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REVIEW

Biocatalysis for the asymmetric synthesis of Active Pharmaceutical Ingredients (APIs): this time is for real

Gonzalo de Gonzalo[a], Andrés R. Alcántara [b], Pablo Domínguez de María[c] and José María Sánchez-Montero[b]

[a]Departamento de Química Orgánica, Universidad de Sevilla, Sevilla, Spain; [b]Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain; [c]Sustainable Momentum, Las Palmas de Gran Canaria, Spain

ABSTRACT

Introduction: Biocatalysis has emerged as a powerful and useful strategy for the synthesis of active pharmaceutical ingredients (APIs). The outstanding developments in molecular biology techniques allow nowadays the screening, large-scale production, and designing of biocatalysts, adapting them to desired reactions. Many enzymes can perform reactions both in aqueous and non-aqueous media, broadening even further the opportunities to integrate them in complex pharmaceutical multi-step syntheses.

Areas covered: This paper showcases several examples of biocatalysis in the pharmaceutical industry, covering examples of different enzymes, such as lipases, oxidoreductases, and transaminases, to deliver active drugs through complex synthetic routes. Examples are critically discussed in terms of reaction conditions, motivation for using an enzyme, and how biocatalysts can be integrated in multi-step syntheses. When possible, biocatalytic routes are benchmarked with chemical reactions.

Expert Opinion: The reported enzymatic examples are performed with high substrate loadings (>100 g L^{-1}) and with excellent selectivity, making them inspiring strategies for present and future industrial applications. The combination of powerful molecular biology techniques with the needs of the pharmaceutical industry can be aligned, creating promising platforms for synthesis under more sustainable conditions.

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Biocatalysis; asymmetric synthesis; active pharmaceutical ingredients; sustainable chemistry

1. Introduction

Inspired by Nature, biocatalysis – the use of natural catalysts, namely enzymes, for organic synthesis – has become a mature field to perform environmentally friendly synthetic reactions. The field has evolved from being an academic curiosity to industrially applied syntheses, in which efficiency and sustainability go hand in intensified processes [1–11]. The exquisite selectivity of enzymes has fascinated chemists, as complex reactions can be handled without needing tedious protection or deprotection steps. Being enzymes composed of a sequence of (chiral) amino acids, the actual place in which (bio)catalysis occurs – the so-called enzyme's active site – results to be a multi-chiral cavity, in which enantio-, regio- and chemo-selective reactions take place. This is particularly important for pharmaceutical chemistry, where highly functionalized molecules demanding delicate approaches are frequently targeted [12–19]

The set-up of a biocatalytic process requires interdisciplinary research, covering areas like molecular biology, organic synthesis, process engineering, bioinformatics, and market analysis. This ensures the holistic management of the entire pipeline, from enzyme discovery to scale-up and market penetration (Figure 1).

Upon identifying a synthetic transformation that an enzyme class may perform; the first step is the selection of suitable enzymes (Figure 1). In particular, for pharmaceutical industry, the need for highly enantio- and chemo-selective biocatalysts is mandatory. The enzyme quest has traditionally started by screening readily available on-the-shelf enzymes (e.g. commercial biocatalysts) for the desired substrate, and analyzing the performance of the enzyme to conduct asymmetric reactions. In recent decades, however, this has been largely complemented (and improved) by using computational tools (e.g. genome databases), and more recently Artificial Intelligence (AI) to define the best protein scaffolds for the intended reaction [20,21]. Once various positive hits are selected – enzymes that can accept the substrate with the desired selectivity –, a genetic design program is executed to upgrade these biocatalysts. Through several rounds of genetic evolution, new variants displaying higher catalytic activity (toward the desired substrate), as well as higher stability in the reaction media, are obtained. These tailored variants will be the actual biocatalysts for the industrial biotransformation [22–24].

Apart from the biocatalyst, the synthetic step must be subjected to research as well (Figure 1). This includes process conditions (temperature, pressure, substrate loadings, etc.), as well as the choice of a reaction media. In nature, enzymes evolved for millions of years in aqueous conditions, and therefore, water is often the first choice for biocatalytic transformations. However, many enzymes can actually display catalytic performances in

CONTACT José María Sánchez-Montero jmsm@ucm.es Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal, s/n. 2804 Madrid, Spain. Tel. no. +34-3941820

Article highlights

- Biocatalysis is nowadays a mature and efficient technology for the synthesis of APIs.
- Biocatalytic reactions can be implemented in both aqueous and non-aqueous media, facilitating the integration of multi-step synthetic procedures.
- Enzymes can be tailored for specific applications, performed at high substrate loadings and with excellent selectivity.
- Education is key to train new generations of scientists to be aware of biocatalysis' advantages for industrial processes.

