)-4-phenylbutan -2- amine) 	a bioactive compound in synthetic precursor of the antihypertensive dilevalol	[10]

Reaction mechanism

The ATs reaction mechanism is defined as a ping-pong bi-bi mechanism, which involves exactly two substrates and two products. All the ATs use pyridoxal 5'- phosphate (PLP) as a cofactor. It is a form of vitamin B6 and is covalently bound in the enzyme active sites. In the initial step, the α-amino acid and water react with a PLP. This transaldimination step consists of the formation of an internal aldimine. The bond in the internal Schiff base between the PLP and a ε-NH_2 group of lysine residues in the enzyme active site dissolves and the PLP is detached and forms an equivalent Schiff base with the amino substrate. In the next step the free ε-NH_2 group acts as a catalyst. During the 1,3 hydrogen shift, deprotonation from the external aldimine and reprotonation to the imine cause the formation of ketamine. This intermediate is hydrolyzed to oxo product and the pyridoxamine-5'-phosphate (PMP) enzyme. In the final step the amino acceptor is produced and the PLP form is regenerated. The transaminase enzyme mechanism including key intermediates is shown in Figure 1 [11].

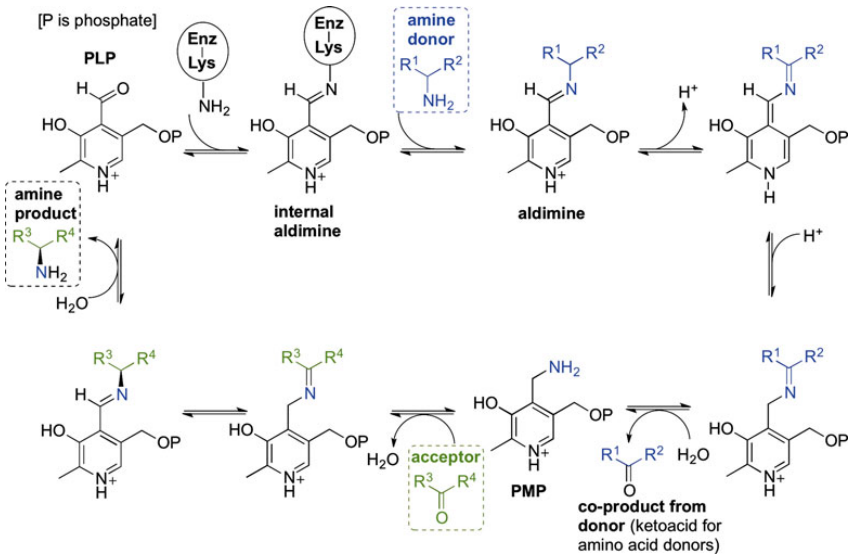


Figure 1. Transaminase enzyme mechanism [1]

Classification of Aminotransferases

According to the Enzyme Commission nomenclature, ATs are classified as transferases (EC 2). As of July 2016, 102 ATs are listed under EC. 2.6.1 in BRENDA, and the numbers on the list have increased by 26 entries over the past 10 years. ATs belong to the diverse group of pyridoxal 5'-phosphate (PLP)-dependent enzymes. The B6 database is a tool for the description and classification of vitamin B6-dependent enzymatic activities and the corresponding protein

families [12]. Historically, Grishin et al. distinguished five different types of B6-dependent enzymes. Currently, PLP-dependent enzymes are divided into seven folds (I-VII). ATs belong to the fold types I and IV. Another classification was introduced in the 1980s and ATs were divided into two groups based on the reaction catalyzes. This led to the division of ATs into: α-ATs, which catalyze a transfer of amino groups at the α-carbon, and ω-ATs, which are able to catalyze transaminations of amino group not adjacent to the carboxyl group [13]. However, it is more convenient to classify ATs based on their substrate specificity, taking into consideration the donors of amino groups. This classification system for ATs was established on the basis of multiple sequence alignments and protein structures in Protein Family Database (Pfam), which is illustrated in Table 2. ATs are divided into six subgroups.

