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Omega transaminases: discovery, characterization and engineering

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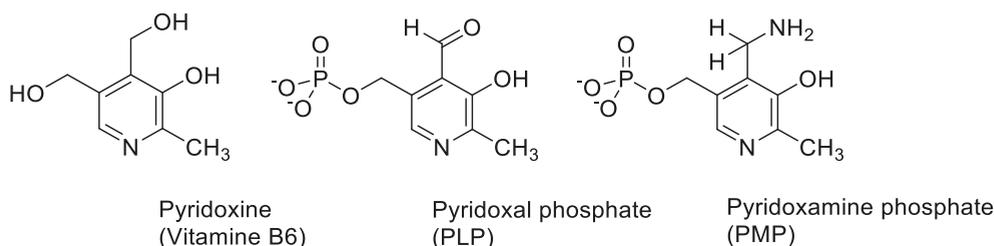
Introduction

Cyntia M. Palacio, Dick B. Jansen

PLP-DEPENDENT ENZYMES

Pyridoxal-5'-phosphate (PLP) is the biologically active phosphorylated derivative of pyridoxine, also called vitamin B6 (Scheme 1). It occurs as cofactor in many biochemical reactions, especially in the metabolism of amino acids. In PLP-dependent enzymes, the cofactor is covalently bound through formation of a Schiff base linkage between its reactive aldehyde group and the ϵ -amino group of an active site lysine, which is topologically conserved within homologous PLP enzymes. An essential catalytic feature of PLP enzymes is imine exchange with the amino group of a substrate replacing the lysine ϵ -amino in the Schiff base linkage. This allows delocalization of negative charge during subsequent reactions of the PLP-bound substrate moiety. This reaction may be proton abstraction, decarboxylation, or an elimination reaction at the C α , C β or C γ of the substrate, C α being the carbon atom carrying the amine group that is coupled to PLP (1, 2). After this, different reprotonation and addition reactions may occur and finally the Schiff base linkage is hydrolyzed or cleaved by imine exchange to release product. Accordingly, enzymes that use PLP as cofactor catalyze a wide variety of conversions including transamination, decarboxylation, β - and γ -elimination, aldol cleavage and racemization (3, 4). This diversity of functions seems to have emerged early in evolution (1).

PLP-dependent enzymes have been classified by reaction type, substrate range, sequence similarity, and by structural features. The widely used EC classification system groups them by reaction and substrate type. However, this does not correlate well to phylogenetic classification and structural conservation. Based on sequence similarities, Mehta *et al.* (5) in an early paper about aminotransferase classification proposed to divide aminotransferases in 4 subgroups (AT-I, AT-II, AT-III and AT-IV; we use the prefix AT to avoid confusion with PLP fold type). Papers on aminotransferases still quite often refer to this classification. Alexander *et al.* (6) grouped PLP-enzymes into α , β , and γ families depending on whether the bond that is formed or cleaved involves a C α , C β or C γ of the amino acid substrate. Another and more complete classification system suggested by Grishin *et al.* (4) is based on three dimensional structures and sequence similarities among a large group of PLP enzymes. It classifies them into five fold types (I to V), each encompassing enzymes with diverse catalytic profiles but evolutionary and structurally related. Since sequence homology and structural similarities coincide, the Grishin classification is similar to the one proposed by Jansonius *et al.* (7), who grouped the enzymes in superfamilies by structural similarities and named the superfamilies after the PLP enzyme of which the crystal structure was determined first. The PLP fold-type classification system of Grishin *et al.* is now most commonly adopted (1, 8), whereas the numbering in AT subgroups has become confusing (see Schirotti and Peracchi *et al.* (9), for a detailed explanation). Recently, two more fold types (VI and VII) were mentioned by Peracchi and coworkers (<http://bioinformatics.unipr.it/cgi-bin/bioinformatics/B6db/fold.pl>). In the discussion below, we adopt the system of Grishin *et al.* (4) as also used by Schirotti *et al.* (9).



Scheme 1. Pyridoxine and phosphorylated derivatives.

Fold type I PLP enzymes, or the aspartate aminotransferase superfamily, encompasses the largest number of biochemically investigated PLP enzymes and the widest variety of activities, including aminotransferases, decarboxylases, and enzymes catalyzing elimination reactions. These are usually dimeric enzymes, with each subunit composed of two domains. The domain organisation and topological position of the PLP group are conserved. In each subunit, an active site with bound PLP is located in a cleft between the two domains (10). Typical conserved motifs along the sequence of these PLP enzymes are a glycine rich loop forming the binding site for the phosphate group of PLP, a conserved aspartate interacting with the pyridoxal nitrogen, and not far from the C-terminus the invariant lysine forming a covalent bond with the PLP (4, 6).

The functional diversity included in the fold type I PLP enzymes is larger than that in other fold types discussed below. The old subgroup AT-I (later divided in class I and class II ATs by Grishin *et al.* (4)), AT-II (later called class III aminotransferases (4)), and AT-IV (later class V ATs (4)) distinguished by Mehta *et al.* (5) all belong to the fold type I PLP enzymes (4). The subgroup AT-I contains the classical aspartate aminotransferase from chicken mitochondria of which several structures have been solved (e.g. pdb 9AAT), providing important mechanistic insight (11), and also alanine-, tyrosine-, histidinol-phosphate-, and phenylalanine/tyrosine aminotransferases (e.g. from *E. coli*, pdb 3FSL). Subgroup AT-II (class III ATs) includes acetylornithine-, ornithine-, 4-aminobutyrate- and also succinyldiaminopimelate aminotransferase (12), and the β -amino acid aminotransferases studied earlier in our laboratory (13, 14). These class III enzymes are sometimes called ω -aminotransferases to indicate that they accept amino acids but preferably not α -amino acids. Subgroup AT-IV of the fold type I PLP enzymes includes serine and phosphoserine aminotransferases.

Fold type II PLP enzymes include all known members of the β family of PLP enzymes (acting on a bond of the $C\beta$ carbon of the substrate) and is also called the tryptophan synthase β superfamily, after the β subunit of tryptophan synthase, which dehydrates and couples serine with indole in a β -elimination/addition reaction. These enzymes are also homodimers comprised of two domains, but unlike in the PLP fold type I enzymes, the lysine forming the Schiff base with PLP is positioned closer to the N-terminus than to the C-terminus. Furthermore, a serine residue rather than an aspartate interacts with

the pyridoxal nitrogen of PLP. Besides the enzymes of the β -family, most members of this fold type II act on α -carbon bonds. Examples are O-acetylserine sulfhydrylase (*Thermotoga maritima* pdb 1O58), threonine deaminase (e.g. from *E. coli*, pdb 1TDJ), L-serine dehydratase (e.g. *Legionella pneumophila*, pdb 4RQO), D-serine dehydratase (e.g. *E. coli*, pdb 3SS7) and cysteine synthase (e.g. *Brucella suis*, pdb 5I7W).

Several racemases and decarboxylases are grouped in the fold type III PLP enzymes, which is also named alanine racemase superfamily (15). Like fold type I and II enzymes, these proteins are homodimers with subunits composed of two domains. However, the structures are clearly different. The lysine forming the Schiff base linkage is situated close to the C-terminus in a loop connecting a strand and a helix, and binding of the pyridine nitrogen is via an arginine residue, indicating that the nitrogen is deprotonated (10).

The group of fold type IV PLP enzymes encompasses several aminotransferases with known 3D structure, including the subgroup AT-III aminotransferases of Mehta *et al.* (5) (called class IV ATs by Grishin *et al.* (4)) termed D-amino acid aminotransferase (DAAT, pdb 1DAA) (16, 17), 4-amino-4-deoxychorismate lyase (18), and branched-chain amino acid aminotransferase (19). Most of these enzymes are homodimers folded into a two-domain structure, except the branched amino acid aminotransferases which in most cases form hexamers. The active site is shaped by residues from both subunits and a glutamic acid interacts with the nitrogen from the PLP pyridine. Although the fold of these enzymes is completely different from that of other PLP-dependent enzymes, the catalytic site somewhat resembles a mirror image of the active site of the typical PLP fold type I aspartate aminotransferase (10).

