CHEMICAL REVIEWS

Review

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Application of ω-Transaminases in the Pharmaceutical Industry

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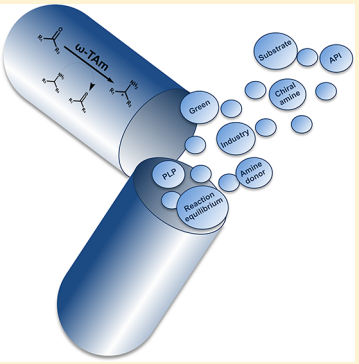
ABSTRACT: Chiral amines are valuable building blocks for the pharmaceutical industry. ω-TAms have emerged as an exciting option for their synthesis, offering a potential "green alternative" to overcome the drawbacks associated with conventional chemical methods. In this review, we explore the application of ω-TAms for pharmaceutical production. We discuss the diverse array of reactions available involving ω-TAms and process considerations of their use in both kinetic resolution and asymmetric synthesis. With the aid of specific drug intermediates and APIs, we chart the development of ω-TAms using protein engineering and their contribution to elegant one-pot cascades with other enzymes, including carbonyl reductases (CREDs), hydrolases and monoamine oxidases (MAOs), providing a comprehensive overview of their uses, beginning with initial applications through to the present day.



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1. INTRODUCTION

1.1. Background

The pharmaceutical industry has benefited from the widespread application of biocatalysis in recent years.[1−5] Chemical approaches to pharmaceutical production often have a number of drawbacks, including harsh reaction conditions, the need for toxic transition metal catalysts and volatile organic compounds (VOCs), primarily solvents, and insufficient stereoselectivity in a single step.[6] As well as possessing excellent enantioselectivity, the ability of enzymes to operate in aqueous media and at ambient temperature and pH[7] mean they are seen as a potential "green alternative" to metal-catalyzed reactions.[8] Increasing awareness of environmental issues and the search for renewable substitutes indicates the growth of biocatalysis in the pharmaceutical industry looks set to continue. The global market for industrial enzymes is expected to reach nearly $7.1 billion by 2018, representing a five-year compound annual growth rate (CAGR) of 8.2%.[9]

Chiral amines are one such example of compounds whose production has benefited from the use of biocatalysis. It is estimated that 40% of current pharmaceuticals contain an amine functionality,[7] making these compounds valuable building blocks in the pharmaceutical industry. The most recognized chemical method for chiral amine production is via hydrogenation of a Schiff base,[10] although other methods have also been employed, including diastereoisomeric crystallization, C−H insertion, and nucleophilic addition.[7] Biocatalytic approaches to the production of optically active amines initially involved hydrolases,[11] although

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more recently other enzymes such as lyases, oxidases, and transaminases (TAms) were studied for this purpose.[12]

TAms are enzymes which catalyze the transfer of an amine group from an amine donor to a ketone or aldehyde.[7] In nature, the transfer of an amine group from a α-amino acids to α-keto acids is the primary role of transaminases, with the ubiquitous occurrence of transaminases showing the vital role they play in the nitrogen metabolism of all organisms.[13,14] Several excellent reviews have examined transaminases from a variety of perspectives,[6,7,12,15−21] showing these enzymes to be highly versatile biocatalysts for the synthesis of chiral amine and α-amino acids.[22] Celgene spearheaded the use of TAms to resolve racemic amines as well as direct chiral synthesis in an industrial setting in the late 1980's.[23] Both (*R*)- and (*S*)-selective TAms, capable of aminating prochiral ketones to a number of phenyl methylamines in >90% yield, were developed. This work was advanced in an academic setting by the work of Shin and Kim, beginning in the 1990's.[24−27]

This review will focus specifically on the use of TAms for the production of chiral amines for the pharmaceutical industry. This includes chiral amines as both active pharmaceutical ingredients (APIs) and as intermediates in API syntheses, providing a comprehensive overview of their uses from their initial application in the late 1980's to the present day.

1.2. Reaction Mechanism

TAms require the vitamin B_6 derivative pyridoxal-5′ phosphate (PLP) **1** as a cofactor. PLP is extremely versatile, with its electron sink nature enabling a vast array of chemistry. This ability to delocalize the excess electron density surrounding the deprotonated α-carbon of the reaction intermediate allows PLP to act as a cofactor in reactions, including transamination, decarboxylation, racemization, elimination, substitution, and ring opening.[28] The TAm reaction is divided into two half reactions: oxidative deamination of an amine donor and reductive amination of an amine acceptor[6] (Figure 1). In the first half of the reaction, the

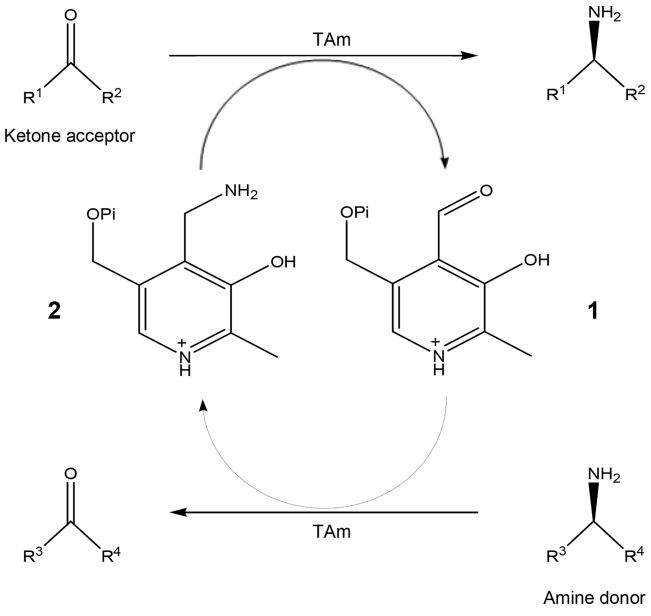

Figure 1. General schematic of a TAm-catalyzed reaction, highlighting the role of PLP as cofactor.[7]

amine group is transferred to an enzyme-PLP (E-PLP) complex, resulting in the generation of a pyridoxamine-5′ phosphate (PMP) **2** form of the enzyme (E-PMP) and corresponding ketone. In the second half of the reaction, the amine group of PMP is transferred to an acceptor substrate, leading to the formation of an amine and the regeneration of PLP.[6] The regeneration of PLP means that only catalytic amounts of this cofactor are required. This reaction is also readily reversible, with the extent of conversion based on which reactants are in excess. A quantum chemical study of the reaction mechanism of ω-TAm from *Chromobacterium violaceum* was carried out by Cassimjee et al., providing an in-depth look at the half-transamination of (*S*)-1-methylbenzylamine (MBA) to acetophenone and characterization of the associated intermediates.[29]

The architecture of the TAm active site was proposed by Shin and Kim in 2002 based on the relationship between substrate structure and reactivity, using a transaminase from *Vibrio fluvialis* JS17.[30] A two-site binding model consisting of a large (L) and small (S) pocket was proposed to explain substrate specificity and stereoselectivity. Key factors for activity were deemed to be the recognition of both hydrophobic and carboxylate groups by the L pocket, while the S pocket repelled carboxylate groups strongly. The S pocket was found to be critical in substrate recognition, as steric constraint at this part of the active site prevented bulky substituents from binding. Further work by Park and Shin in 2011 suggested active site residues in the S pocket were involved in binding but not significantly in the catalytic step.[31] Using this reasoning, the S pocket could be redesigned to relieve steric constraint and thereby accept more bulky substituents for catalysis.

1.3. Classification

TAms (EC 2.6.1.x) can be classified as either α-transaminases (α-TAms) or ω-transaminases (ω-TAms) based on the position of the amine group being transferred relative to the carboxyl group of the substrate.[32] α-TAms require the presence of a carboxylic group in the α- position to the carbonyl functionality.[7] Hence, α-TAms only allow for the formation of α-amino acids.[33] ω-TAms represent all other TAms (i.e., those in which at least one of the two substances is not an α-amino acid or α-keto acid).[34] ω-TAms are able to aminate keto acids, aldehydes, and ketones.[30] In fact, they can in principle accept any ketone or amine and are therefore considered much more useful and generally of higher interest to the pharmaceutical industry.[7,35] They have the advantage of a higher equilibrium constant versus their alpha- counterparts and possess many benefits over hydrolase and dehydrogenase enzymes, including broad substrate specificity, high enantioselectivity, and no requirement for cofactor regeneration.[24,26,30] ω-TAms have been subdivided into two further groups, β-TAms and amine transaminases (ATAs), the latter proposed by the Bornscheuer group and often used as a synonym for ω-TAms.[36] ω-TAms have been shown to possess excellent regioselectivity, exhibiting the ability to selectively convert a single ketone moiety in a di- or triketone system in a more complex molecule.[37,38]

TAms can also be classified under the umbrella of PLP-dependent enzymes, based on fold-type and alignment of amino acid sequences.[39−41] PLP-fold types I (aspartate aminotransferase superfamily) and IV (D-alanine transaminase family) contain TAms. As well as using PLP-fold types, TAms can be divided into six classes based on structural features and sequence similarity.[42] Subgroups I, II, and IV are all α-TAms, whereas subgroup III contains only ω-TAms.

The following sections will focus exclusively on ω-TAms and their application in the production of APIs and their intermediates. A number of concepts will be explored regarding

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process considerations for TAm-catalyzed reactions. The combination and application of these concepts will be illustrated using pertinent examples from the pharmaceutical industry, with ω-TAms playing a pivotal role in each case.