Table 2. Aminotransferases subgroups according to Pfam. Comparison of the substrate and acceptor spectra of selected ATs, modified table from [2]

ATs subgroup	Pfam ID	Enzyme	Amino donor	Amino acceptor	α/ω ATs
I and II	00155	AspAT	L-Aspartate	2-Ketoglutarate	α
		AlaAT	L-Alanine		
		AroAT	L-Phenylalanine		
		HisPAT	L-Histidinol-phosphate		
III	00202	AcornAT	N-Acetyl-L-Ornithine		α
		OrnAT	L-Ornithine		
		ω-AaAT	β-Alanine	Pyruvate	
		GaBaAT	4-Aminobutyrate	2-Ketoglutarate	
IV	01063	D-AlaAT	D-Alanine	2-Ketoglutarate	α
		BCAT	L-Leucine		
V	00266	SerAT	L-Serine	Pyruvate	
		PSerAT	3-Phospho-L-serine	2-Ketoglutarate	
IV	01041	ArnB	L-Glutamate	UDP-2-acetamido-4-keto-2,6-dideoxyglucose	
		TylB		TDP-3-keto-6-deoxy-D-glucose	
		StsC		Scyllo-Inosose	

Industrial biotransformations using transaminases

Examples of AT reactions for the production of valuable products

Generally, all types of ATs have been widely applied to the large-scale biosynthesis of unnatural amino acids. Rapid reaction rates, broad substrate specificity and no requirement for additional cofactor regeneration make them effective biocatalysts. Fuchs et al. developed a highly stereoselective and short chemoenzymatic synthesis of (S)-rivastigmine, which represents one of the most potent drugs for the treatment of Alzheimer's disease at early stages and also exerts a beneficial influence on dementia of Parkinson patients. The key building block was derived via enzyme catalyzed asymmetric transamination of a structurally tuned ketone using ω-transaminase from *V. fluvialis* [14]. (S)-repaglinde used in the treatment of type 2 diabetes [15] and (R)-levocetirizine, which is an antihistamine agent [16] are other examples of important drugs prepared by using ATs. Also the industrial usage of ATs is demonstrated by the synthesis of optically active D-glutamate, which is a compound of a CCK-receptor antagonist, a drug used in bowel disorder treatment [17]. It is known that ATs are indirectly involved in the biosynthesis of L-tert-leucine [18], D-phenylalanine [19]. Aromatic aminotransferase (PheAT, EC 2.6.1.57) is valuable for industry to produce some analogues of aromatic amino acids, such as L-homophenylalanine, a starting material for synthesizing angiotensin-converting enzyme inhibitor like enalaprilat [20]. A collaboration between Codexis and Merck resulted in a development of an (R)-specific transaminase mutant (ArRMut11) from *Arthrobacter* sp. to generate an (R)-chiral amine used for the synthesis of sitagliptin, the active ingredient in the commercially available oral drug used in the treatment of type II diabetes [21]. The company Cambrex applies transaminase to the asymmetric synthesis on an industrial scale of various chiral amines, such as aminotetralins, benzylamines, methylbenzylamines, phenylpropylamines, polyfunctional amines and other linear, branched chain and aromatic amines. A substituted (S)-aminotetralins is an example of an active pharmaceutical ingredient (API), which has potential in the treatment of depression and Parkinson disease. The wild type gene of aminotransferase has been isolated from *Athrobacter citreus*, overexpressed in *Escherichia coli* MG1655 strain and engineered by a directed evolution strategy. This synthesis requires substituted tetralone and isopropylamine as amine donors, and the by-product obtained at the same time is acetone. The best mutant was generated after three rounds of error-prone PCR. A 5-fold increase in the product concentration has been observed and the activity and thermostability of recombinant (S)-aminotransferase have been improved [22]. The use of aminotransferases is demonstrated at commercial scale also in the production of (S)-7-methoxy-2-aminotetralin (7-MAT), an intermediate in several commercially important APIs. Cambrex has manufactured it at multi-tone scale and it is isolated as the HCl salt, with enantiomeric excess > 99% [23].