non-aqueous media – the so-called non-conventional media – and this has stimulated the implementation of biotransformations in solvent-free, in organic solvents, in neoteric media (e.g. ionic liquids or deep eutectic solvents), and even in supercritical fluids [10,25,26]. With the incorporation of the non-aqueous media, the enzymatic reaction can be coordinated with the entire (industrial) production pipeline, as well as with the solubility of the substrates, ease of downstream, etc. Moreover, the biocatalyst can be used either in the form of a free enzyme – which can be more or less purified – or as whole-cells, that is, using directly non-growing microorganisms hosting the desired enzyme. The final decision on whether to use enzymes or whole-cells is usually made on a case-by-case basis. Whereas whole cells tend to be cheaper and easier to be stored and used, in some cases secondary reactions (triggered by other enzymes present in the cell) may occur, as well as mass transfer limitations of the substrate or the product to enter or exit the cellular membranes. On the other hand, free enzymes do not present those challenges, but may become less stable in the reaction media, and their higher purification costs – namely cell disruption, protein precipitation, chromatographic steps, etc. – need to be considered. Moreover, when cofactors are needed (e.g. for oxidoreductases), their regeneration cycle must be taken into account, as the extremely high prices of cofactors hamper its use in stoichiometric amounts. Both, free enzymes and whole cells, can be immobilized to be heterogenized, to reuse them a certain number of cycles, enhancing their economic advantage and the process sustainability [25].

Once the enzyme, its form (free or whole-cells), and the reaction media are selected, the biocatalytic reaction is optimized and scaled-up (Figure 1). Optimizations are related to substrate and enzyme loadings, downstream processing and product recovery. Industrially sound reactions must be performed with high substrate loadings (for biocatalysis, typically higher than 100 g L^{-1}), with minimized enzyme loadings, and with an adequate cofactor regeneration. The downstream unit must be straightforward to achieve the purified product while generating the lowest amounts of wastes. For scaling-up, while enzymatic reactions have often been performed at (paid-off) in-house facilities, such as batch reactors, the last decade has witnessed the implementation of continuous flow-based systems, which lead to higher efficiency and less wastes [27]. From a different angle, the mild conditions applied for enzymatic processes may enable multi-enzymatic reactions, involving several biotransformations in the same reactor, minimizing the wastes associated to the downstream units [27,28].

To close the loop, an industrial biotransformation is developed because there is a commercial incentive, in the form of a market opportunity (Figure 1). The new process may either intend to replace a current existing one (e.g. one which uses hazardous chemicals, or one that it is not efficient enough, etc.), or may be created as a novel commercial route. Regardless the case, when envisioning modern synthetic methods – fully aligned with the Green Chemistry –, the new reactions cannot be assessed only from the commercial and economic standpoint. Rather, a holistic perspective must analyze also environmental metrics, the possible biogenic origin of the substrates and solvents, as well as the efficiency of the process and its inherent wastes. Furthermore, when it comes to API production, health issues associated with these compounds must be taken into account as well. Remarkably, the combination of highly intensified biocatalytic processes, using high substrate loadings and integrated conditions, lead to synthetic paths that can gather both economic and environmental aspects [10,18,29].

In particular, modern pharmaceutical synthesis is currently a complex picture in which holding a strong IP position may be necessary (e.g. fully covering the synthetic route, possible alternatives, and the drug use as well), but it is not sufficient to reach commercial success. The raw material costs, reaction efficiency, and waste production need to be considered too. Herein, biocatalysis has much to offer, and a broad number of industrial applications related to the synthesis of Active Pharmaceutical Ingredients (APIs) have been extensively documented [12,19]. Rather than being comprehensive, in the following sections selected successful cases of industrial biocatalysis adapted to production of APIs will be highlighted, emphasizing the required optimizations in each example (from enzyme identification and upgrade to process conditions and scale-up).

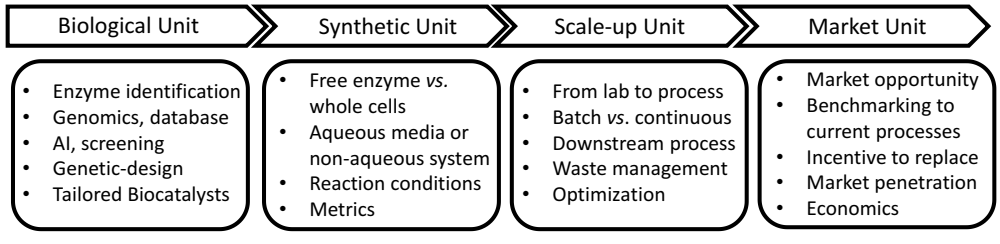

Figure 1. Conceptual path and units to be addressed to set up an industrial biocatalytic process.