2. PROCESS CONSIDERATIONS FOR USING TAMS IN CHIRAL AMINE SYNTHESIS

2.1. Types of TAm-Catalyzed Reaction

Production of chiral amines by ω-TAms can be brought about via different approaches, namely kinetic resolution (KR) of a racemate and asymmetric synthesis from the corresponding prochiral ketone.[5]

Using an enzyme's enantiopreference to selectively act on one isomer of a racemic mixture, KR can be used to produce enantioenriched compounds (Figure 2A). This results in a maximum theoretical yield of 50% of the desired isomer. Direct asymmetric synthesis of a chiral amine from its corresponding prochiral ketone however can produce a theoretical yield of up to 100%[34] (Figure 2B). As such, asymmetric amination of ketones should be preferred to the KR of amines[22,43,44] and direct synthesis is highly desired in pharmaceutical manufacture.[45] ω-TAms can be employed for either asymmetric synthesis of amines from the corresponding ketone or for the KR of amines.[7] ω-TAms are among a small number of enzymes capable of the stereoselective amination of ketones, further emphasizing their value in this area.

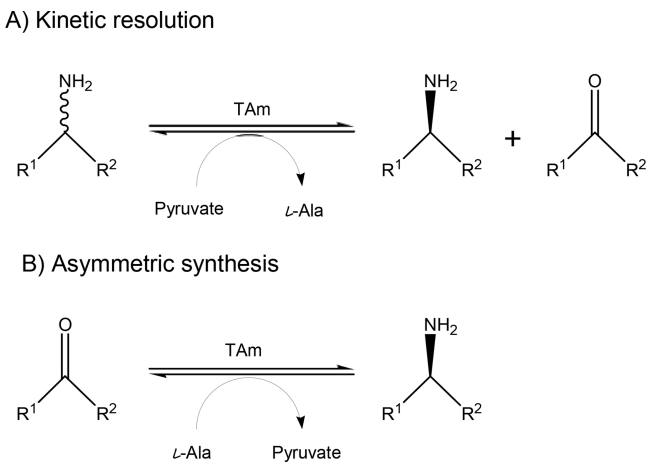

Figure 2. Reaction schematic showing (A) TAm-catalyzed KR of a racemic amine using pyruvate as an amine acceptor, and (B) asymmetric synthesis of a chiral amine from a prochiral ketone using L-alanine as an amine donor.

There are a number of recent examples involving ω-TAms in asymmetric synthesis of pharmaceutical intermediates. 17-α-Amino steroids are interesting non-natural products used as intermediates in the preparation of pharmacologically relevant steroids. An (R)-selective ω-TAm derived from *Arthrobacter* sp. was used to aminate a prochiral ketone substrate **3** to an amine intermediate **4** (Table 1), enabling a 9-fold yield increase and a reduction in processing steps from three to one versus the existing chemical approach.[46]

The same evolved variant of this enzyme was used in the asymmetric synthesis of the dual orexin receptor antagonist, suvorexant **5** (Table 1). Orexin A and B neuropeptides play a central role in sleep cycle regulation and antagonism has been found to promote sleep in a number of species.[47] Amination of a prochiral ketone **6** catalyzed by ω-TAm with subsequent ring annulation, afforded the diazepane ring shown in structure **7**, a key feature of suvorexant (Table 1). This obviated the need for a toxic transition metal catalyst and the use of dichloromethane as a solvent, significantly reducing the environmental impact of the process.[47] The intermediate for another dual orexin receptor antagonist, MK-6096, was prepared using an (R)-selective ω-TA. ATA-117 catalyzed the reaction for the synthesis of the required chiral lactam intermediate from a prochiral keto-ester. This reaction proceeded with full conversion to the (R)-amine **8** but perhaps more importantly demonstrated the scalability of a ω-TAm-catalyzed process to a multikilogram scale[48] (Table 1).

(R)-Selective ω-TAms have been employed in the asymmetric synthesis of a number of other intermediates for APIs, among them the antihypertensive dilevalol **9** and the potent bronchodilator formoterol[49] **10** (Table 3).

2.2. Choice of Amine Donor

Owing to its widespread acceptance by enzymes and the various options to remove the pyruvate coproduct, alanine has proven popular as an amine donor for TAm-catalyzed reactions.[16] However, the use of alanine results in an unfavorable reaction equilibrium, which is far on the side of the starting material.[16]

This has, in part, driven the search for other suitable amine donors, such as isopropylamine (IPAm) (Figure 3A). Its acceptable chemical price[50−52] and the ease with which byproducts can be removed, means the use of IPAm as a sacrificial amine donor represents a significant advance in improvement of conversion rates.[4]

The basicity of IPAm can lead to unwanted side reactions, as observed in the production of the antiallergic drug ramatroban[53] **11**. The subsequent use of the more sterically demanding (R)-(+)-α-methylbenzylamine (MBA) as amine donor led to a decrease in side product formation in the preparation of intermediate **12**, also avoiding the need for lipases and oxidoreductases in the process[54] (Table 1).

Recently, the O'Reilly group have developed a new generation of "smart" amine donors.[55,56] These diamine donors have the ability to form aminoaldehydes which can dimerize or cyclize following transamination. This effectively removes one reaction product from the mixture, helping to overcome product inhibition and shift reaction equilibrium toward further product formation. With one such donor, cadaverine **13** (Figure 3B), 94% conversion of acetophenone to MBA was achieved using enzyme ATA256, compared with only 44% when IPAm was employed as amine donor.[56]

Similar work was reported by Baud and Almac using the amine donor 2-(4-nitrophenyl)ethan-1-amine **14** (Figure 3B). On deamination to the corresponding aldehyde, an imine is formed which tautomerizes as a red precipitate.[57] As well as an improved reaction equilibrium, the color change brought about enables this reaction to be used as a high-throughput colorimetric assay for the detection of TAm activity.

In a recent study by Voges et al., the reaction equilibrium of the ω-transamination of the commonly applied amine donor (S)-MBA was investigated.[58] This characterized the influence of a number of factors on reaction equilibrium, including temperature, pH, and reactant concentrations.

2.3. Protein Engineering and in Silico Design

The bulky side groups[59] and inherently low reactivity of many ketones,[25] along with narrow substrate scope of wild type enzymes,[7] present other challenges for ω-TAm-mediated asymmetric synthesis. Such problems have led to the emergence of protein engineering of ω-TAms as a solution. Several excellent

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reviews have highlighted the advances in protein engineering of biocatalysts, among them TAms.[60−65]

One of the most successful example of ω-TAm use in the pharmaceutical industry, encompassing many of the concepts mentioned previously, is that of the antidiabetic drug sitagliptin **15**. In this case, a homologue of the (*R*)-selective ω-TAm ATA-117 from *Arthrobacter* sp. was used in the direct asymmetric synthesis of chiral amine sitagliptin from its corresponding prochiral ketone, prositagliptin[59] **16** (Table 1). Initially the enzyme was unable to bind to the substrate due to steric constraints caused by the bulky side groups of the prositagliptin ketone. Through use of in silico design and directed evolution, a substrate walking approach was

Table 1. Examples of Asymmetric Synthesis of Chiral Amine-Containing Pharmaceuticals from Prochiral Ketones Involving ω-TAms

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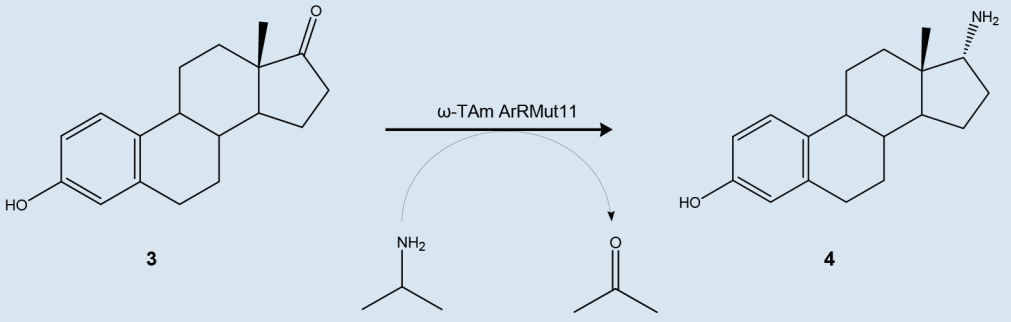
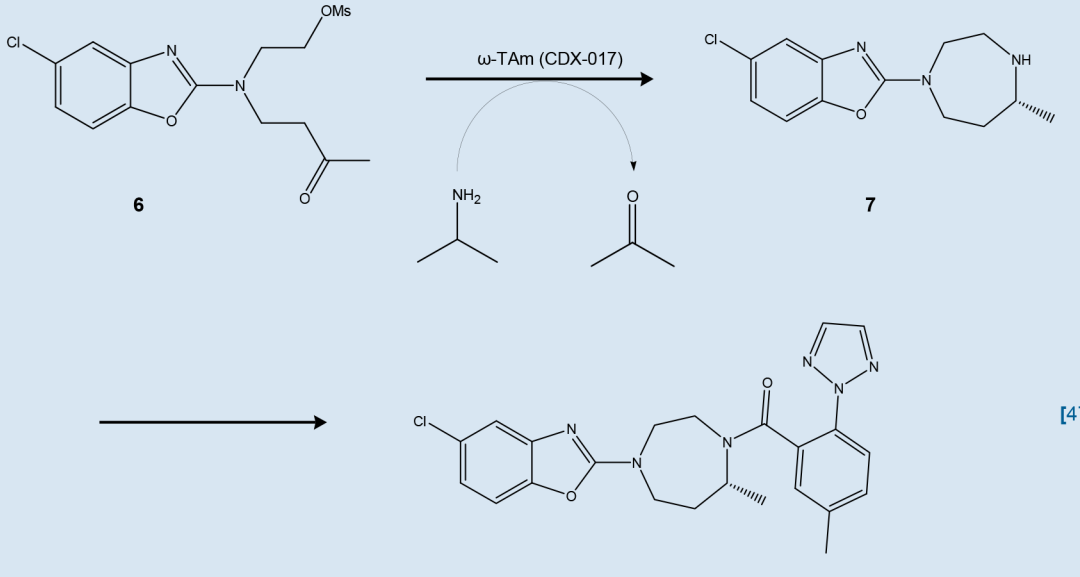
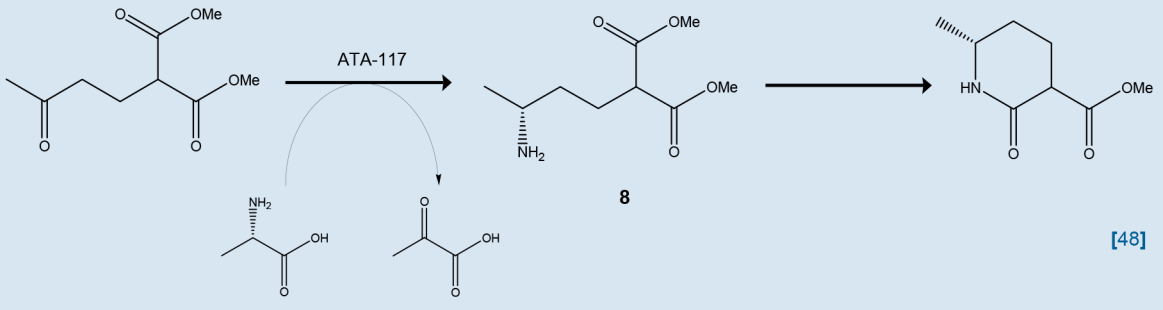
	[15]
	[47]
	[48]