Limitations in efficient production by use of ATs

According to the reversible character of the transamination mechanism, a development of ATs on an industrial scale unfortunately has also some limitations. The main disadvantage that needs to be overcome is a substrate/product inhibition caused by the unfavourable equilibrium for the reaction. The most important requirement for application of a selected AT in biotransformations is a high enantioselectivity of the enzyme. So, the equilibrium must be shifted by reaction engineering to make the product synthesis efficient. By using various strategies, the process can be improved in order to overcome the product inhibition, thus increasing the possibility of ATs practical application. A selective product removal by the reactive solvent extraction with di-(2-ethylhexyl) phosphoric acid (D2EHPA) can be used for the selective separation of amino acids as the function of the pH value of aqueous solution and the character of each amino acid [24]. The product inhibition by the ketone by-product can be overcome by the coupling reactions. AlaAT from *E. coli* K-12 and ω-AT from *V.fluvialis* JS17 coupled reaction system was designed to produce enatiomerically pure (S)-amino acids and (R)-amines [25]. This method was also used to overcome product inhibition of ω-TA by the ketone product in the kinetic resolution of racemic amines at high concentration. Yun et al. used an ω-AT, alcohol dehydrogenase, and glucose dehydrogenase in a coupled reaction to achieve a simultaneous synthesis of (*R*)-1-phenylethanol and (*R*)-α-methylbenzylamine [26]. ω-AT from *B. thuringiensi*s JS64 was highly enantioselective for (S)-enantiomer of α-MBA but showed severe product inhibition by acetophenone. An aqueous/ organic two-phase system was introduced to overcome this problem [27]. In the next years, Shin et al. developed the kinetic resolution process for the production of chiral amines with ω-AT from *V. fluvialis* JS17 and *Bacillus thuringiensis* JS64, using an enzyme-membrane reactor (EMR) and a hollow-fiber membrane contactor [28].

Engineering of ATs

Since transaminases are essential enzymes for the unnatural amino acids and chiral amines synthesis, researchers would like to overcome limitations in the application of ATs to biotransformations. Transaminases as industrially relevant enzymes can be engineered to fit the process of interest. Both the directed evolution and the rational design by site-directed mutagenesis have been employed in order to modify and expand substrate spectrum, change the enantioselectivity, overcome substrate/product inhibitions or improve in enzyme stability. Those strategies allow us to design more valuable ATs for the pharmaceutical and chemical industry. The achievements in transaminase engineering for bioprocess development have been reviewed in Table 3. The selected examples show that the alteration of recombinant ATs by mutagenesis can benefit product yields and is increasingly important for a development of new biotransformation processes.

Table 3. Transaminase engineering and characteristic futures

biocatalysts	mutations	influence	methods	Ref.
ω-AT from *Vibrio fluvialis* JS17	P233L, V297A	reduced product inhibition by aliphatic ketone	directed evolution	[29]
	W57G, W147G	enhanced activity toward aliphatic amines		
	R415K	enhanced activity toward aromatic α-amino acids		
ω-AT from *Caulobacter crescentus*	N285A, V227G	enhanced activity toward phenylpropionic acid 3-fold and 11-fold respectively	site-directed mutagenesis	[30]
ω-AT from *Athrobacter citreus*	17 mutations	improved activity and thermostability for substituted aminotetralin	directed evolution (error-prone PCR)	[22]
	E326D, Y331C E326D, Y331C	enhanced enantioselectivity toward 4-fluorophenylacetone	single point mutation	[31]
	V328A	enantioselectivity shifted from (S) to (R) for 4-fluorophenylacetone		
ω-AT from *Vibrio fluvialis* 117	27 mutations	enhanced activity toward prositagliptin ketone	directed evolution	[21]
AT from *Enterobacter* sp. BK2K-1	Y66L	ability to synthesize L-diphenylalanine	site-directed mutagenesis	[7]
aspartate AT from *Escherichia coli*	V39L, T47I, N69L, N297S, K41Y, T109S	redesign of the substrate specifity of aspartate to tyrosine transaminase	site-directed mutagenesis	[32]
ω-AT from *Vibrio fluvialis*	2 mutants	3-fold activity increase towards β-phenylalanine comparted to the wild type	directed evolution (error-prone PCR)	[33]
(S)-selective ω-ATfrom *Ochrobactrum anthropi*	W58L	activity improvements for structurally diverse ketons (340-fold increase in kc_{at}/K_M for acetophenone)	partial saturation mutagenesis	[34]

Transaminases from extreme environments

Many industries are making efforts to move away from the use of harmful chemical processes. As a result, there is an increasing biotechnological demand for biocatalysts that are stable and active under extreme conditions (temperature, pH, ionic strength), such as those found in industrial processing. Extreme

environments on Earth (e.g. hydrothermal vents, hypersaline lakes and pools, alkaline soda lakes, dry deserts, cold oceans, and volcanic areas) are expected to yield novel microbial diversity and unknown proteins with interesting properties and new catalytic activities. Over the past decade, one of the major areas of biotechnological research are extremophiles. This organisms are a source of "extremozymes". These enzymes are adapted to perform the same enzymatic functions as their non-extreme mesophilic counterparts, but with greater adaptability to extreme physical and/or chemical conditions. It is worth of mention that the use of transaminases from extremophilic organisms for the synthesis of enantiopure amino acids might increase the benefits for industrial applications. Examples of transaminases from extreme environments are known and given below.