2. Selected examples of relevant enzymatic methods in the preparation of APIs

2.1. Hydrolases: selected examples in aqueous or in organic media for the synthesis of APIs

Hydrolases (E.C. 3.x.x.x) are enzymes able to perform hydrolytic transformations as well as the formation of amides and esters. These enzymes are the most frequently employed biocatalysts, in particular for pharmaceutical chemistry, because these enzymes do not require cofactors, are robust, readily available, and very versatile both in their substrate recognition pattern (catalytic promiscuity [30]) and in their capability for working in organic [31,32] and neoteric solvents [26,33].

An outstanding example of hydrolases applied to pharmaceutical chemistry is the synthesis of (*S,S*)-Reboxetine mesylate (Edronax®), a selective norepinephrine reuptake inhibitor [34], used as an antidepressant. Edronax® is marketed as a mixture of (*R,R*) and (*S,S*)-enantiomers ((*R,R*) and (*S,S*)-**1**, Figure 2), although the (*S,S*)-(+)-eutomer of reboxetine (named esreboxetine) is 24-fold more effective than the correspondent (*R,R*)-(-)

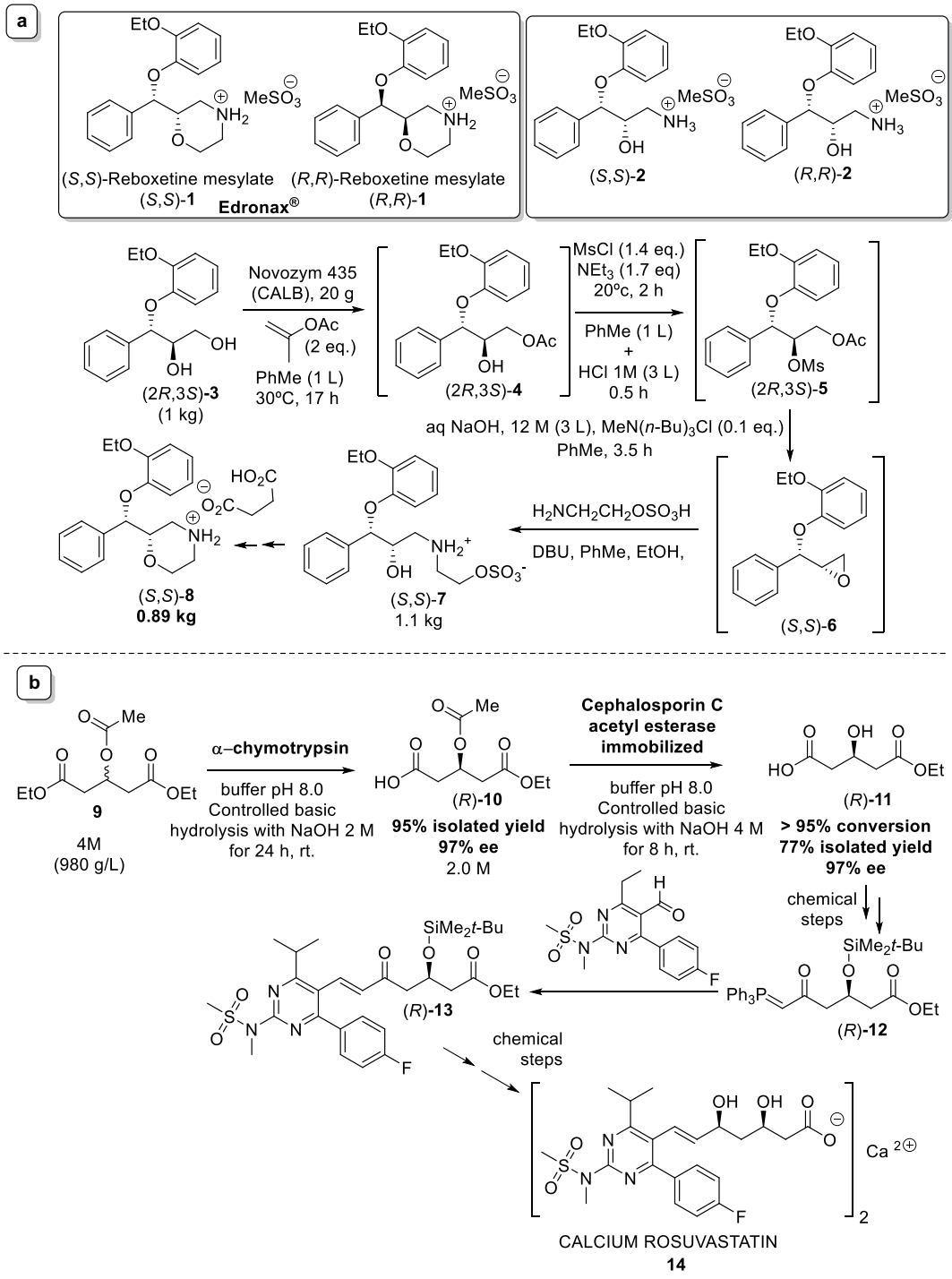

Figure 2. Some examples of hydrolase-based preparation of drugs. a) Schematic chemoenzymatic synthesis of (*S,S*)-reboxetine succinate (Esreboxetine).[38] b) Chemoenzymatic synthesis of Rosuvastatin[51]