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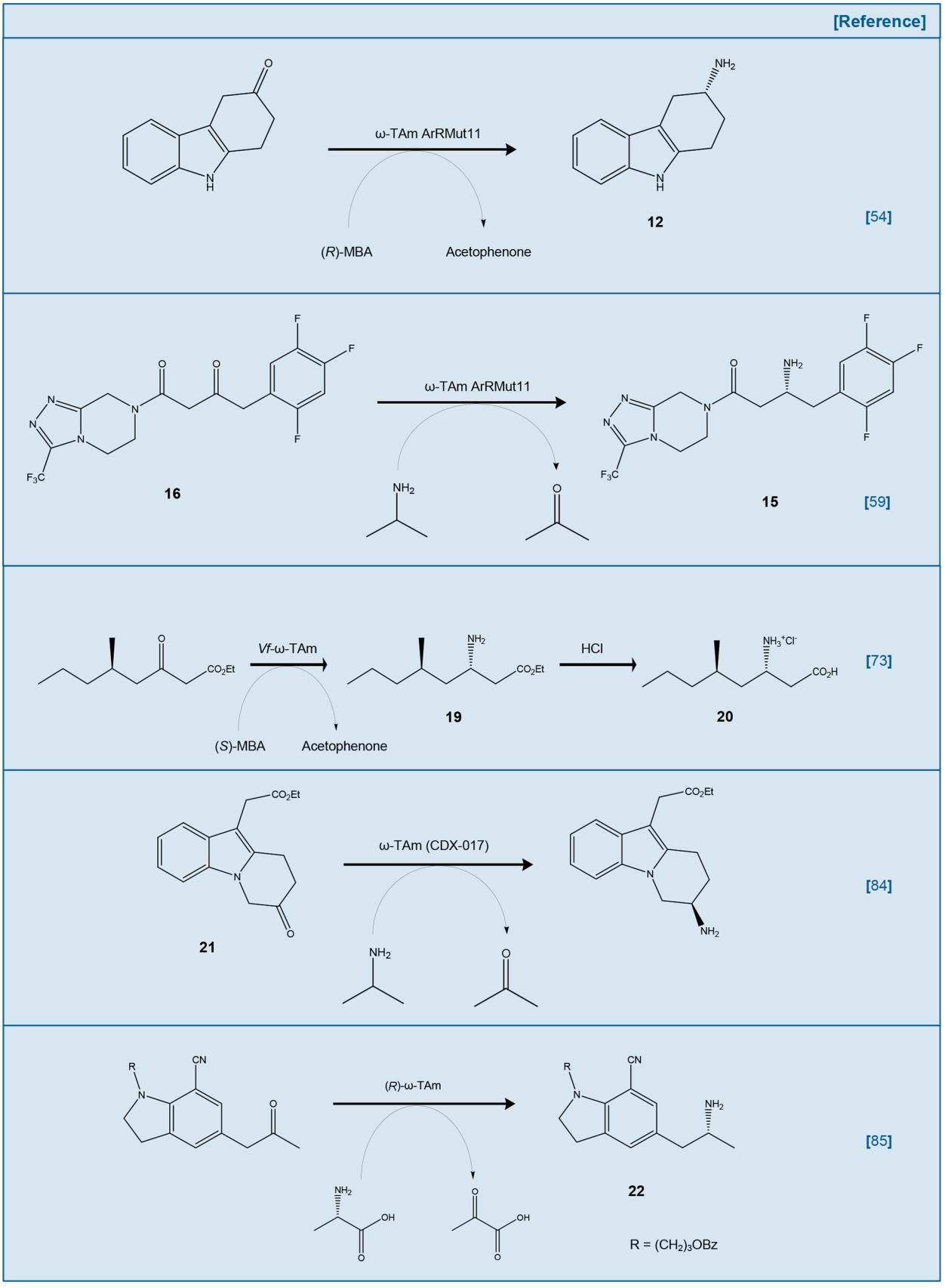

employed to engineer the large binding pocket of the enzyme's active site, with further evolution of the enzyme directed at improving activity toward prositagliptin.

The resultant effect was a reaction which proceeded with 92% yield (>99.95% *ee*), contributing to a process providing sitagliptin with a 10−13% increase in overall yield, 53% increase

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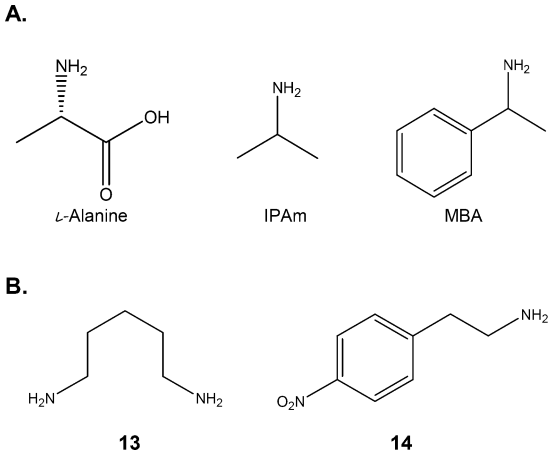

Figure 3. (A) Commonly used amine donor molecules. (B) Examples from the new generation of "smart" amine donor donor molecules.[56,57]

in productivity and 19% reduction in total waste. This ω-TAm-catalyzed system was able to replace the existing rhodium-catalyzed asymmetric enamine hydrogenation, negating the need for toxic heavy metals and expensive high pressure specialized hydrogenation equipment.[59]

This example also illustrates the use of IPAm as a sacrificial amine donor and subsequent removal of acetone coproduct, creating a more favorable reaction equilibrium.

This process was developed further by the same group with immobilization of this enzyme on a polymer resin. Again, activity in organic solvents was demonstrated, along with multiple rounds of enzyme reuse with minimal loss of activity.[66] Further investigation of the rational design involved in this example was undertaken by Guan et al.,[67] highlighting a specific target for rational design as a means of altering substrate specificity of ATAs.

Work by the Moody group demonstrated the applicability of rational protein engineering to an industrially relevant (*S*)-selective TAm from *V. fluvialis* (*Vf*-ω-TAm).[68] The wild-type enzyme showed no catalytic activity toward the bulky ketone 2-acetylbiphenyl. Using a combination of computational modeling and rational mutagenesis, *Vf*-ω-TAm was engineered to convert **17** to its corresponding amine **18** with 42% (>99% *ee*), representing >1716-fold increase in activity (Figure 4). By modeling the enzyme in the presence of the PMP intermediate and focusing on enlarging the large binding pocket, in total only 7 mutations were required to bring about these improvements. As well as being larger than the substrates studied in previous research, no activity had previously been shown toward ketone **17** by the well-characterized *Vf*-ω-TA. Critically, this work shows that initial weak promiscuous activity toward a substrate is not always necessary for enzyme evolution. Instead, selecting functionally relevant mutations and combining these with multistep rational mutagenesis can provide a more efficient pathway for protein engineering.[68] As well as providing an excellent example of expanding substrate scope in a currently scarce list of successes, this study could have a significant influence on the approach adopted for enzyme engineering for the biocatalysis industry going forward. Similar work was carried out by the Bornscheuer group, with rational protein engineering of key motifs allowing for increased activity of (*S*)-selective TAms toward substrates bearing bulky substituents.[69,70] The Berglund group have also demonstrated the ability of rational design to alter the enantiopreference of given TAms.[71,72] Such an approach could be used to help alleviate the need for novel (*R*)-selective TAms.

There are a number of other recent examples of the use of protein engineering of TAms to improve asymmetric synthesis of pharmaceuticals.[73−75] In one such example, *Vf*-ω-TAm was subjected to protein engineering in order to improve synthesis of