The first crystallographic structure of the thermophilic archaeal transaminase was solved by Sayer et al. (2012). *Sulfolobus solfataricus* isolated from hot volcanic region has been found to be a source of a thermostable transaminase enzyme. Thermozymes are stable and active at elevated temperatures. Moreover, the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Also, the risk of contamination is reduced to minimum at higher temperature. The *Sulfolobus* transaminase is involved in the non-phosphorylated pathway for the serine synthesis and thermostable for 10 min at 70°C and at pH 6.5. Moreover, the broad substrate range was observed. The activity towards methionine, asparagine, glutamine, phenylalanine, histidine, and tryptophan was detected. Finally, the *S. solfataricus* enzyme has been classified as a serine: pyruvate aminotransferase, due to the absence of activity towards α-ketoglutarate (the most frequently used as amino acceptor of all transaminases) [35]. Chen et al. (2006) developed a transketolase-ω-transaminase two-step one-pot aminotriol/aminodiol synthesis reaction model. Based on the simulations, the enzyme can be used in combination with transketolase for the synthesis of chiral drug intermediates [36].

Another example of enzymes which are valuable and desirable for industry are psychrozymes. Due to economic and ecological reasons it is very important to lower the heating temperature during the process. Enzymes isolated typically from the Antarctic regions, in contrast to their mesophilic homologues, are able to catalyze the reaction at lower temperatures and tolerate the low water activity environments [37]. This unique properties make them especially interesting also in biotransformation reactions. The psychrofilic cold-active transaminase from *Psychrobacter* sp. B6 has been characterized. The activity assay shows the dual substrate specificity for both aromatic amino acids and aspartate. Also the crystallographic studies confirm the dual functionality of this protein. The role of the aminoacid residues responsible for substrate binding in the active site of the enzyme was explained. These results give a good grounding for further research on the modification of the catalytic center of the protein in order to increase its productivity and selectivity in the production of useful chiral building blocks [19].

The next group of extremophiles that have unique features and produce enzymes adapted to work under the particular extreme conditions are halophilic archaea. Halozymes are stable in the presence of high salt concentrations, heat resistant, organic solvent tolerant and frequently requiring a low-water environment. Halophilic amine ω-aminotransferase from *Halomonas elongata* was characterized by Paradisi et al. (2015). This halo-adapted enzyme shows an unusually broad substrate scope and naturally accepts isopropylamine as amino donor in asymmetric synthesis providing a 41% conversion of pyruvate in 24 h at 37°C starting with 1:1 molar ratio between the reagents. Moreover, it accepts ortho-xylylenediamine as amino donor in amine synthesis, in particular, with benzaldehyde yielding high conversions between 90 and 95%. This novel enzyme is also tolerant to the presence of cosolvents up to 20%, which makes it a promising candidate for industrial applications. It has been successfully immobilized on an epoxy-resin, which allows for reusability of the biocatalyst over 10 times. The research focusing on the potential of this enzyme for synthetic applications is still ongoing [38].

Despite the studies described below, any aminotransferase identified in methanogenes has been shown to have some application in biotransformations. ATs are found in methanoges, but researchers focus on their role in the central metabolism of amino acids and in metabolic pathways [39, 40].

Summary

The use of ATs for the biocatalytic preparation of pharmaceutical compounds is expanding at a great rate and is being increasingly studied because of the advantages that these catalysts present over chemically catalyzed processes. The range of novel products are available, therefore they are highly enantio-, chemo- and regioselective. Improvement of the biocatalyst is very often required for industrial application There are many challenges inherent in transaminase processes that need to be dealt with. For instance, overcoming substrate/product inhibition, expansion of substrate specificity, changes in enzyme stability – all of them must be handled to make ATs more attractive enzymes. The origin of ATs from extreme environments makes these enzymes promising candidates for industrial biocatalysis. Moreover, with increasing interest in extremophiles and their applications, the alteration of ATs will become a very important strategy for a development of novel routes to produce valuable chiral building blocks. The engineered ATs offer novel opportunities for biotechnological exploitation. The redesign allows us to understand the enantioselectivity of the reaction, which is an important imperative for further biochemical and structural studies of recombinant ATs.

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