Glycogen phosphorylase (20, 21) is a member of the fold type V PLP enzymes. This enzyme uses the PLP cofactor in a very different way. Despite a Schiff base with the ϵ -amino of a lysine being formed, the PLP does not participate in the catalytic cycle by acting as an electron sink like in aminotransferases (22). Instead, according to the mechanism proposed by Helmreich and coworkers (23–25), the phosphate group of PLP plays a role in acid/base catalysis. The nitrogen of the pyridine ring also lacks the hydrogen bonds with residues in the active site, whereas other PLP-enzymes form hydrogen bonds with aspartic acid, serine, glutamic acid or arginine as discussed above (10).

AMINOTRANSFERASES

As mentioned above, the aminotransferases of fold type I PLP proteins can be further classified into subgroups (5) or classes (4) (AT-I/Class I & II, AT-II/Class III and AT-IV/Class V), based on the degree of similarity in their primary (and three-dimensional) structures (9, 26). All fold type I PLP aminotransferases bind the cofactor at a topologically conserved position and share four invariant residues: Glu314 (numbering of the pig cytosolic aspartate transaminase, pdb 1AJR), located in a turn at the interface of the dimer;

Lys385, which forms a Schiff base with PLP; Asp340, interacting with the nitrogen of the pyridoxal ring; and Arg562, which interacts with the α -carboxylate group of the substrate (2, 5). Furthermore, the transaminases of subgroups AT-I, AT-II, and AT-IV have a similar organization of the active site, and a distinguishing feature is that the conserved lysine involved in abstracting the proton from the aldimine intermediate points towards the *Si* face of the C4' of the PLP (usually called the *Si* face of the PLP ring) (26, 27).

The substrate selectivity of aminotransferases only partially coincides with the division in these subgroups. Aminotransferases of subgroup AT-I tend to have a broad substrate scope and accept L-alanine, aromatic L-amino acids, and also dicarboxylic amino acids (28). The substrate range of AT-II enzymes is even more diverse, including acetyloronithine, ornithine, ω -amino acids, 4-aminobutyrate and diaminopelargonate. Sequence analysis indicates that this subgroup includes enzymes catalyzing other reactions, such as decarboxylation. Several of these PLP fold type I subgroup AT-II enzymes have been studied in detail (9). Activity with L- α -amino acids is often lacking. Some members accept molecules that contain two amino groups of which one reacts, e.g. L-lysine ϵ -aminotransferase. Also the well-studied aminotransferases from *Chromobacterium violaceum* (CvAT) (29) and *Vibrio fluvialis* (VfAT) (30), which poorly accept L- α -amino acids, belong to this AT-II subgroup. The latter transaminases have been the subject of multiple studies during the last decade, mainly because of their use in biocatalytic processes toward enantiopure compounds (31). They show high preference for aromatic amines, such as benzylamine and (*S*)-methylbenzylamine (32). CvAT and VfAT both are (*S*)-enantioselective with aryl-substituted substrates and lack detectable activity with (*R*)-enantiomers of the same compounds (32–34). Aliphatic amines and most of the α -amino acids, except for L-alanine, give very poor activity and pyruvate is the only amino acceptor showing good activity (30, 32, 35). The β -amino acid aminotransferases investigated in our laboratory also belong the subgroup AT-II enzymes (13, 14). Within the fold type I PLP enzymes, the subgroup AT-IV enzymes catalyze the transamination of substrates with related chemical structures such as L-serine and 3-phospho-L-serine, utilizing 2-oxoglutarate or pyruvate as amino acceptors (4, 5).

The subgroup III aminotransferases (AT-III) do not group with the fold type I PLP enzymes; instead they belong to the fold type IV PLP enzymes (4, 5). A comparison between the structures of the dimeric D-amino acid aminotransferase from *Bacillus* sp. (16) (pdb 1DAA) and the hexameric branched chain amino acid aminotransferase from *E. coli* (pdb 1A3G, (19)) shows that the same fold type can occur in different multimeric assemblies. The stereochemical preference of some of these enzymes is opposite that of the fold type I enzymes, which is explained by the inverted way of PLP binding, i.e. with the *Re* face of the PLP C4' towards the β -barrel present in the N-terminal domain of the protein (36). The conserved lysine forming the Schiff base in the internal aldimine thus points towards the *Re* face of the C4' of the external aldimine intermediate (37). Enzymes of subgroup III accept a variety of substrates, including 4-aminobutyrate, branched amino acids, and D-alanine.

Transaminase discovery

Early studies on transaminases used whole cells, cell-free extracts, and enzymes isolated from microbial cultures or tissue. More than 50 years ago, Rudman *et al.* (38) reported a transamination reaction between aliphatic amino acids and α -ketoglutarate catalyzed by cell extracts from *E. coli*, and Campbell *et al.* (39) described the transamination of amino acids catalyzed by a *Pseudomonas* strain. The latter paper reported the production of glycine from glyoxylic acid utilizing aspartic acid, glutamic acid, asparagine and glutamine as amino donors (40).

Later studies employed microbiological enrichment cultures with a substrate of interest as a strategy to find desired transaminases. The target substrate is added as a sole source of nitrogen or/and carbon. This selection method requires expression of the enzyme under the applied cultivation conditions, which may be the case both in fungi and bacteria. Fincham *et al.* (41) reported an increase of the transaminase production by *Neurospora crassa* when valine and isoleucine were added to the culture medium. Similarly, transaminase activity towards taurine and α -ketoglutarate was enhanced when *Achromobacter superficialis* and *Achromobacter polymorph* were cultured in medium containing β -alanine (42). It was found that to detect aminotransferase activity in cell lysates of such cultures, the cofactor pyridoxal phosphate often had to be added (40), which suggested that loss of the cofactor under enzyme preparation and assay conditions can easily occur (41). Soil samples are often used as a source of microbial diversity in aminotransferase enrichment experiments. For example, the well-known ω -aminotransferase from *V. fluvialis* was found by selective enrichment with (S)-methylbenzylamine as the sole source of nitrogen and with soil as inoculum (33, 43).

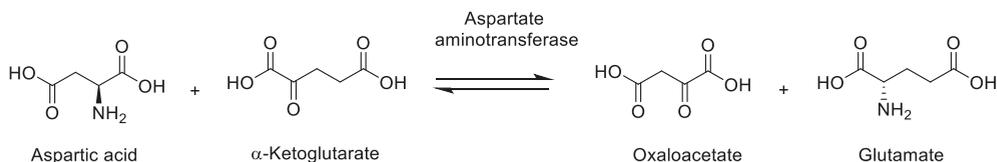
New tools have delivered additional and faster procedures for the discovery of aminotransferases. Functional or sequence-based analysis of metagenomic libraries, genome sequencing of microbial isolates, and bioinformatics mining of genomic databases can reduce the number of steps necessary for identifying and expressing a new enzyme. Bioinformatics can be used for selecting enzymes that are expected to exhibit a certain performance, such as substrate range or thermostability. For example, the well-studied aminotransferase from *C. violaceum* DSM30191 was found through sequence analysis of the genome, based on homology to the published sequence of the aminotransferase from *V. fluvialis* (32). Furthermore, Wilding *et al.* (44) reported the identification of 14 aminotransferases from *Pseudomonas* AAC, a strain from a collection of soil and water bacteria. Eleven of these enzymes could be expressed in *E. coli* and 3 of them showed activity with 12-aminododecanoic acid, indicating that they could be promising biocatalyst for the production of nylon precursors. Computational methods to predict aminotransferase selectivity and to design variants have been explored. Höhne *et al.* described the discovery of 17 (R)-selective aminotransferases by a computational approach which included structure analysis to predict amino acid motifs and a subsequent in-silico screening of sequence databases to identify those

variants carrying the key motifs (45). This indicated that approaches which combine rational design and computational screening will gain importance for the discovery and engineering of aminotransferases with specific substrate preferences (45).

Aminotransferase mechanism

Aspartate aminotransferase from chicken mitochondria (AATA, pdb 1AAT) is a mechanistically well-studied PLP-dependent enzyme. The enzyme belongs to subgroup AT-I (fold type I) and catalyzes the reversible transfer of an amino group from L-aspartate to 2-oxoglutarate, producing oxaloacetate and L-glutamate (Scheme 2). As most of the fold type I aminotransferases, it is a homodimer composed of two subunits of ca. 45 kDa with the active site at the dimer interface. Its crystal structure was the first aminotransferase structure to be elucidated (11) and it served as model to study the reaction mechanism of PLP enzymes. More than 30 years ago Kirsch *et al.* (46) described the catalytic cycle in detail, including the role of the PLP cofactor acting as an electron sink.