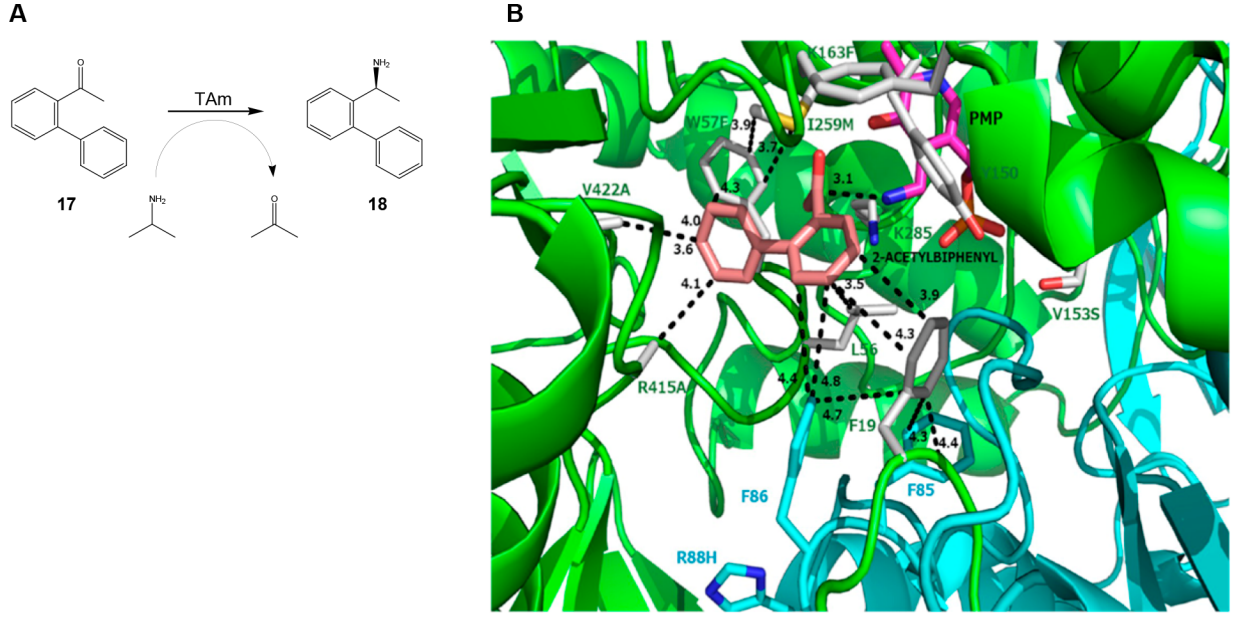

Figure 4. (A) Schematic showing (*S*)-selective TAm-catalyzed reaction converting 2-acetylbiphenyl **17** to (1*S*)-1-(1,1′-biphenyl-2-yl)ethanamine **18** employing IPAm as amine donor. **B.** MD reference structure of ketone **11** docked to *V. fluvialis* TAm with mutations W57F/R88H/V153S/K163F/I259M/V422A/R415A in the presence of PMP. The active center residues are represented by sticks with the carbons of chain A (green ribbon) colored in gray and the carbons of chain B (cyan ribbon) colored in cyan. Relevant distances are shown (in units of Å). The MD reference structure corresponds to the structure with lowest root-mean-square deviation (RMSD) (α-C atoms), relative to the average structure of the simulation. No significant changes were observed in the MD replicas.[68]

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(3*S*,5*R*)-ethyl 3-amino-5-methyloctanoate **19**, an intermediate for the anxiolytic imagabalin **20** (Table 1). The resultant effect was a significant optimization in selectivity and activity, shown by a 60-fold increase in initial reaction.[73]

Computational data mining has aided the discovery of novel biocatalysts. Most TAms published to date are (*S*)-selective, but an in silico approach using the Brookhaven protein database has yielded a number of (*R*)-selective TAms,[36] with experimental confirmation and application in asymmetric synthesis, demonstrating their usefulness as biocatalysts.[36,76,77] Undoubtedly in silico design of enzymes is a significant advance for the biocatalysis industry. Its limitations lie in the difficulty of accurately predicting function based on structure alone. This is pointedly true of PLP-dependent enzymes such as TAms. Christen and Mehta (2001) proposed that PLP binding developed first, and reaction specificity was evolved before substrate specificity.[78] Furthermore, genomic analysis of PLP-dependent enzymes showed there are fewer genes encoding PLP-dependent enzymes than there are PLP-catalyzed reactions required by an organism's metabolism.[79] Further studies confirmed reaction and substrate promiscuity occur across all PLP fold types,[80−82] highlighting that structure−function prediction can be complicated. In other words, some closely related enzymes do not always accept the same substrates, and some enzymes of very low sequence similarity sometimes do.[79] In that sense, while in silico data mining has proven to be extremely useful, its associated difficulties mean it is not as yet the silver bullet for novel biocatalyst discovery.

2.4. Further Improvements to the Process

A number of additional techniques have been incorporated into TAm-catalyzed syntheses with a view to optimizing systems for pharmaceutical production. In one such example, a ω-TAm-catalyzed process was developed for the production of a prostaglandin receptor CRTH2 antagonist, a potential therapeutic for allergic inflammatory diseases such as asthma and atopic dermatitis.[83] Screening of an in-house library of TAms developed for sitagliptin as discussed previously, allowed Merck to uncover an enzyme (CDX-017) capable of transaminating the relevant ketone intermediate **21** with 80−81% yield (98−99% *ee*)[84] (Table 1).

Optimization of this process was promoted by the use of a constant nitrogen sweep of the reaction vessel. This eliminated the formation of an oxidation byproduct arising from α-hydroxylation of ketone under basic conditions. As seen in other examples, the acetone byproduct was removed to improve reaction equilibrium. Furthermore, the reaction mixture was acidified following conversion in order to precipitate the enzyme and facilitate its removal via filtration. Use of ω-TAm contributed to a reduction in processing steps from 18 to 9, with an improvement in overall yield from 10% to 49%.[84]

The ability of TAms to retain activity in organic solvents is a commonly observed characteristic of these enzymes. Pro-drug silodosin **22** is used in the treatment of dysuria associated with benign prostatic hypertrophy (BPH). Biocatalytic asymmetric synthesis of a silodosin intermediate using ω-TAm from *Arthrobacter* sp. offered a more economically viable alternative to the current inefficient approaches requiring multiple crystallization steps.[85] However, as the ketone substrate was almost insoluble in water, DMF was used as a cosolvent to improve the reaction process and substrate loading. The stereochemical outcome was unaffected, affording the desired (*R*)-enantiomer in high yield (≥97%) and enantioselectivity (≥97%)[85] (Table 1).

2.5. Product Inhibition and Pyruvate Removal

Transamination reactions are not without drawbacks. Accumulation of coproduct inhibits the reaction from proceeding, rendering the process unsuitable at high concentrations.[24,27,32] In order to overcome this issue, strategies for removal of the ketone byproduct were investigated. The first technique introduced involved the use of an aqueous/organic biphasic reaction system, with the extraction capacity and biocompatibility of different organic solvents investigated for their ability to resolve α-methylbenzylamine (α-MBA).[24] A hollow-fiber membrane contractor was employed to further advance ketone extraction for the preparation of enantiopure arylalkyl amines.[27] Strategies employing other enzymes to remove the ketone coproduct have also been investigated. Many of these strategies focused on the removal of pyruvate, the ketone byproduct formed with the use of alanine as amine donor. The removal of pyruvate shifts the reaction equilibrium toward product formation and allows the reaction to proceed to completion. Lactate dehydrogenase (LDH) was used to convert pyruvate to lactate. However, as LDH requires the cofactor NADH, glucose dehydrogenase (GDH) and glucose were added to regenerate the cofactor and allow the reaction to continue[86] (Figure 5A). This system was modified by Truppo et al. to create a rapid and high throughput screening technique for ω-TAm activity.[87] In this example, a pH indicator dye (phenol red) was added, the gluconolactone produced from glucose and GDH caused a drop in pH and subsequent color change. The degree of color change correlated closely (within 5%) with the product formation from the ω-TAm-catalyzed reaction.[87] A number of similar examples have been developed which act as high throughput screens for TAm activity and have recently been reviewed by Mathew et al.[88]

Fuchs et al. developed a chemoenzymatic method for the preparation of (*S*)- rivastigmine **23** (Table 3), involving ω-TAm from *V. fluvialis* (*Vf*-ω-TAm), incorporating such an LDH/GDH + NADH recycling system for the removal of pyruvate.[89]

Rivastigmine is one of the most effective medications used to treat patients with Alzheimer's disease as well as dementia associated with Parkinson's disease, with the desired cholinesterase inhibition brought about by the (*S*)-enantiomer. The use of (*S*)-selective *Vf*-ω-TAm allowed for the desired isomer in optically pure form to be prepared with the shortest route, replacing previously more complex syntheses.

Also in this study, an (*R*)-selective ω-TAm (ATA-117) was used in place of *Vf*-ω-TAm to produce (*R*)-rivastigmine, demonstrating the flexibility in enantioselectivity ω-TAms can afford. Moreover, this example highlights the approach of combining biocatalysis with common chemical transformations, a phenomenon which is on the increase.[90−95] Several reviews focus on chemoenzymatic applications in the pharmaceutical industry.[4,96,97]

The same group improved (*S*)-rivastigmine production via introduction of a carbamate pharmacophore early in the process, avoiding the need for protective group strategies. In this case, a novel ω-TAm from *Paracoccus denitrificans* was needed to convert the prochiral ketone substrate, 3-acetylphenyl ethyl-(methyl)carbamate, to the corresponding amine intermediate in 99% *ee* and >80% conversion.[98]

Alanine dehydrogenase (AlaDH) has also been extensively employed to convert pyruvate back to alanine,[51,92,99−102] having the double effect of not only removing pyruvate to create a more favorable reaction equilibrium but also regeneration of the amine donor. A recent publication combining an alanine racemase (AlaR) enzyme with AlaDH, allows for recycling of pyruvate to