diastereomer [35]. Likewise, esreboxetine succinate has been explored for the treatment of fibromyalgia in clinical phase studies [36]. Herein, hydrolases applied in organic media – to perform an enantioselective transesterification –, are demonstrative of the enzymatic capabilities to afford (*S,S*)-reboxetine succinate ((*S,S*)-**8**, Figure 2A) [37]. But before applying biocatalysis, industry explored other more classical synthetic paths for its preparation. The first attempt followed a chemical resolution starting from the racemic Edronax® **1**, through the crystalline enantiopure (*S*,S)-reboxetine-(*S*)-mandelate [38]. Recrystallization and further transformation into the succinate afforded the desired product (overall yield of 31% and enantiomeric excess of 92.95%). The route allowed the production of more than 100 kg of (*S,S*)-**8**, but it resulted in an expensive and not resource-efficient process, requiring 3.0 kg of racemic Edronax® **1** – whose synthesis is not trivial –, to yield 1.0 kg of (*S,S*)-**8**, leading to unacceptable waste generation [37]. Subsequent modifications of the route did not lead to significant improvements with respect to the waste formation and low overall yields [38].

Another alternative started from the open racemic mesylate **2** (Figure 2), through further construction of the morpholinic ring. For the racemic resolution, (*S*)-camphanic acid proved to be robust, leading to a scaled process of 150 kg with overall yield (starting from racemic **2**) of 47% with an enantiomeric excess of 99% [38].

Herein, biocatalysis entered into motion by setting-up a chemo-enzymatic approach starting from the enantioenriched diol (2 *R*,3*S*)-**3** – prepared *via* a sharpless epoxidation of cinnamyl alcohol – and incorporating six chemical transformations *via* two synthetic steps with an isolated intermediate. The regioselective protection of the primary alcohol with TMSCl resulted in moderate yield and regiocontrol, and thus a more suitable alternative was the substitution of the primary hydroxy group with an acetyl group. To that end, the biocatalytic acylation of diol (2 *R*, 3*S*)-**3** with isopropenyl acetate mediated by a commercially available immobilized lipase B from *Candida antarctica* (Novozym 435, CALB) in non-aqueous media was performed. By working in organic solvents, hydrolases do not perform hydrolysis (absence of water), but synthesis by using alcohols as nucleophiles. Thus, this led to the selective monoacetylation of the primary alcohol to furnish the corresponding acetate (2 *R*,3*S*)-**4** with 99% *in situ* yield and 98% regioselectivity. The immobilized biocatalyst was filtered and reused for several cycles. The main impurity obtained, acetylated secondary alcohol, could be minimized by operating at low temperature and short reaction times (T < 25°C, 16 h). Importantly, the entire process was conducted in toluene (PhMe), thus minimizing costs and wastes associated with solvents. Toluene is not considered, though, a sustainable option, according to modern standards [39] (see Expert Opinion). Looking beyond, the use of greener solvents improves the sustainability of the chemical processes, and can be used also when employing biocatalysts [40,41]. In a subsequent step, the activation of the secondary alcohol *via* mesylation to produce (2 *R*,3*S*)-**5**, followed by an S_N2 reaction with the primary alcohol after acetyl deprotection proceeded quantitatively to furnish the terminal epoxide (*S,S*)-**6**. This last step was carried out by treating the solution containing (2 *R*,3*S*)-**5** with NaOH 12 M in the presence of a phase transfer catalyst (methyltributylammonium chloride). However, the product was obtained as an oil (with cumbersome handling), and thus it was transformed into (*S,S*)-**7** by treatment with 2-aminoethyl hydrogen sulfate (2-AEHS). The final product was obtained by formation into morpholine from (*S,S*)-**7** and subsequent formation of the succinate salt. This involved the combined use of solid NaOH (or KOH) in THF with alcohol-type additives (MeOH, EtOH, *t*-BuOH, IPA, *t*-AmylOH) and 1% water. In this way, a highly pure API (pharma-grade quality) was obtained with an overall yield of 67.7% in six chemical transformations, compared to the previously optimized process (33%). The process was successfully scaled up to 1 kg of the chiral diol **3**, A detailed chemometric analysis illustrating the advantages of the chemoenzymatic procedure was reported [42]. Hence, the implementation of this route allowed a significant reduction in costs and wastes (more than 1300 tons [37,38]) compared to the initial process involving the resolution of racemic **1**. The capability of the lipase to conduct a transesterification in organic media resulted in key to success, as the same solvent (toluene) could be used throughout the entire pipeline, reducing costs, wastes, and simplifying the overall synthesis.