The catalytic cycle of aminotransferases consists of several steps, which can be grouped in 2 half reactions. The first half reaction includes a series of steps in which the substrate amino group is transferred to PLP, yielding the pyridoxamine enzyme (PMP) and the keto product (3). In the second half reaction, the amino group of PMP is transferred to a keto (or aldehyde) acceptor, yielding an amine and regenerating the PLP in the pyridoxal form. Mechanistically, the second half reaction is the reverse of the first half reaction, so it also involves multiple steps (Scheme 3).

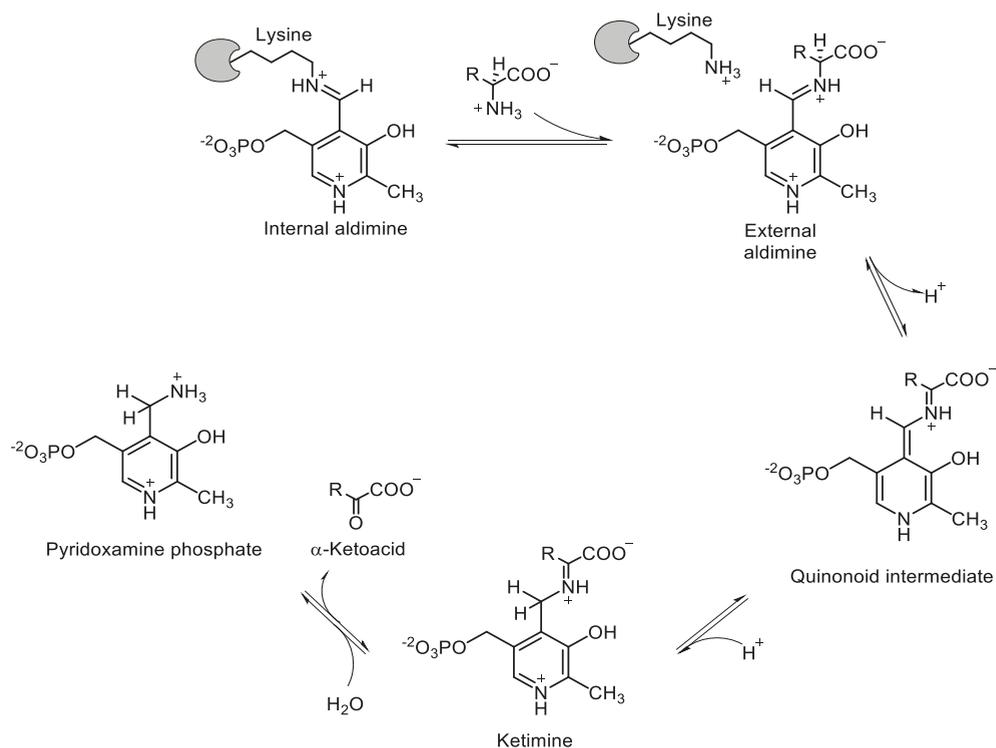


Scheme 2. Transamination of aspartic acid catalyzed by aspartate aminotransferase.

In the substrate-free form of an aminotransferase, the ϵ -amino group of the conserved lysine has formed a Schiff base linkage with the aldehyde group of PLP (47). This invariable complex in the holoenzyme is known as the internal aldimine, the formation of which involves several intermediates and the release of one molecule of water. After formation of a Michaelis complex by substrate binding, in the first chemical step of the first half reaction the internal aldimine is converted into an external aldimine, in which the substrate amine group forms a Schiff base linkage with PLP and the side chain ϵ -amino group of the lysine is released. Next, a proton is abstracted from the α -carbon of the substrate-PLP complex, resulting in the formation of the quinonoid

intermediate by transfer and delocalization of negative charge to the pyridine ring. Subsequently, the ketimine intermediate is formed through protonation of the former aldehyde carbon (C4') of the coenzyme. This conversion of the quinonoid to the ketimine intermediate is a critical step in the reaction mechanism of aminotransferases, since subsequent hydrolysis of the ketimine yields the keto product. In racemases, the quinonoid intermediate can be reprotonated on the opposite face of the substrate carbon, in which case racemisation occurs upon hydrolysis at the PLP 4' carbon. Since different intermediates may be generated from the quinonoid by transfer of protons, the particular configuration of acid and basic residues in the active site seems to determine the substrate specificity and the type of PLP-dependent reaction that is catalyzed (26). Hydrolysis of the ketimine is accompanied by deamination of the substrate and conversion of the PLP to a pyridoxamine, which in turn can transfer the amino group to a ketoacid or another carbonyl compound that acts as acceptor. In case of aspartate aminotransferase, the acceptor is 2-oxoglutarate and the enzyme produces L-glutamate.

The mechanism is similar in many other aminotransferases, including other fold type I enzymes, and within that group similarities are high enough to identify residues involved in cofactor binding and catalysis from sequence- or structural alignments (47). Furthermore, the overall mechanistic features are conserved in aminotransferases from other fold types.



Scheme 3. Catalytic mechanism of PLP-dependent aminotransferases. Only the PLP-amination reaction with an L-amino acid as donor is shown. Transfer of the amino group from the pyridoxamine phosphate intermediate to a keto acceptor follows the inverse sequence of reactions.

Function of aminotransferases

Scientific interest in aminotransferases not only stems from their mechanistic features and evolutionary origins, but is also stimulated by their industrial relevance and important functions in biology. Aminotransferases are key enzymes of central amino acid metabolism, e.g. by catalyzing the deamination reaction during the catabolism of amino acids (3). As mentioned above, aspartate aminotransferase transfers the α -amino group to α -ketoglutarate producing glutamate, connecting central intermediates of carbon metabolism to amino acid metabolism (Scheme 2). Transamination provides the nitrogen group for the synthesis of various amino acids (48). Glutamate is a key component of amino acid metabolism because it plays two important roles: it is the nitrogen donor for the synthesis of most amino acids, and it acts as the precursor for the synthesis of proline and arginine (3, 49). Aminotransferases also carry out the deamination of branched-chain amino acids during catabolism. In humans, these amino acids need to be acquired through the diet since they cannot be synthesized endogenously. When taken in excess they are metabolized with the deamination

reaction catalyzed by aminotransferases (48). In bacteria, aminotransferases also have a crucial role in the amino acid catabolism, for example in the conversion of β -alanine and L-tryptophan (50).

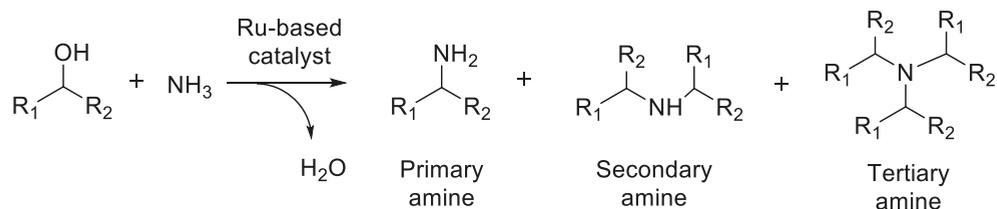
Many synthetic pathways towards amino acids and other aminated compounds in bacteria, animals and plants include aminotransferases as crucial enzymes for the amination of keto-precursors. In bacteria, the last step of the synthesis of the aromatic amino acids phenylalanine and tyrosine is the aminotransferase-catalyzed amination of the precursors phenylpyruvate and *p*-hydroxyphenylpyruvate, respectively, using glutamate as the amino donor. A tyrosine aminotransferase is involved in the biosynthesis of leucine, phenylalanine and tyrosine (51–55). An aspartate aminotransferase catalyses the amination of oxaloacetate to produce aspartate (54, 56) and the aminotransferase B is involved in the synthesis of the branched-chain amino acids leucine, isoleucine, and valine (3, 57).

During our studies on the enzymes responsible for the biodegradation of caprolactam described in Chapter 3, a new ω -aminotransferase was found and characterized in *Pseudomonas jessenii*. It is involved in the transamination of 6-oxohexanoate to produce 6-aminohexanoic acid and represents one enzyme in the caprolactam degradation pathway that remained unidentified so far (57).