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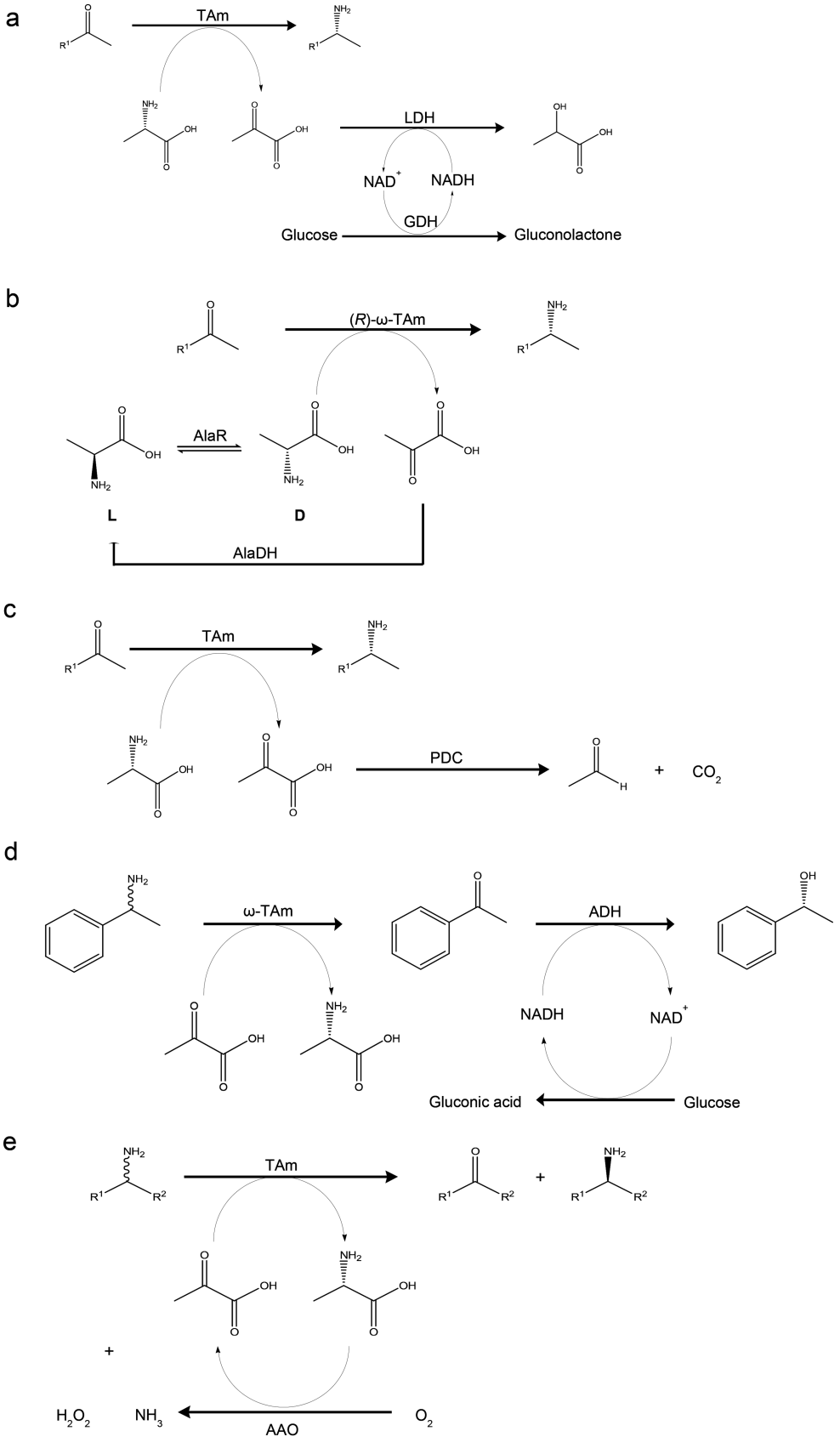

Figure 5. (A) Pyruvate removal employing a lactate dehydrogenase (LDH)/glucose dehydrogenase (GDH) system.[77] (B) Pyruvate recycling and alanine racemization using alanine dehydrogenase (AlaDH) and alanine racemase (AlaR), respectively.[92] (C) Pyruvate conversion to acetaldehyde and carbon dioxide via a pyruvate decarboxylase-catalyzed (PDC) reaction.[93] (D) Conversion of acetophenone product to (*R*)-1-phenylethanol using an alcohol dehydrogenase (ADH)/glucose dehydrogenase (GDH) coupling reaction.[94] (E) Resolution of a racemic amine using a TAm with catalytic quantities of pyruvate and an amino acid oxidase.[95]

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L-alanine, followed by conversion to racemic alanine[103] (Figure 5B). In this way, the more expensive D-alanine can be generated from the racemization of the cheaper L-alanine, allowing aminations catalyzed by (*R*)-ω-TAms.[103]

The combination of ω-TAms with pyruvate decarboxylase (PDC)[104] for asymmetric synthesis represents another option for improving product formation. This system possesses the added advantages that cofactor recycling is not required, as well as an irreversible shift in equilibrium due to loss of the carbon dioxide byproduct[104] (Figure 5C). A three enzyme system consisting of ω-TAm, alcohol dehydrogenase (ADH), and GDH was also employed for the KR of α-MBA, resulting in optically pure (*R*)-MBA and (*R*)-1-phenylethanol[105] (Figure 5D).

Another elegant approach to minimize pyruvate concentration was reported by Truppo et al., employing an amino acid oxidase (AAO) along with an ω-TAm[106] (Figure 5E). This example differed from those previously mentioned, in this case actively adding pyruvate for use as an amine acceptor to the reaction mix, as opposed to its formation as a byproduct. A high concentration of pyruvate would still inhibit the enzyme and lead to an unfavorable equilibrium. Through use of an AAO, the alanine formed could be recycled back to pyruvate, meaning only catalytic amounts of pyruvate would be needed.

Undoubtedly enzymatic removal of inhibitory byproducts is a highly useful and successful method of manipulating reaction equilibria to increase product formation. This has not only been applied to TAm-catalyzed reactions but also in a multitude of biocatalytic reactions with other enzyme classes.[107] As we have seen when IPAm is used as an amine donor, there are other methods for removal of unwanted side products. Shin and Kim introduced a biphasic reaction system for removal of coproduct in the asymmetric synthesis of unnatural amino acids using an ω-TAm from *V. fluvialis* JS17.[108] Using 2-oxobutyric acid as a starting point, L-2-aminobutyric acid was synthesized utilizing benzylamine as an amine donor. The choice of benzylamine meant benzaldehyde was formed, consequently leading to potent product inhibition. This was removed through use of a biphasic reaction system, with hexane employed as an extractant for benzaldehyde, allowing the reaction to continue and greatly increasing conversion rates from 39 to 96%.[108] The unnatural amino acid produced could be applied in the synthesis of levetiracetam **24** (Table 3), an anticonvulsant drug used for the treatment of epilepsy.

2.6. Simple KR and Deracemization (Dynamic Kinetic Resolutions and Other)

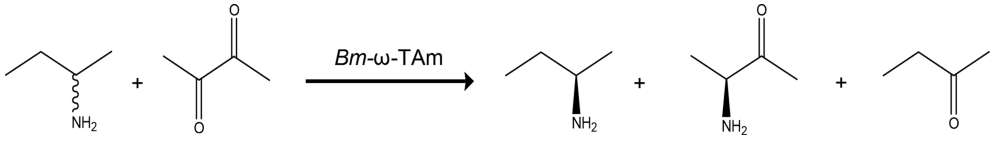
The relatively modest yields associated with KRs (max theoretical yield of 50% vs up to 100% by asymmetric synthesis) represent an economically more undesirable use of ω-TAms. Despite this drawback, simple KRs have been applied to the production of chiral amines from a racemic starting point, such as the production of (*R*)-*sec*-butylamine 25 (Figure 6) and (*R*)-1-cyclopropylethylamine.[109] These molecules are important intermediates for corticotropin releasing factor (CRF-1) antagonists, which have been proposed as novel therapeutics for the treatment of depression and anxiety.[110−113] Use of an (*S*)-selective TAm from *Bacillus megaterium* was able to produce the above (*R*)-isomers via KR, proceeding with 46% yield (theoretical max 50%) and 99% *ee*.[109]

Development and improvement of these processes to enable full conversion of a racemate to a single enantiomer has led to important applications in the pharmaceutical industry.[114−116] Conversion of the unreacted isomer back to the racemate, either actively or spontaneously, allows further resolution, producing the desired enantiomer. This process, known as dynamic kinetic resolution (DKR), is an example of deracemization, producing a theoretical yield of up to 100%.[117] DKR has been used to produce intermediates of 3-arylGABA (γ-aminobutyric acid) derivatives,[114] which are known to play an important role in the central nervous system. DKR of 4-oxo-3-phenylbutyric acid ethyl ester **26** by the (*R*)-selective ATA-117 TAm was used to form (*R*)-4−22.5 phenylpyrrolidin-2-one **27**, an intermediate to a 3-arylGABA derivative[114] 28 (Figure 7).

DKR was also employed in the production of an intermediate **29** to niraparib, a poly(ADP-ribose)polymerase inhibitor currently under development for the treatment of ovarian cancer.[115] As well as being the first kilogram-scale production of the niraparib molecule, this example also shows the use of ω-TAm in combination with C−N coupling, as well as using an aldehyde as a substrate for the transaminase reaction (Figure 8).

The application of DKR in conjunction with asymmetric synthesis is exemplified by a process developed by Pfizer, in which a TAm is used to produce an intermediate molecule **30** for a smoothened receptor (SMO) inhibitor[118] **31**. SMO is a receptor in the hedgehog (Hh) signaling pathway, and its inhibition represents a viable therapeutic target in the treatment of a range of human cancers. Despite the compound's excellent potency and bioavailability as an oral preparation, production processes suffered from both safety concerns and difficulty in scale-up. Enzymatic amination once again provided an alternative to traditional chemical synthesis and an ability to circumvent these shortcomings. The commercially available ω-TAm, ATA-036, was able to aminate a 4-piperidone precursor **32** with DKR, producing the penultimate intermediate to an SMO inhibitor. The reaction proceeded with an 85% yield and excellent enantioselectivity for the desired (2*R*,4*R*)-amine (>99% *ee*)[118] (Figure 9).