Another remarkable industrial example of hydrolases applied to APIs is the synthesis of rosuvastatin (Figure 2B, **14**). This drug belongs to the statin family, and is widely used for the pharmacological treatment of hypercholesterolemia and dyslipidemia [43,44] by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. Since their introduction in 1987, the life expectancy and quality of life of millions of people have increased [45]. The initial patent on rosuvastatin synthesis (purely chemical) was reported by Shionogi Research Laboratories [46], and subsequently sold to AstraZeneca, who launched it under the trade name Crestor®. Although the patent expired in June 2016, the great sales volume has remained (in the range of billions of USD per year) [47,48].

The patent expiration has triggered the development of more efficient and sustainable processes for its production, where biocatalysis has taken over. Different enzymatic asymmetric processes leading to statins, involving several enzyme classes, have been established at lab scale [49]. In a particularly successful case, a seven-step synthesis was proposed without intermediate isolation by incorporating two highly efficient biotransformations. The multi-step process is a great step forward compared to other routes in which isolation and purification of intermediates generates wastes and tedious downstream units. The waste reduction created by multi-step reactions follows the premises of the ACS pharmaceutical round table, created by the Green Chemistry Institute (GCI) [50].

Furthermore, the approach demonstrates once again the compatibility of biocatalysis with classical organic synthesis, leading to a highly efficient synthesis of rosuvastatin with excellent yields, high substrate loadings, and straightforward biocatalyst, while avoiding isolation and purification steps [51]. In this case, two hydrolases were used in aqueous media – to

perform hydrolyses – native α-chymotrypsin (from Biozym, Hamburg, Germany) for catalyzing the regioselective hydrolysis of racemic diester **9** and immobilized cephalosporin C acetyl esterase (CCAH from Sandoz GmbH, Kundl, Austria) for the subsequent deacetylation to obtain (*R*)-**11**.

Based on the extraordinary chemo-selectivity of the acetyl group, it was possible to afford the chiral monoester (*R*)-**11** with substrate loadings of 980 g L^{-1} (4.0 M, one of the highest substrate concentrations reported in biocatalysis), with an overall yield of 95% in two steps from diester **9** and an enantioselectivity of 97%. No isolation steps were required, as α-chymotrypsin was removed by ultrafiltration and immobilized cephalosporin C acetyl esterase was simply filtered out; remarkably, both catalysts could be re-used up to five cycles. The desired chiral monoester (*R*)-**11** was obtained with a final concentration of 220 g L^{-1}. Further chemical steps depicted in Figure 2B led to the preparation of rosuvastatin **14**. The (bio) synthetic route was transferred to Sandoz for its industrial implementation [52].

2.2. Redox enzymes: Selected examples in the synthesis of APIs

Ketoreductases (E.C. 1.1.1.1) catalyze a wide range of redox processes with exquisite chemo-, regio- and/or enantioselectivities, while working under environmentally friendly reaction conditions [53,54]. Thus, the enzymatic approach has demonstrated to be an excellent alternative to 'classical' redox procedures in which harsh reagents and severe reaction conditions are required. Within ketoreductases, one of the most applied biocatalysts are the alcohol dehydrogenases (ADHs), which catalyze the reversible transformation of carbonyl compounds into the corresponding alcohols. The redox equivalents required for the bioreduction of aldehydes or ketones are provided by a reduced nicotinamide cofactor [NAD(P)H]. This cofactor must be recycled, as its use in stoichiometric amounts is hampered by the high price and biocatalyst inhibition. Two main approaches for the recycling are either using *iso*propanol (IPA) as co-substrate that is oxidized by the ADH to acetone or coupling a secondary enzymatic system to the bioreduction reaction [55].