SYNTHESIS OF AMINES

I. Chemical synthesis

Amines can be synthesized via many chemical methods, with alkylation and reduction as the main reactions. In case of synthesis of amines by alkylation, ammonia or an organic amine acts as a nucleophile and reacts with a haloalkane or an alcohol to give a primary amine product. The disadvantage of this process is the low purity of the product, which is caused by the reactivity of the amine product as a nucleophile. This may lead to subsequent reactions that produce secondary, tertiary and quaternary amines or ammonium salts (Scheme 4).

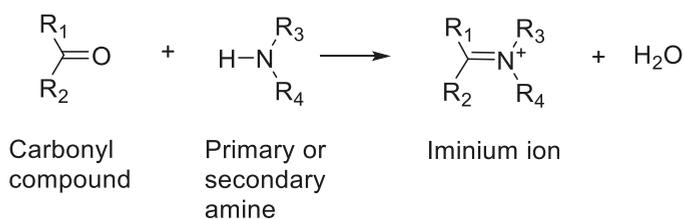


Scheme 4. Synthesis of amines by alkylation with alcohols. During the first steps, a primary amine is formed. This two-step sequence may continue, forming secondary, tertiary and quaternary amines (58).

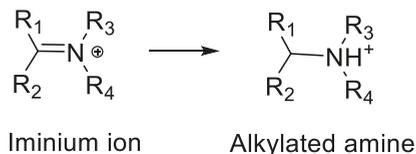
The second most used method is the reductive amination of aldehydes and ketones, which comprises two steps. In the first step, an amine undergoes condensation with a carbonyl compound to produce an imine. In the second step, the imine double bond is reduced by catalytic hydrogenation. This process can be carried out as one-pot reaction with the reducing agent triacetoxyborohydride or with hydrogen in industrial processes (59) (Scheme 5).

An example of a synthetic pathway involving both alkylation of an organic halide and reductive amination is the route towards the drug fentanyl, a compound used to induce anesthesia for surgery and to treat pain (Scheme 6).

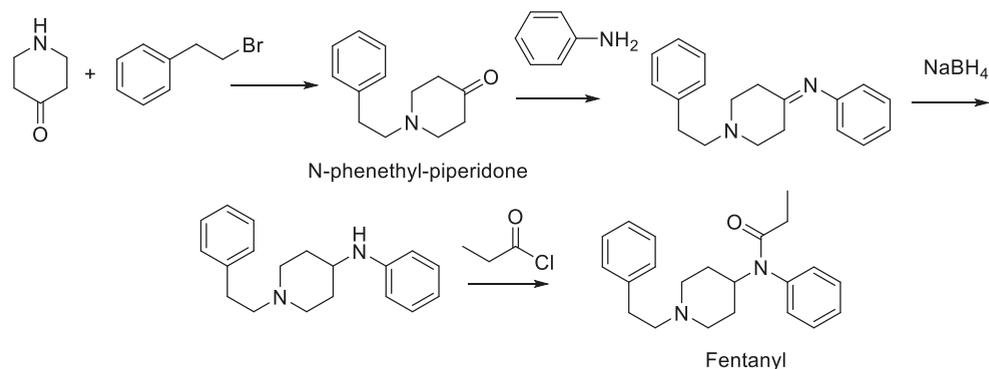
Condensation



Reduction

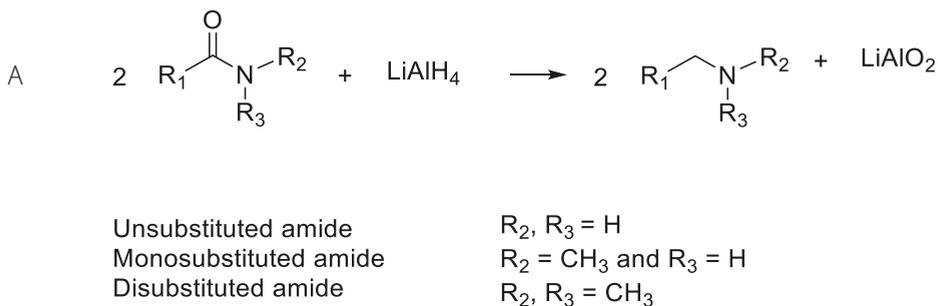


Scheme 5. Reductive amination of ketones and aldehydes. Adapted from Abdel-Magid *et al.* (59).



Scheme 6. Synthesis of fentanyl, a commercial drug containing a piperidine moiety. C-N bond formation (amination) occurs by alkylation of an organobromide, and by a condensation reaction followed by reduction (60).

Two reductive methods often used for the preparation of amines are the reduction of amide moieties and the reduction of nitriles. The carbonyl group of an amide is reduced by lithium aluminum hydride to give an amine. This reaction produces primary amines from *N*-unsubstituted amides, secondary amines from monosubstituted amides, and tertiary amines from disubstituted amides (61) (Scheme 7A). Lithium aluminum hydride is also used as the reducing agent for the reduction of nitriles where the triple carbon-nitrogen bond is reduced to give a primary amine (Scheme 7B). Metal catalysts such as rhodium (62) and lithium (63) can also serve to reduce the nitrile moiety.



Scheme 7. A. Amine reduction with lithium aluminum hydride. **B.** Preparation of amines by reduction of nitriles.

II. Aminotransferases for the production of chiral and non-chiral amines

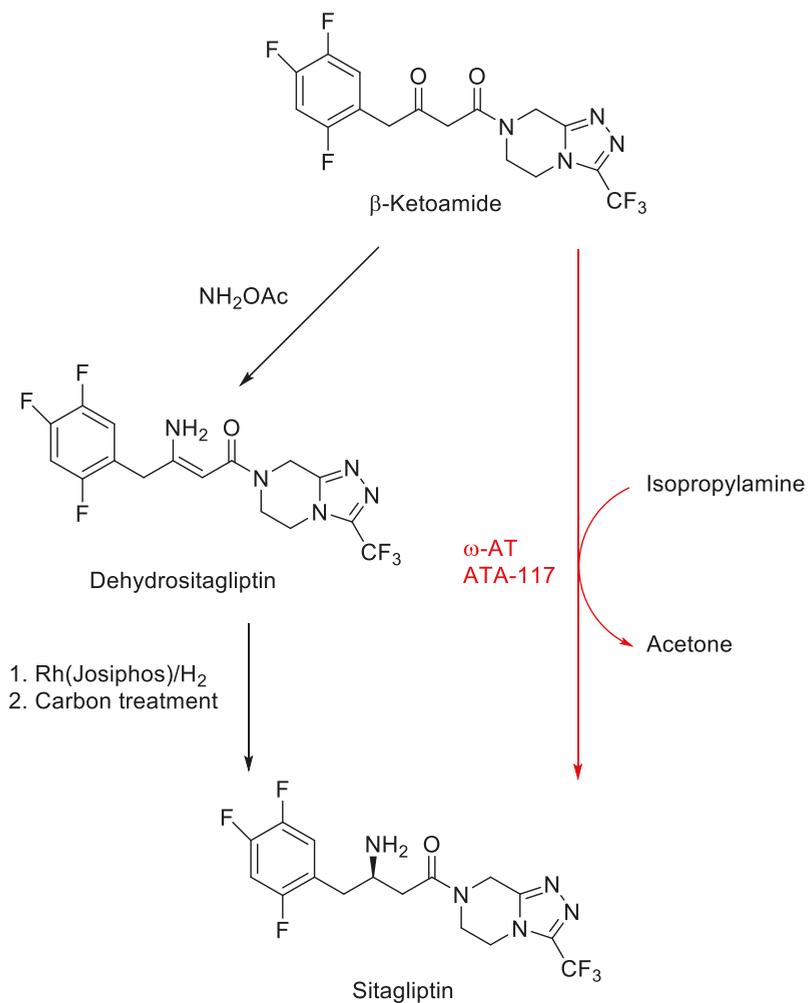
Biocatalysis offers alternative methods for the production of amines, especially primary amines. These enzyme-mediated conversions often are environmentally friendly and can avoid the necessity to use potentially toxic heavy metal catalysts. Enzymatic reactions can be carried at room temperature and atmospheric pressure, thereby decreasing energy usage and avoiding problems such as molecular rearrangement of substrates and products (64). Additionally, enzymes are regio-, chemo- and enantioselective catalysts. These important features allow selective conversion of target compounds in complex mixtures, like it happens in living cells.