Deracemization can also be brought about by employing stereocomplementary enzymes. For example, an (*R*)-selective TAm acts on a racemic amine mixture forming a ketone product and the unreacted (*S*)-isomer. A similar enzyme with the opposite enantiopreference [i.e. (*S*)-ω-TAm] then converts this ketone product back into the (*S*)-isomer. In this way, the initial mixture has been deracemized, producing an enantioenriched (*S*)-isomer solution. Such a concept has been developed by a number of groups.[116,119]



25

Figure 6. KR of *rac-sec*-butylamine to (*R*)-*sec*-butylamine using ω-TAm from *B. megaterium*.[97]

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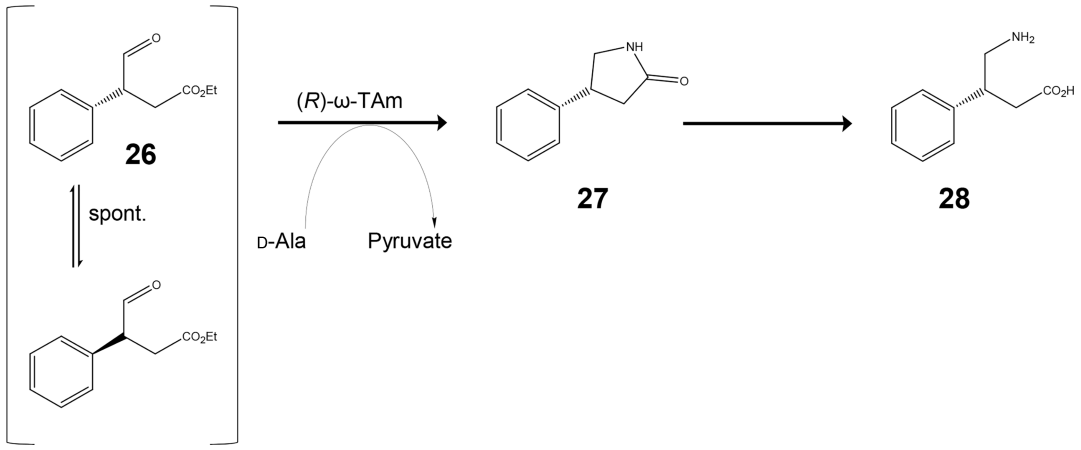

Figure 7. DKR by ω-TAm of 4-oxo-3-phenylbutyric acid ethyl ester to phenylpyrrolidin-2-one, an intermediate to a 3-arylGABA derivative.[102]

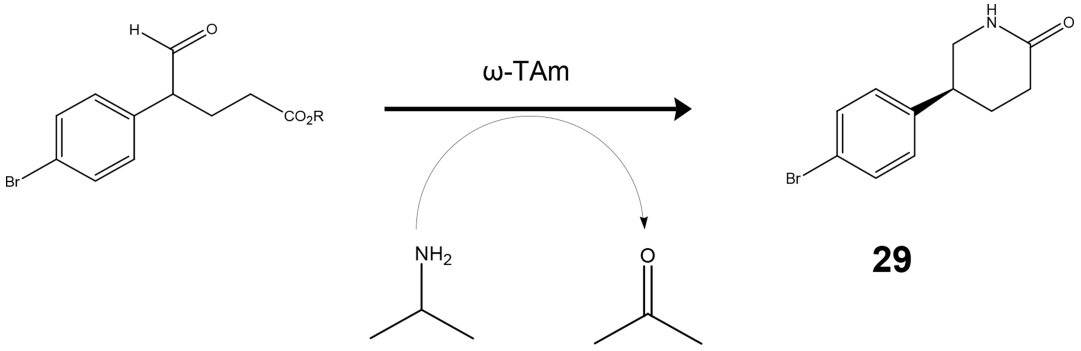

Figure 8. Formation of niraparib intermediate via ω-TAm-catalyzed DKR.[103]

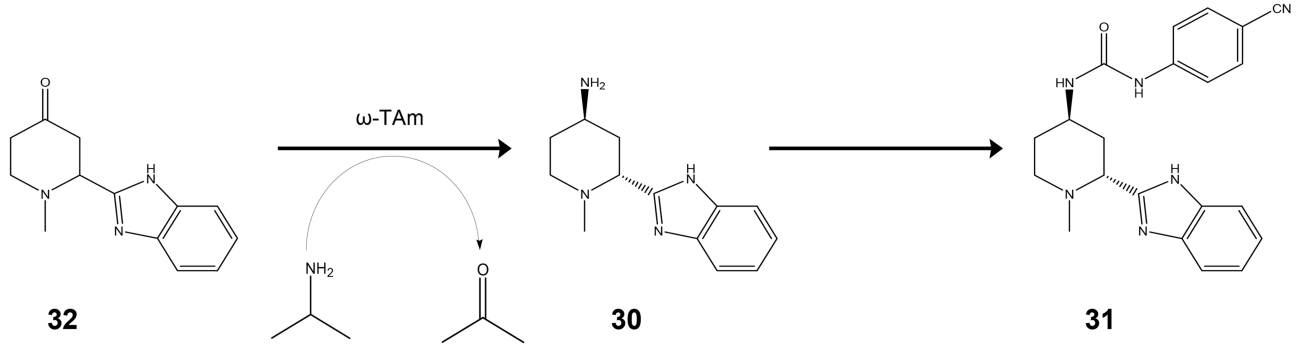

Figure 9. Preparation of an intermediate molecule for a smoothened receptor inhibitor via ω-TAm-catalyzed DKR.[118]

The Kroutil group developed a method using this approach for the deracemization of the chiral orally effective antiarrhythmic agent, mexiletine[52] **33**. Both the (*S*)- and (*R*)-isomers were prepared from the commercially available racemate 1-(2,6-dimethylphenoxy)-2-propanamine. Only (*R*)-mexiletine binds preferentially to cardiac sodium channels, and it was prepared in 99% *ee* with 97% isolated yield. ATA-117 was employed as an (*R*)-selective ω-TAm, with a number of (S)-selective ω-TAms used, from species including *C. violaceum*, as well as *V. fluvialis* and *B. megaterium*.

In order to produce (*R*)-mexiletine, an (*S*)-selective ω-TAm was used to produce the (*R*)-enantiomer from *rac*-mexiletine by KR, as well as forming the corresponding ketone via reaction with the (*S*)-enantiomer. This ketone was then converted back to (*R*)-mexiletine using the (*R*)-selective ω-TAm, ATA-117. If desired, (*S*)-mexiletine could be formed using the same method but by reversing the order in which the stereocomplementary ω-TAms were employed (Figure 10).

For the first reaction, pyruvate was used as an amine acceptor. The alanine formed was converted back to pyruvate using amino acid oxidase (AAO), meaning only small amounts of pyruvate would be needed. For the second reaction, when alanine was needed as an amine donor, two complementary systems were employed for pyruvate removal. AlaDH and LDH systems were both used in combination with an NADH recycling system. After the first reaction, a heat treatment was performed in order to avoid interference from stereocomplementary ω-TAms, before the enzyme for the second reaction was added. The efficiency of this step was improved further through immobilization of the ω-TAm used in the first step by encapsulation in a sol–gel/Celite matrix, and simply removing it prior to the second step.[120]

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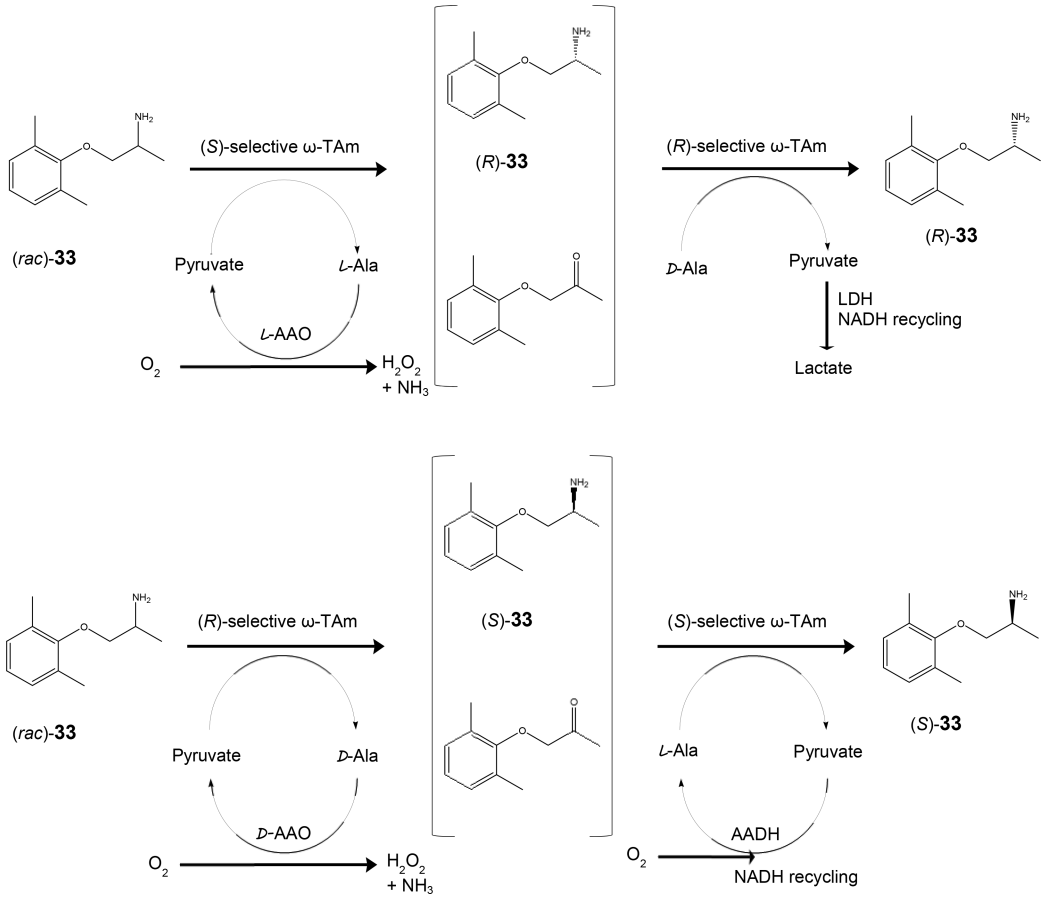

Figure 10. Deracemization of 1-(2,6-dimethylphenoxy)-2-propanamine (Mexiletine) to either the (*S*)- or (*R*)-isomer using ω-TAms.[49]

The combination of ω-TAms with other enzymes in multistep reactions can also be used for deracemization of amines. Using a stereoselective AAO, a racemic amine can be converted to a ketone and an unreacted enantiomer. A ω-TAm with opposite stereopreference can aminate the ketone product of the first reaction, resulting in a sample of optically active amine. A similar approach was used in the preparation of a key intermediate in glucagon-like peptide-1 (GLP-1) mimics, potential therapeutics for type II diabetes.[121] Using an (*R*)-AAO from *Frigonopsis variabilis* and an (*S*)-ω-TAm from *Burkholderia* sp., the desired (*S*)-amino acid product was prepared in 73% yield and 99.9% *ee*.[121]