Several industrial examples of ADH-catalyzed reactions in the synthesis of APIs have been described, including valuable drugs such as Aprepitant, Atazanavir, Atorvastatin, Eslicarbazepine acetate, Ezetimibe, Ticagrelor, and others [56–59] (see Figure 3). Of particular relevance to illustrate how biocatalysis can improve the preparation of a valuable API is the synthesis of Montelukast sodium (Singulair, Figure 4, **17**), a leukotriene receptor antagonist discovered by Merck in 1991, patented in 1996, and used in the treatment of asthma and symptoms of seasonal allergies. Its annual production exceeds 66,000 kg with worldwide sales of >1500 million USD. Montelukast is prepared in a multistep procedure involving the assembling of four blocks including quinolone, a *meta*-disubstituted aromatic moiety, another aromatic ring harboring a tertiary alcohol and a side chain formed by a thiol-carboxylate unit. Being an enantiopure compound, a resolution step was required, which could not be performed as end-of-pipe (late) option, because the recycling of the non-desired enantiomer was costly and waste-intensive. Thus, the introduction of the chirality was conducted earlier in the synthetic route, through the asymmetric hydrogenation of ketone **15** to chiral alcohol (*S*)-**16**, which is the key-step of the synthetic procedure. Due to the presence of several different reducible moieties in **15**, the developed methodology requires an excellent chemo-selectivity in addition to a high enantioselectivity. The original chemical synthesis was carried out through (-)-β-chlorodiisopropinolcampheylborane [(-)-DIPCl]-based asymmetric reduction in which the desired alcohol was enantiopure after recrystallization in 90% yield. However, the use of the borane reducing agent presents some drawbacks for its application at large scale. First, stoichiometric amounts (1.5–1.8 equivalents) were needed, challenging the atom efficiency and creating large waste quantities. In addition, (-)-DIPCl is corrosive and air sensitive, forcing it to anhydrous conditions. To overcome this, catalytic methods were considered. The metal-catalyzed asymmetric hydrogenation of **15** in presence of ruthenium catalysts was assessed [60], yielding excellent enantioselectivity for the chiral alcohol (99% *ee*) at the gram level, with low catalyst loadings 5000:1, which led to reduced wastes when using ((*R*)-Xyl-BINAP)((*R,R*)-DPEN)RuCl. Yet, the hydrogenation had to be performed at high hydrogen pressure, requiring the (costly) appropriate equipment and an exquisite control of the reaction conditions. Likewise, the inherent high toxicity of metal-based compounds should be addressed too.

On that basis, there was a strong incentive to implement biocatalysis. In 2010, Codexis Inc. disclosed the bioreduction of (*E*)-methyl-2-(3-(3-(2-(7-chloroquinolyn-2-yl)vinyl)phenyl)oxopropyl)benzoate to the optically pure (*S*)-**16** catalyzed by the KRED CDX-026 [61]. A panel of these biocatalysts which showed activity on the starting material were subjected to directed evolution; this is a consecutive methodology of random mutations and high-throughput screening used to identify the best mutants, which are used for further rounds of mutagenesis, picking out the best example from each generation, and so gradually evolving the enzyme's structure [22–24]. In this case, the desired target was to improve the enzymatic activity and (thermal) stability. Beneficial mutations in the KREDs were recombined in three iterative rounds of evolution until it was possible to improve the activity of the biocatalysts by 1000-fold compared to the initial activity. As stated in the introduction, not only the enzyme must be tailored but also reaction conditions can also be adapted. Due to the hydrophobic character of **15**, organic cosolvents were required to enable a proper substrate solubilization. Reactions were performed at substrate loadings of 100 g L^{-1}, analogous to the (-)-DIPCl-mediated reduction, in a mixture of toluene/*iso*propanol/triethanolamine-HCl buffer pH 8.0 1:5:3, in which *iso*propanol was also employed for the cofactor regeneration. In this medium, the (*S*)-alcohol **16** readily crystallizes, and complete conversion can be achieved upon product precipitation. Yields were around 90–98% (24 h), slightly higher than those achieved for the (-)-DIPCl procedure with the additional advantage that the enantiopure alcohol could be recovered without any further separation step. The superiority of the enzymatic process is observed when process mass intensity (PMI) is compared (the ratio between the quantity of the