As described above, aminotransferases catalyze the transfer of an amino group from an amino donor to an amino acceptor (an aldehyde or a ketone). Therefore, these enzymes are widely explored for the production of amines. Attractive features are the broad substrate specificity covered by the diversity of known aminotransferases, the often observed excellent stereoselectivity, and the high turnover rates. Furthermore, unlike with dehydrogenases, no (reducing) external cofactor is needed. Thus, aminotransferases are employed in the synthesis of fine chemicals including building blocks for the pharmaceutical industry (65).

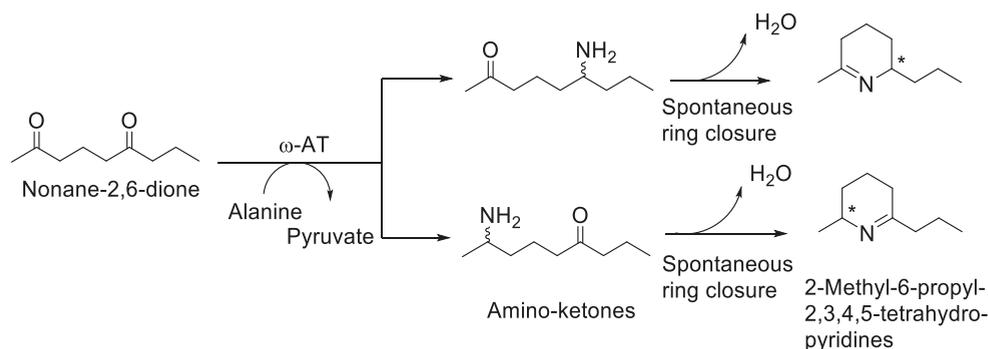
During enzymatic asymmetric synthesis, amines might be produced starting from a ketone with the desired carbon skeleton. In the presence of a good amine donor and biocatalyst, conversion can proceed to near 100% product yield. Not surprisingly, aminotransferase-mediated ketone amination has become a highly attractive step in synthetic routes for the production of non-enantiopure and enantiopure amines (35, 66). One famous example is the synthesis of sitagliptin, the active ingredient of Januvia, an oral medicine for the treatment of type 2 diabetes (67). When this compound is synthesized via an enzymatic step catalyzed by an enantioselective aminotransferase (Scheme 8, right branch) two time- and resource-consuming steps part of the chemical synthesis route (Scheme 8, left branch) are eliminated. The aminotransferase catalyzed reaction is performed under mild conditions and fewer steps are needed to obtain the desired product.

Although less intensively studied, aminotransferases also have potential as catalysts for the production of non-chiral amines. Simon *et al.* (68), described the preparation of pyrrolidines and piperidines via the amination of diketones to produce cyclic amines (Scheme 9). Several ω -aminotransferases, including CvAT (32) and VfAT (28), showed excellent conversion of 1,5-diketones to amino-ketones. These molecules are valuable precursors for the synthesis of the alkaloids dihydropinidine and epi-dihydropinidine (69); both are naturally occurring defense compounds produced by certain plants to repel predator insects or animals.

Furthermore, due to their regioselectivity, aminotransferases are useful catalysts for the synthesis of non-chiral products when using as substrates with two equal functional groups, as in 1,5-diketones. An example of this is the production of 2,6-disubstituted piperidines in one-pot cascade reactions, described by France *et al.* (70). Aminotransferases appeared promising for the production of aromatic pyrazines, using 4-phenyl-2-butanone as amino acceptor and 1,2-aminodiamines as amino donors (Scheme 10) (71).

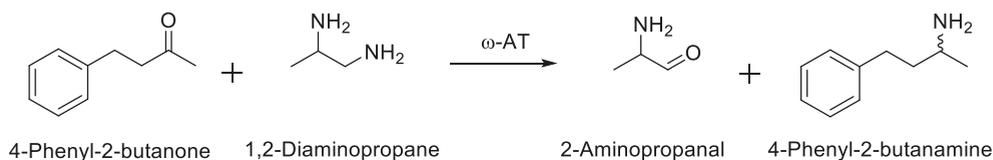


Scheme 8. Routes for the synthesis of sitagliptin. Left: chemocatalytic route. Right: biocatalytic route with an engineered aminotransferase. Adapted from Desai *et al.* (67).



Scheme 9. Enzymatic monoamination of diketones catalyzed by ω -aminotransferases in the synthesis of 2,6-disubstituted pyridines. Adapted from Simon *et al.* (68).

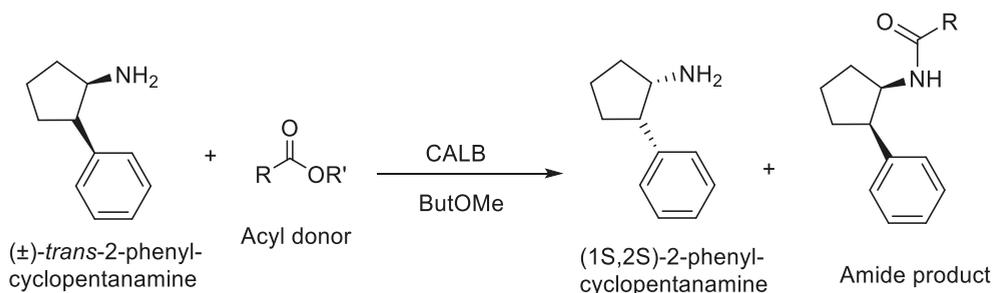
Besides the possibility of using aminotransferases as isolated enzymes in single or multi-enzyme reactions, they also may play an important role in the *in vivo* synthesis of amines, e.g. in artificial pathways constructed by metabolic engineering. An example is the assembled pathway for the production of 6-aminohexanoic acid utilizing an aminotransferase from *Bacillus subtilis* (72). This product is the precursor in the synthesis of nylon-6, one of the most used polymers for manufacturing of fibers, utensils, mechanical parts and household products.



Scheme 10. Enzymatic amination of ketones utilizing 1,2-diamines as amino donors. Based on Payer *et al.* (71).

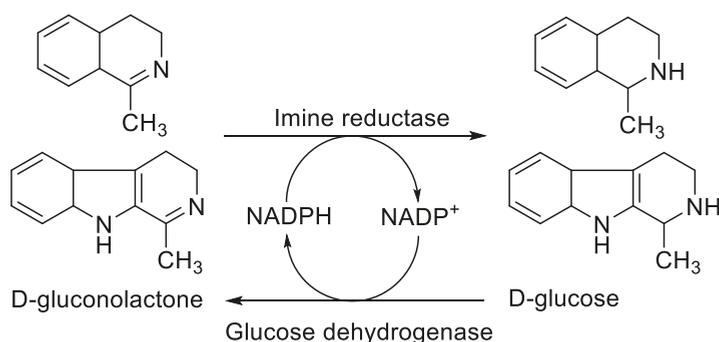
III. Alternative enzymes for the production of amines

Besides aminotransferases, hydrolases and oxidoreductases have been used for the preparation of enantiopure amines. The most important group of hydrolases used in biocatalysis are the lipases, enzymes with a broad substrate spectrum which catalyze the cleavage of esters and occasionally also of amides. The high activity in organic solvent makes lipases attractive for the conversion or production of compounds that are poorly water soluble (73). The lipase from *Candida antarctica* (CALB) is an efficient biocatalyst for the enantioselective acylation of amines (74–76). González-Sabín *et al.* (77) reported an enzymatic resolution of (\pm)-*trans*- and (\pm)-*cis*-2-phenylcyclopentanamine by CALB which catalyzed an aminolysis reaction (Scheme 11).



Scheme 11. Enzymatic kinetic resolution of (±)-*trans*-2-phenylcyclopentanamine by lipase CALB-catalyzed aminolysis of esters into amides. *Trans*-2-phenylcyclopentanamine is an antidepressant, and can be used for the synthesis of compounds with hypoglycemic activity. Modified from González-Sabín *et al.* (77).