An (*S*)-AAO from *Proteus mirabilis* in conjunction with an ω-TAm from *Bacillus thuringiensis* has been used to produce D-amino acids from a racemic mixture, using racemic alanine as an amine donor.[122] Such an approach has the potential to yield high value pharmaceuticals, with D-amino acids forming the building blocks of a number of therapeutic agents, including β-lactam antibiotics and calcitonin gene-related peptide antagonists for migraine treatment.[122]

3. CASCADE REACTIONS WITH OTHER ENZYMES

Examples combining TAms with common chemical steps (i.e. chemoenzymatic synthesis) have been discussed. TAms can also be combined with other enzymes, not only for process enhancements such as byproduct removal but for synthesis of desired compounds such as APIs as well. The coupling and combining of ω-TAms with other enzymes has progressed beyond mere multistep operations, replaced in many cases by elegant one-pot cascade systems. These processes have been refined to a high level of efficiency and are opening doors to new and exciting biocatalytic opportunities.[44] Such enzyme-initiated domino reactions have been reviewed in detail, including those where TAms play a pivotal role.[15,123−127]

One-pot cascade reactions allow for much greater versatility in the starting material used in the biocatalytic production of chiral amines. Instead of using chemical synthesis to manufacture a prochiral ketone, the advantages of biocatalysis can be exploited for this step as well. For example an alcohol **34** could be used as a starting material and oxidized to form a ketone using a galactose oxidase enzyme, as shown by Fuchs et al.[101] This afforded the prochiral ketone **35** at the expense of molecular oxygen, with only water formed as a byproduct (Table 2). Use of horseradish peroxidase (HRP) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) ensured over-oxidation was avoided. Using alanine as an amine donor, this provides another instance of pyruvate removal using AlaDH coupled with an NADH/FDH/GDH recycling system.

Depending on the alcohol substrate, aldehydes were often produced as a result of the oxidation reaction. These intermediates were duly aminated, further demonstrating the versatility of TAms. Due to the relative lack of information on amination of aldehydes versus their ketonic counterparts, a study of benzaldehyde amination was carried out to assess the suitability of a number of ω-TAms.[101] *Vf*-ω-TAm was deemed to be the most suitable, converting benzaldehyde to benzylamine with 96% yield. A series of benzyl and cinnamic alcohols were screened for oxidation and subsequent amination. The applicability of the process to pharmaceutical manufacture was demonstrated by the formation of the chiral amine 2-phenylallylamine, an intermediate molecule for the potent antifungal naftifine[101] **36** (Table 3).

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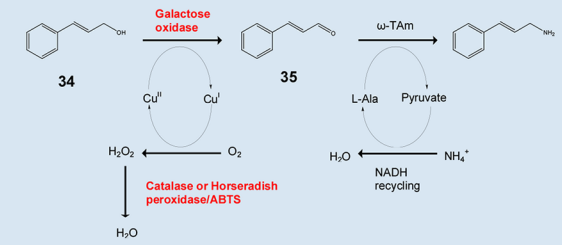
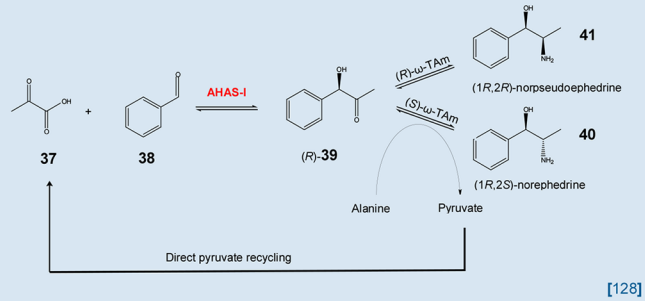
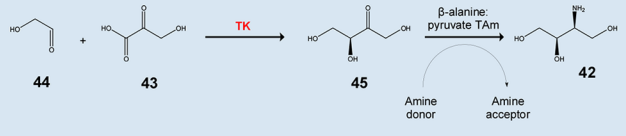
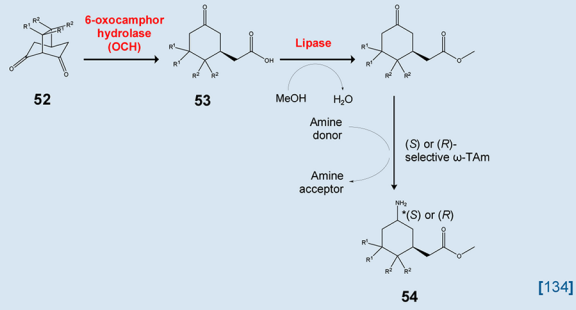
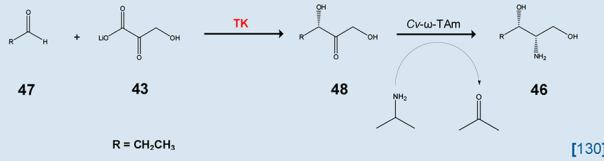
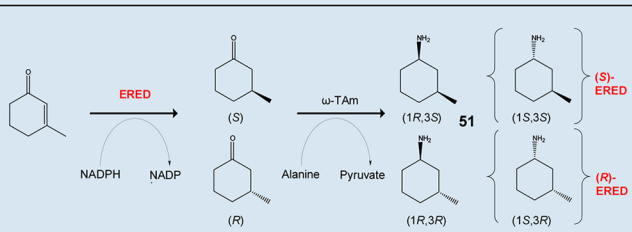
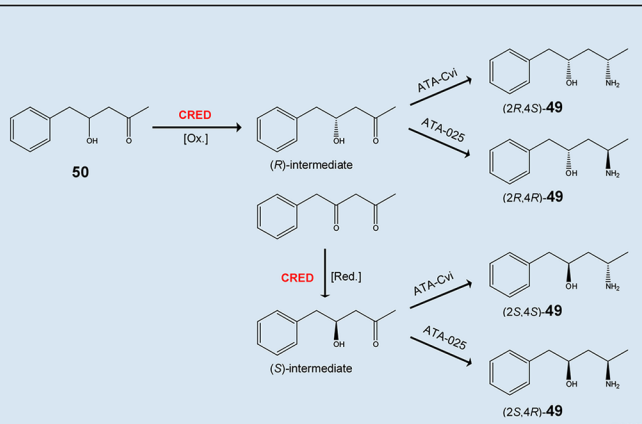
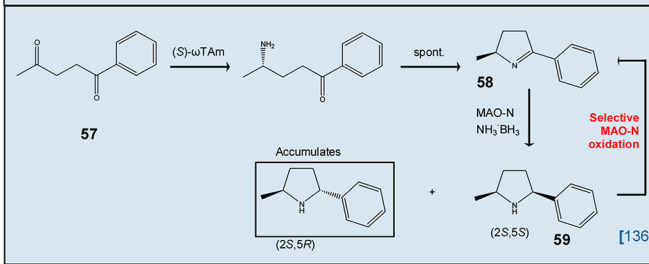
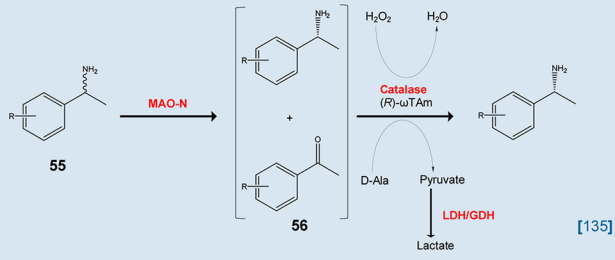
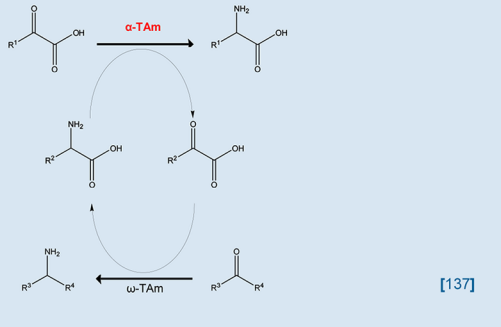
Table 2. Cascade Reactions in the Production of Pharmaceuticals Involving ω-TAms[a]

[Reference]	[Reference]
 [101]	 [128]
 [129]	 [134]
 [130]	
 [133]	 [132]
 [136]	
 [135]	 [137]

[a]Other enzymes in the cascade are highlighted in red.

The amination of starting materials other than prochiral ketones using one-pot cascades represents an important step forward in biocatalysis, providing exciting opportunities for chiral amine synthesis.