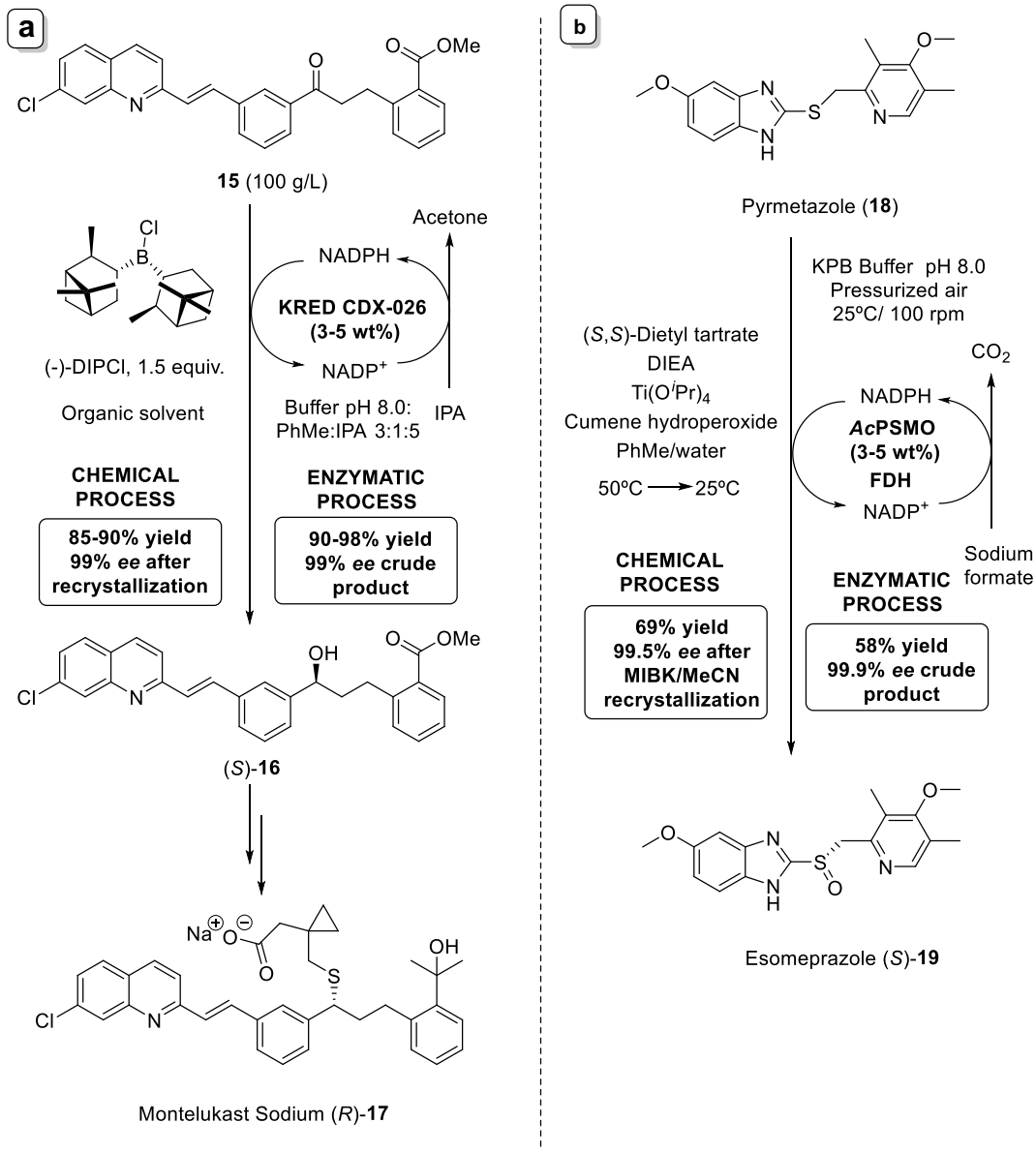

Figure 3. (a) Bioreduction of prochiral ketone **15** to yield the Montelukast chiral intermediate (*S*)-**16** employing a chemical or a biocatalytic approach; (b) Oxidation of pyrmetazole (**18**) to esomeprazole (*S*)-**19** by using a chemical approach or by a biocatalytic method in presence of a BVMO.

material inputs by the quantity of the product output), PMI is around 30-fold lower with the enzymatic methodology while reducing the organic solvent consumption 25-fold. The biocatalytic procedure was scaled up to 250 kg batches, demonstrating its applicability for this crucial reaction step in the preparation of Montelukast sodium.

Among all oxidative biocatalysts, monooxygenases perform the insertion of one atom of molecular oxygen into different substrates, usually with exquisite chemo-, regio- and/or enantioselectivity. Baeyer-Villiger monooxygenases (BVMOs) are a class of these enzymes catalyzing the Baeyer-Villiger oxidation as well as the oxygenation of different heteroatoms including sulfur [62,63]. These enzymes are flavoproteins, in which the flavin is non-covalently bound to the catalyst, which require NADPH to carry out their activity. The cofactor is recycled through a secondary enzymatic system as formate dehydrogenase (FDH), glucose or glucose-6-phosphate dehydrogenase (GDH or G6PDH), or a KRED. Their large-scale application has some drawbacks, as the requirement of stoichiometric O_2, what makes the mass transfer of oxygen a critical parameter, or the high price of the NADPH cofactor. One example has been recently shown in the BVMO P1/D08 catalyzed sulfoxidation step in the synthesis of Ceralasertib (see Figure 5) developed by AstraZeneca [64]. The optimization of the reaction allowed using low enzyme loadings and the improvement of the oxygen mass transfer by sparging of air and continuous feeding of 1-propanol. A 74% yield was achieved to obtain the chiral sulfoxide with >99% enantiomeric excess when the oxidation was carried out in 60 kg batches, which improves to a great extent the chemical synthesis.