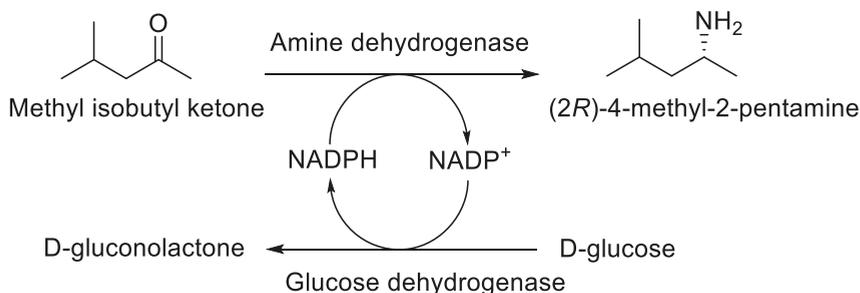
Oxidoreductases, including imine reductases, amine dehydrogenases and amine oxidases, also can be efficient catalysts for the production of enantiopure amines (78). Imine reductases are NADPH-dependent enzymes which catalyze the reduction of imine moieties to amines. Huber *et al.* (79) reported the enzymatic reduction of 3,4-dihydroisoquinolines and 3,4-dihydro-β-carbolines by the *S*-selective imine reductases from *Streptomyces sp.* GF3546 and *Streptomyces aurantiacus* (Scheme 12).



Scheme 12. Reduction of 3,4-dihydroisoquinoline and 3,4-dihydro-β-carboline catalysed by imine reductases. A cofactor regeneration system to recycle the reducing cofactor was set up with glucose dehydrogenase (79).

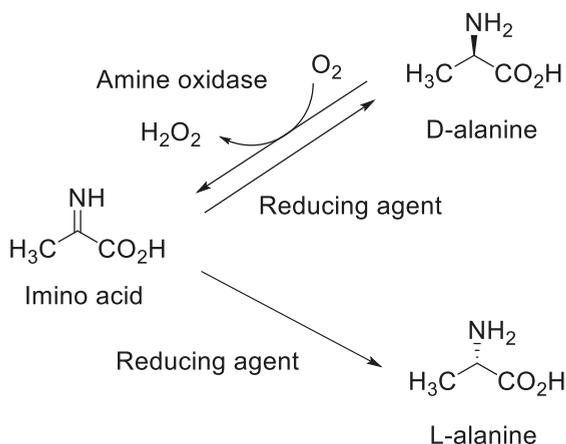
Amine dehydrogenases are also enantioselective enzymes of the oxidoreductase family. They catalyze the reductive amination of ketones, in particular ketoacids, to produce chiral amines using NADPH as electron donor. As for the imine reductases, the cofactor regeneration system can be a glucose dehydrogenase or a formate dehydrogenase, both accepting cheap substrates as electron source (80, 81). Abrahamson *et al.* (82) altered

the substrate specificity of a leucine dehydrogenase from *Bacillus stearothermophilus* in order to create a novel amine dehydrogenase that is independent of the presence of a carboxylate functionality in the substrate. This enzyme reduces the carbonyl function of methyl isobutyl ketone (Scheme 13).



Scheme 13. Reduction of methyl isobutyl ketone by an engineered leucine dehydrogenase converted to an amine dehydrogenase (82).

The final group of enzymes of the oxidoreductase family with the potential to produce enantiopure amines mentioned here are the amine oxidases. These enzymes catalyze the oxidation of amines to imines with the simultaneous reduction of oxygen to hydrogen peroxide. Alexeeva *et al.* (83) and coworkers reported the deracemization of α -amino acids by D- and L-amino acid oxidases. A cycle of oxidation of the amine to imine catalyzed by the amine oxidase and the nonselective reduction by a strong reducing agent, as an amine-borane complex, is repeated until a stereoinversion of one enantiomer to the other is reached (84) (Scheme 14).



Scheme 14. Stereoinversion of D- to L-alanine by the porcine kidney D-amino acid oxidase and sodium tetrahydroborate (NaBH_4). Adapted from Beard *et al.* (84).

ENZYMATIC CASCADE FOR THE BIOTRANSFORMATION OF ALCOHOLS TO AMINES

Alcohols are widely available for industrial usage since they can be conveniently produced either from renewable resources or via petrochemistry, both on large scale. Due to the low cost of many alcohols and the large production scale, alcohols would be suitable substrates for the production of amines. The chemical production of amines from alcohols and ammonia can occur at high temperature and pressure in the presence of a catalyst to form primary amines (85). Such chemical amination processes take place under harsh conditions and make use of organic solvents and metal-based catalysts because alcohols are not very reactive (86–88). The use of metal catalysts such as ruthenium, copper (89), iron (90) and palladium (91) in combination with high pressure and temperature and the long reaction times often cause formation of side products, i.e. secondary and tertiary amines (92–95). Therefore, the chemical production of amines with alcohols as starting compounds suffers from unfavorable sustainability characteristics, which justify a search for more efficient alternatives, including biocatalysis.

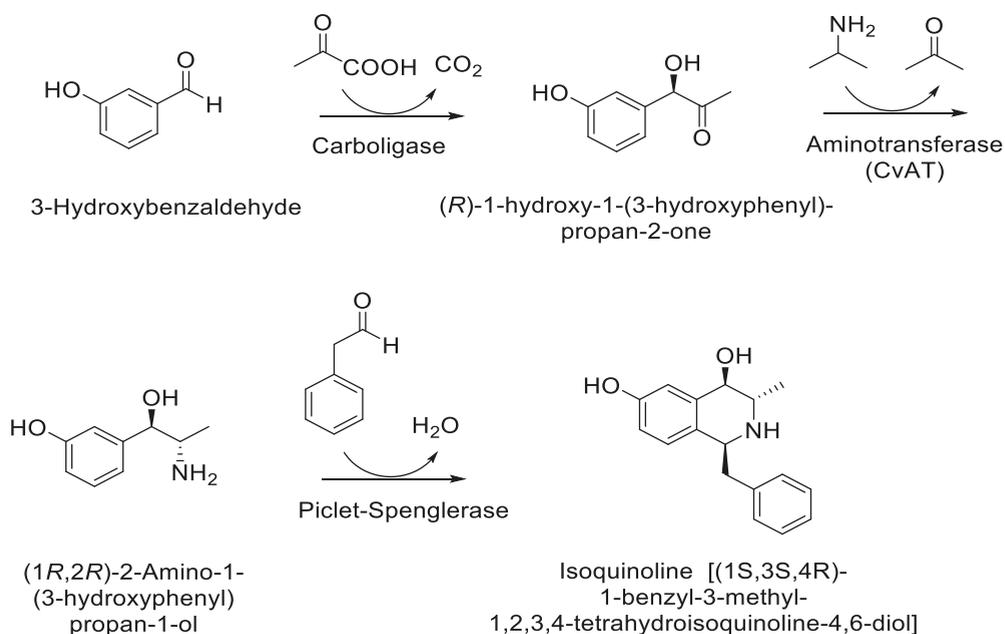
Advantages of the use of enzymes for alcohol amination may not immediately be evident since most aminating enzymes do not act on alcohols but on aldehydes or ketones. Enzymatic processes for alcohol amination thus imply substrate oxidation (hydroxy to oxo), followed either by amination with an organic amine catalyzed by an aminotransferase or by amination with ammonia catalyzed by amino acid (or amine) dehydrogenase. The use of a combination of enzymes is attractive, especially in case of an alcohol dehydrogenase and an amine dehydrogenase, where the overall reaction is redox neutral. Chemical conversion of oxo compounds to amines is also possible, but as a side reaction the substrate's carbonyl group might be hydrogenated forming alcohols at the expense of substrate (95). Furthermore, both in chemical and enzymatic processes the carbonyl substrate might react with the amine product, forming unwanted imines or amines (96). This causes the need of extra purification steps, which can be expensive and technically difficult to perform.

Biocatalytic cascade reactions of alcohols to amines via aldehydes or ketones may be feasible if the problems described above can be avoided, especially through a high selectivity of enzymes and rapid turnover of reactive intermediates. Therefore, enzymatic cascade reactions where two or more enzymes are used in a single mixture can be attractive. Multienzymatic systems can achieve high yields, increase reaction rates, and avoid purification steps. This reduces production costs and decreases the amount of waste that is produced (97, 98).

The keto intermediates that undergo amination reaction in enzymatic cascades may be formed by dehydrogenases and/or transketolases (99, 100). Sehl *et al.* (101) proposed a combination of reactions resulting in a recycling cascade, meaning that the deaminated product of the aminotransferase served as substrate for the transketolase.