A similar concept was applied in the synthesis of both norephedrine and norpseudoephedrine, molecules used as a stimulant and decongestant.[128] With the use of benzaldehyde and pyruvate as substrates, an acetohydroxyacid synthase I

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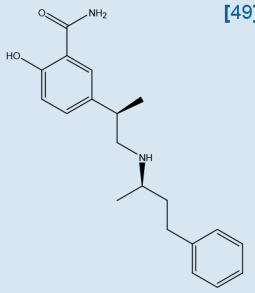
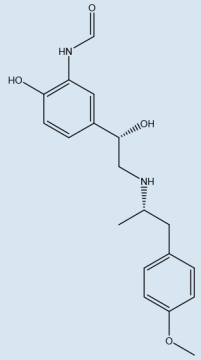
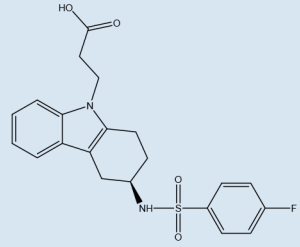
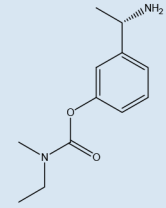
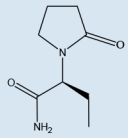
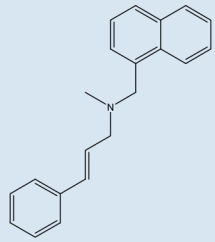
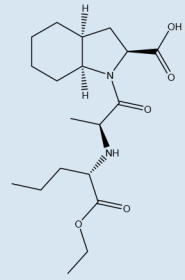
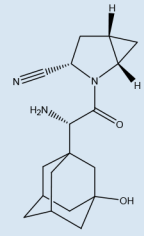
Table 3. Examples of APIs in Whose Syntheses TAms Have Been Applied

[Reference]	[Reference]	[Reference]	[Reference]
[49]  Dilevalol **9**	[49]  Formoterol **10**	[53]  Ramatroban **11**	[89]  (*S*)-Rivastigmine **23**
[108]  Levetiracetam **24**	[101]  Naftifine **36**	[137]  Perindopril **60**	[137]  Saxagliptin **61**

(AHAS-I) enzyme was combined with a TAm in a one-pot, two-step reaction. In the first step, pyruvate **37** is decarboxylated and subsequently ligated to benzaldehyde **38**, yielding (*R*)-phenylacetylcarbinol **39**. In the second step, an (*S*)-selective *ω*-TAm can be used to prepare (1*R*,2*S*)-norephedrine **40**, whereas an (*R*)-selective *ω*-TAm can be used to form (1*R*,2*R*)-norpseudoephedrine **41** (Table 2). By employing alanine as an amine donor for the amination reaction, an elegant system is created whereby the pyruvate byproduct, instead of being removed, is simply recycled for use in the lyase-catalyzed reaction.[128]

Using similar starting materials but employing a different enzymatic approach, a single diastereomer of 2-amino-1,3,4-butantriol (ABT) **42** was synthesized, starting from hydroxypyruvate **43** and glycoaldehyde[129] **44**. Amino alcohols such as ABT make excellent building blocks for many syntheses due to their dual amino-alcohol functionality, with the ABT moiety found in such pharmaceuticals as the protease inhibitor Nelfinavir.[129] In this case, a transketolase (TK) enzyme was combined with a TAm in a single *Escherichia coli* host. The TK-catalyzed C−C bond formation yielded L-erythrulose **45**, from which a *β*-alanine:pyruvate TAm could form the desired ABT[129] (Table 2).

A TK has also been combined with a TAm from *C. violaceum* for the formation of a pharmaceutically relevant amino alcohol[130] **46**. TK from *E. coli* was able to convert achiral substrates propanal **47** and hydroxypyruvate **43** to (3*S*)-1,3-dihydroxypentan-2-one **48**, which was subsequently converted to (2*S*,3*S*)-2-aminopentane-1,3-diol **46** in a TAm-catalyzed reaction using IPAm as amine donor[130] (Table 2).

Other amino alcohols have been employed as intermediates for antiviral glycosidase inhibitors and antibiotics such as chloramphenicol.[131] As well as TKs, carbonyl reductase enzymes (CREDs) have been combined with TAms in order to form these valuable building blocks. Kohls et al. reported a biocatalytic method using a CRED/*ω*-TAm cascade, allowing selective access to all four diastereomers of 4-amino-1-phenylpentane-2-ol[132] **49**. Using either a 1,3-diketone or a 1,3-hydroxy ketone (*β*-hydroxy ketone) **50** as a starting material, this method provided a step efficient approach to 1,3-amino alcohol production, avoiding the need for a transition metal catalyst. Depending on the selectivities of the respective CRED and *ω*-TAm chosen, any of the four diastereomers could be prepared[132] (Table 2).

The ability to manipulate individual stereogenic centers with carefully chosen biocatalysts is further exemplified in the work by the Bornscheuer group. In a cascade involving enoate reductases (EREDs), the enantiopreference of *ω*-TAms can be exploited to access the desired isomers of ring-substituted exocyclic amines.[133] It was shown that protein engineering of the active site of the *V. fluvialis* ATA brought about substantially improved selectivity toward either diastereomer, via amino acid mutation at a single site. Initially, the TAm used in this cascade showed only a modest preference toward the (*R*)-configuration (14% *de*). Mutation of the leucine residue at position 56 caused substantial changes in selectivity, with mutant Leu56Val exhibiting and higher (*R*)-selectivity (66% *de*), while mutant Leu56Ile caused a switch in preference toward the (*S*)-configuration (70% *de*). Moreover, addition of 30% DMSO increased selectivity further,

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producing (1*R*,3*S*)-1-amino-3-methylcyclohexane **51** with 87% conversion and 89% *de*[133](Table 2).

ω-TAms have been combined with both hydrolases in the production of 2-(3-aminocyclohexyl)acetic acid derivatives, valuable synthons for potential modulators of the multidrug resistant protein, MRP1.[134] In work carried out by the Kroutil group, bicyclic diketones **52** were hydrolyzed to keto acids **53**, which underwent lipase-catalyzed esterification before subsequent amination using a TAm to the desired 3-substituted cyclohexylamine derivatives[134] **54** (Table 2). As seen in previous examples, the enantiopreference of ω-TAm determined which diastereomer was formed, providing much more control versus chemical means involving an imine reduction. (*R*)-selective ArMut11-ω-TAm was able to produce the *cis* diastereomer with up to 91% conversion in 24 h (>99% *ee*). (*S*)-selective His-*Vf*-ω-TAm was used to prepare its trans counterpart, albeit at a slower rate of 71% over 96 h (>99% *ee*). Additionally, this study reported the ability of ArMut11-ω-TAm to provide up to 81% conversion over 24 h (>99% *ee*) in the presence of organic solvent diisopropyl ether (DIPE).[134]

The progression of such cascades to include multiple enzymes is illustrated exquisitely in a one-pot method for the deracemization of primary amines[135] **55**. In this example, primary amines are deracemized using (*S*)-selective monoamine oxidase (MAO-N) enzymes, in combination with an (*R*)-selective ω-TAm to aminate the ketone product **56** of the first reaction. In addition, a catalase enzyme is used as a peroxide scavenger, as well as employing the well-established LDH/GDH system for pyruvate removal and cofactor regeneration respectively[135] (Table 2). In all, this represents an elegant one-pot system combining five biocatalysts, without affecting conversion or stereoselectivity.

A number of examples illustrating the application of enzymes to produce prochiral ketones for TAm-catalyzed amination have been discussed. However, cascades have also been developed in which the TAm is involved in the initial step of the cascade, producing an intermediate which undergoes subsequent transformation by a different enzyme class.

The Turner group has developed another cascade involving an MAO-N and an ω-TAm. In this example, however, an ω-TAm aminates an achiral 1,4-diketone **57** the first step, with the resulting pyrroline **58** undergoing a chemoenzymatic conversion to a pyrrolidine **59** by MAO-N/NH_3·BH_3 in the second step[136] (Table 2). MAO-N also acts selectively on the (*S*)-C2 center of the pyrrolidine, converting it back to the imine for further nonselective reduction. In this way, the (*R*)-diastereomer at the C2 center accumulates, producing an optically pure pyrrolidine following repeated cycles. The diastereomer formed at the other stereocenter is determined entirely by the enantiopreference of the ω-TAm chosen for the first step. This approach allows for control of which diastereomer is formed, circumventing the difficulties associated with using imine reduction.[136]

ω-TAms have also been combined with α-TAms for the amination of α-keto acids in the production of unnatural amino acids[137] (Table 2). Challenges included finding a substrate which could simultaneously act as an amine donor for the α-catalyzed amination as well as an amine acceptor for ω-TAm-catalyzed reaction. Upon investigation, L-homoalanine and its deaminated derivative 2-oxobutyric acid were deemed to be the most effective option. Access to L-amino acids was achieved by combining branched-chain TAm from *E. coli* with an (*S*)-selective ω-TAm from *Ochrobactrum anthropi*, with the D-enantiomer accessed with D-amino acid transaminase from *Bacillus sphaericus* and (*R*)-selective ArRmut-ω-TAm.

A number of important bioactives were formed using this approach, including intermediates to the ACE inhibitor perindopril **60** and antidiabetic drug saxagliptin **61** (Table 3), as well as building blocks of antibiotics and drugs for the treatment of HIV.[137]

4. CONCLUSION AND OUTLOOK

It is clear that the use of TAms in pharmaceutical production has progressed significantly in recent years, evolving from simple KRs and asymmetric syntheses, through more complex multistep processes, eventually expanding to elegant multienzyme one-pot cascade systems. Ongoing research has facilitated the development of innovative methods for overcoming product inhibition, as well as systems with increased economic viability and the discovery of novel biocatalysts with previously unreported capabilities. Challenges within this area remain, however, with unfavorable reaction equilibria and limited substrate scope continuing to prove problematic. Rational protein engineering of biocatalysts and the advent of "smart" amine donors have contributed further to the potential of TAm-catalyzed production of pharmaceuticals. Novel enzyme discovery, encompassing both a metagenomic approach[138] and culture-based searches from previously untapped environments,[139,140] may help to expand the substrate scope and subsequent industrial applications of TAms. The need for an expanded toolbox of (*R*)-selective enzymes remains a challenge which may be overcome with continuing developments in rational design and the discovery of new biocatalysts. The increasing demand for efficient and green production of chiral amines in the pharmaceutical industry suggests that the growth in TAm use is set to continue. Currently, concepts for process-optimization have been combined and refined to create efficient enzyme-driven systems, ensuring TAms will continue to provide exciting opportunities for the biocatalytic industry for years to come.

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Stefan Mix was born in Berlin/East Germany, where he also completed his secondary education. After graduation with a Diploma in chemistry, he received his doctorate in 2004 from the Technical University of Berlin after working in the group of Prof. Siegfried Blechert on stereoselective synthetic methodology and olefin metathesis. He is the author of several publications and has been working with Almac Group since 2005. He has gained broad industrial experience including expertise in the fields of biocatalysis, crystallization development, process development for chiral building blocks and APIs, and technology transfer to manufacturing network partners. His hobbies include sailplane gliding, kayaking, and gardening.

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