This one-pot two-step process included a pyruvate decarboxylase that formed (*R*)-phenylacetylcarbinol ((*R*)-1-hydroxy-1-phenylacetone) from pyruvate and benzaldehyde. This product was transformed by aminotransferase activity either to (1*R*,2*R*)-nor(pseudo)ephedrine or (1*R*,2*S*)-nor(pseudo)ephedrine, dependent on the enantioselectivity of the used aminotransferase; *Aspergillus terreus* AT, or *Chromobacterium violaceum* AT, respectively. The transamination was driven by L-alanine, and the formed pyruvate could be used directly or via enzymatic formation of acetolactate as a substrate for the transketolase reaction. To avoid unwanted side reactions the aminotransferases were added after the synthesis of the keto intermediate was completed.

Another example that employs a pyruvate decarboxylase as a carboligase in order to synthesize the amine acceptor was recently reported for the synthesis of a tetrahydroquinoline (Scheme 15). In the three-enzyme cascade, the transaminase step was catalyzed by the enzyme from *C. violaceum* with isopropylamine as amine donor (102). The tetrahydroisoquinoline moiety occurs in a number of bioactive compounds with therapeutic applications.

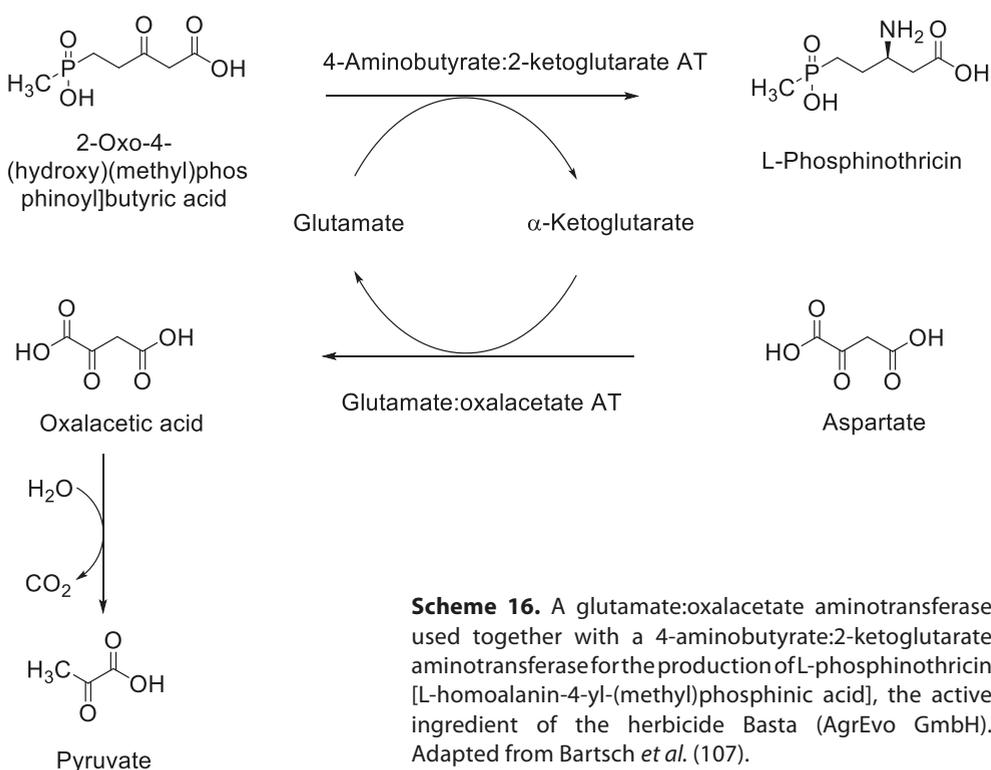


Scheme 15. Synthesis of a tetrahydroquinoline by a three-enzyme cascade (102). Piclet-Spenglerase is a norcoclaurine synthase from plant benzyl-isoquinoline alkaloid biosynthesis.

Both in single-enzyme reactions and in multi-enzyme systems that involve an aminotransferase step, an unfavorable reaction equilibrium may be an issue. Dependent on the properties of the amine donor and acceptor, thermodynamics may favor the aminotransferase reaction in the direction towards the ketone form of the substrate

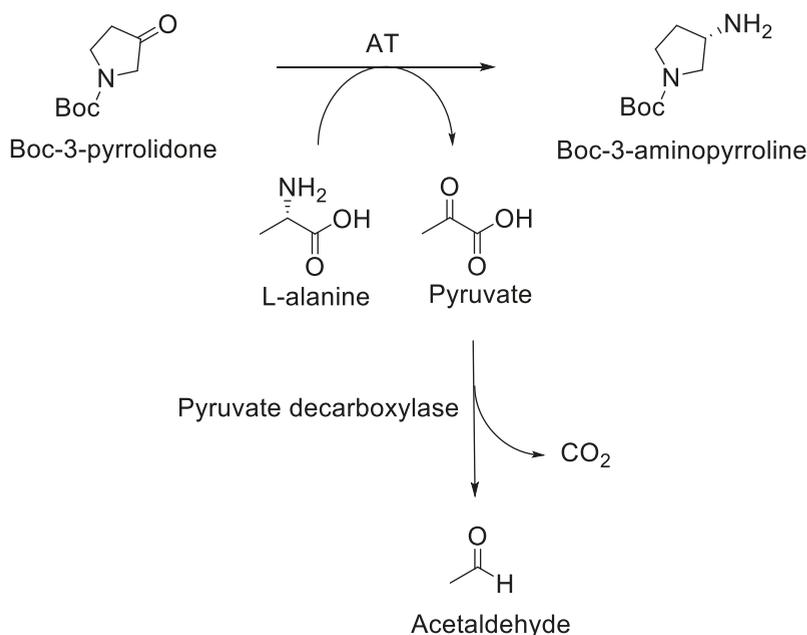
rather than to the amine product of interest (43, 103). Besides this equilibrium issue, kinetics of enzymatic transamination may be problematic, especially due to product inhibition. Shin and Kim (104) demonstrated that, although thermodynamically favorable, the kinetic resolution of (*R,S*)- α -methylbenzylamine with the ω -transaminase of *Vibrio fluvialis* did not reach completion due to the inhibition of the enzyme by the products alanine and acetophenone formed during conversion.

A possible strategy to shift the unfavorable equilibrium of aminotransferase reactions towards production of a desired amine is to couple the reaction to a thermodynamically more favorable conversion (105, 106). In an early paper, Bartsch *et al.* (107) reported the synthesis of the herbicide L-phosphinothricin by a two-enzyme process of a glutamate:oxaloacetate aminotransferase and a 4-aminobutyrate:2-ketoglutarate aminotransferase (Scheme 16). The proposed conversion is a one-pot reaction in which glutamate, the primary amino donor, is regenerated from aspartate, the secondary amino donor, obtaining oxaloacetic acid as by-product. Oxaloacetate decarboxylates spontaneously to pyruvate, forcing the equilibrium towards the synthesis of the desired product, L-phosphinothricin. When both transaminases were coupled in a two-step reaction the yield was significantly higher in comparison with an earlier one-enzyme reaction.



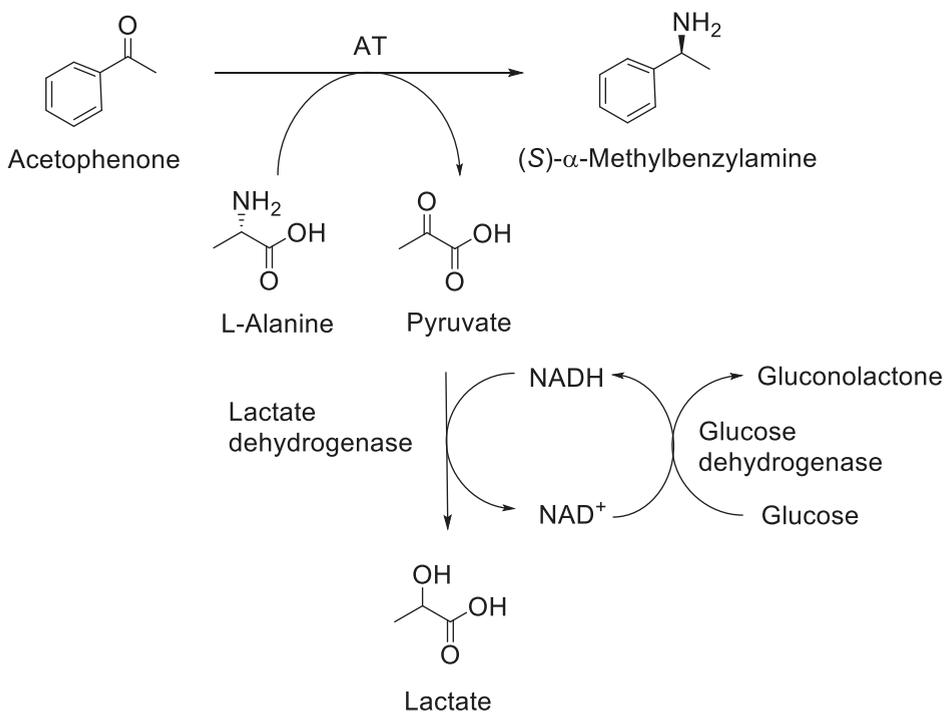
Scheme 16. A glutamate:oxalacetate aminotransferase used together with a 4-aminobutyrate:2-ketoglutarate aminotransferase for the production of L-phosphinothricin [L-homoalanin-4-yl-(methyl)phosphinic acid], the active ingredient of the herbicide Basta (AgrEvo GmbH). Adapted from Bartsch *et al.* (107).

When using alanine as the amino donor in aminotransferase reactions, a feasible strategy to shift the equilibrium is the addition of an enzymatic step for the removal of the deaminated product pyruvate. Enzymatic cascades employing two or three enzymes have been designed to produce amines using this strategy. One possibility to remove pyruvate is by adding a pyruvate decarboxylase (Scheme 17) (108). Another option is to use a lactate dehydrogenase that reduces pyruvate to lactate with regeneration of the required reduced nicotinamide cofactor by addition of a glucose dehydrogenase (Scheme 18) (109–111). The use of a dehydrogenase to shift the equilibrium was also described by Cassimjee *et al.* (112), in this case for the oxidative removal of acetone formed when isopropylamine was used as the amine donor, with cofactor regeneration by formate dehydrogenase. Occasionally, an amine donor may be used that rearranges upon being converted to a ketone, forming a compound that cannot act as amine acceptor and preventing any reverse reaction. This principle was demonstrated with 3-aminocyclohexa-1,5-dienecarboxylic, accepted as donor by a variant of *C. violaceum* aminotransferase (113).



Scheme 17. Removal of pyruvate in the amino transferase reaction by a pyruvate decarboxylase. The product of the pyruvate decarboxylation is acetaldehyde, a volatile compound which together with the production of gaseous CO₂ makes the reaction irreversible. Adapted from Höhne *et al.* (108).

Other possible strategies for shifting the aminotransferase reaction towards the amine product are the removal of the amine product by continuous extraction with an organic solvent at alkaline pH (114, 115), the removal of acetone by evaporation when 2-propylamine is used as amino donor (116), and the addition of an excess of the amino donor (43). Shin *et al.* (117) performed a kinetic resolution of *sec*-butylamine under reduced pressure, driving the reaction to higher yields through the removal of the inhibitory deaminated product 2-butanone. When adding one substrate in excess it needs to be considered that very high concentrations might not be tolerated by the aminotransferases. The ATs are known to be sensitive to substrate inhibition (118), which can be explained by binding of the amino donor to the pyridoxamine intermediate instead of the ketone.



Scheme 18. Enzymatic cascade reaction including an aminotransferase, a lactate dehydrogenase and a glucose dehydrogenase (109).

Besides the advantages of cascade reactions related to more environmental friendly reaction conditions and the shifting reaction equilibria, there is a third reason to explore cascades for alcohol amination. The necessity to first convert alcohols to keto compounds or aldehydes that can undergo the actual amination reaction implies an oxidative step preceding amination. A transketolase reaction will also yield a keto substrate that may

act as acceptor (discussed above), but that changes the carbon skeleton and starts with a keto compound. Although oxidase reactions for alcohol oxidation which use molecular oxygen and generate hydrogen peroxide are possible, most alcohol oxidation reactions employ dehydrogenases that use an electron-accepting nicotinamide as cofactor. Cofactor recycling is then a must, which can be done by coupling to an reductive conversion or mediated by an oxidase activity (126, 127).

Strategies to overcome enzyme-related kinetic or stability problems in aminotransferases are directed evolution and rational redesign (119). Impressive studies aimed at engineering more stable and active aminotransferases for use in production processes have appeared in recent years. For example, Savile and coworkers (120) used multiple rounds of directed evolution to drastically widen the substrate range of a PLP fold type IV aminotransferase from *Arthrobacter*. The enzyme active site was gradually widened to allow binding of a bulky ketoamide shown in Scheme 18, which is not at all accepted by the wild-type enzyme. Resistance to harsh process conditions was also improved. The result was an aminotransferase that can be used for the production of the antidiabetic compound sitagliptin.

Engineering studies aimed at broadening the aminotransferases of the PLP fold type I enzymes have also been carried out. Prominent examples are the engineering of the selectivity of the aminotransferases from *V. fluvialis* to obtain an enzyme for the synthesis of the neuroactive compound imagabalin, which is a potential drug for treating anxiety disorders (121). The *V. fluvialis* AT was engineered by the group of Bornscheuer (122) who also investigated a somewhat related AT from *Ruegeria* (123). Remarkable progress also has been made with respect to stability improvement. Whereas single mutations discovered by FoldX energy calculations gave some improvement of a β -amino acid aminotransferase from *Variovorax paradoxus* that was studied earlier in our lab (124), larger increases in stability were achieved by introducing mutations that improve cofactor binding in a Fold type I class III AT that was discovered in a metagenomic library (125). Such stabilized enzymes may be more suitable than wild-type variants in cascade reactions employing high concentrations of amine donor to shift reaction equilibria. The use of high substrate concentrations is challenging but important for success in many biocatalytic conversion processes.

AIMS AND OUTLINE OF THIS THESIS

Aminotransferases are highly useful enzymes for the production of non-chiral and chiral amines. Many potential advantages are associated with the use of enzymes as catalysts in amination reactions, such as benign process conditions and high catalytic selectivity. The implementation of aminotransferases in industrial processes can also reduce the number of required purification steps and waste production, increasing the yield and contributing to overall process efficiency.

As with other biocatalytic processes, the screening of existing enzymes and the discovery or engineering of known enzymes are key steps in the development of new conversions. The work described in this thesis aims to identify and characterize novel aminotransferase activity, to explore computation-supported engineering of an aminotransferase for improving stability, and to gain insight in the application of aminotransferases in an enzymatic cascade reaction for alcohol amination. The work is devoted to ω -aminotransferases that act on aldehydes, which poses additional challenges because of their reactivity.

Chapter 2 of this thesis describes an exploration of the caprolactam degradation pathway. Microbiological enrichment of soil organisms with caprolactam as growth substrate was performed to find organisms possessing a degradation pathway that includes ω -aminotransferase activity. Two novel enzymes involved in the caprolactam degradation were identified in a strain of *Pseudomonas jessenii* that grows on caprolactam. The activity and putative function of both enzymes are discussed.

Chapter 3 is dedicated to a detailed biochemical characterization of the PLP fold type I aminotransferase from the caprolactam degradation pathway found in *P. jessenii* described in Chapter 2. The substrate profile of the novel enzyme was studied and the crystal structure was elucidated.

A computational approach to improve the stability of the aminotransferase of *P. jessenii* is presented in **Chapter 4**. Because of the low storage and operational stability of the enzyme at higher temperatures and high salt concentrations, the FRESCO protocol was applied to find stabilizing mutations. Although variants with improved stability were found, the strategy is not yet optimal.

In **Chapter 5** we examine the thermodynamic aspects of an ammonia-driven enzymatic cascade, which includes the aminotransferase of *Chromobacterium violaceum*. Equilibrium calculations were carried out finding that the cofactor recycling reaction and high ammonia concentration drive the overall conversion of ether alcohols towards the aminated product.

Finally, in **Chapter 6** a summary of the results obtained in this thesis is presented. We discuss the importance of aminotransferases in industrial processes and the features of the enzymes which still need further improvement.

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