Esomeprazole (Nexium®, (*S*)-**19**), the (*S*) enantiomer of omeprazole, is the first drug of the class of proton pump inhibitors (PPIs). Since its development in the 70s it is one of

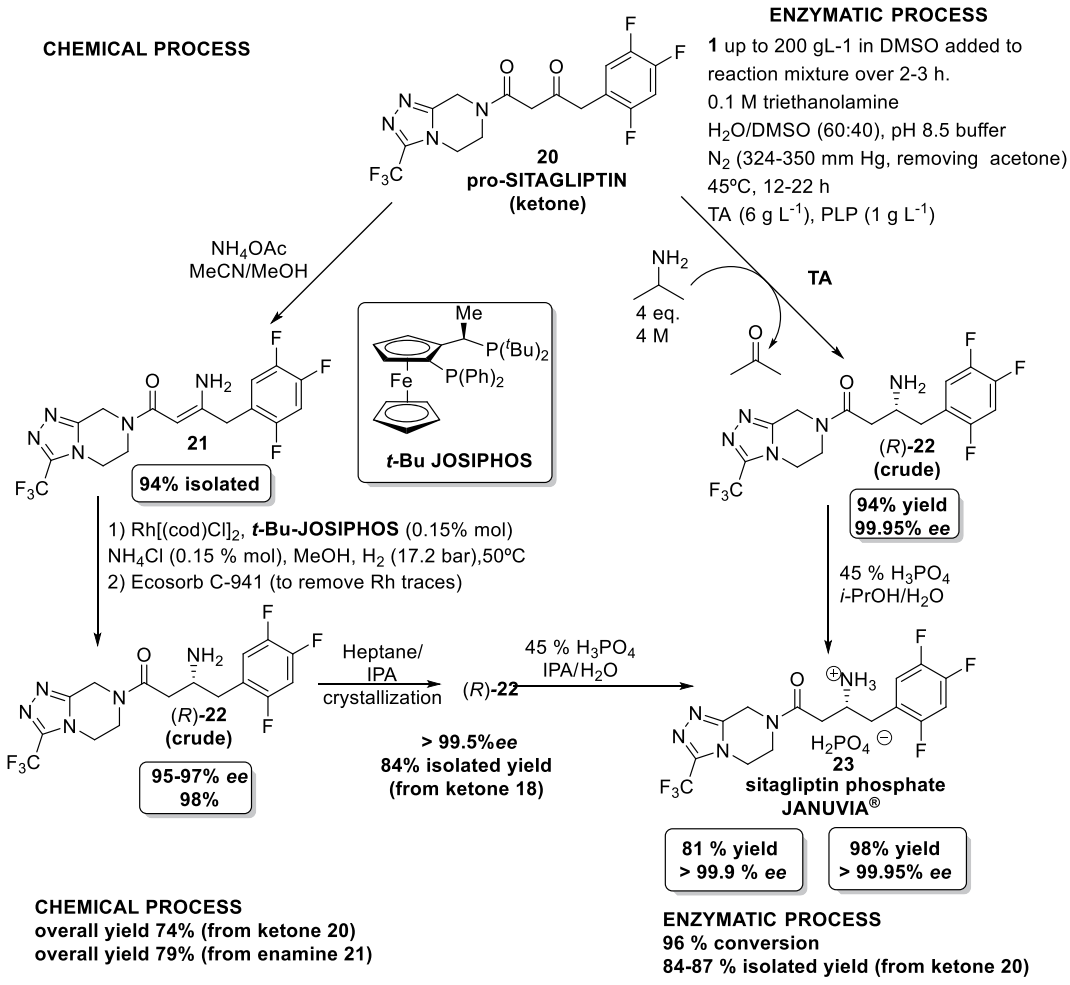

Figure 4. Chemical *vs*. enzymatic synthesis of sitagliptin (*R*)-**22** starting from the precursor prochiral ketone prositagliptin **20.**

the world's best-selling drugs, with over 800 million patients treated and annual sales reaching 6 billion USD. This compound has been prepared in a multistep procedure starting from a trimethyl pyridine, which after different transformations was treated with a benzimidazole thiol in a basic medium leading to pyrmetazole (**18**), thioether-5-methoxy-2-[((4-methoxy-3,5-dimethyl-2-pyridinyl)methyl)thio]-1 *H*-benzimidzole. Then, omeprazole is obtained through the oxidation of the prochiral sulfide.

Similar to Montelukast, the introduction of chirality in esomeprazole can be end-of-pipe through a (late) resolution step, but the separation and reuse of the non-desired enantiomer is cumbersome, consuming large quantities of chiral auxiliaries and solvents. Thus, the asymmetric oxidation of **18** was selected as the key step to the desired enantiomer. The chemical sulfoxidation was a slight modification of the Kagan method, through a titanium-mediated process with cumene hydroperoxide and *N,N*-diisopropylethylamine (DIEA) in the presence of (*S,S*)-diethyl tartrate. The desired sulfoxide was recovered with 69% yield and 94% enantiomeric excess, which was increased by up to 99.5% after crystallization in methyl *iso*butyl ketone and acetonitrile. [65]. It must be noted, though, that the oxidation is water-sensitive, and some over-oxidation to sulfone occurs, complicating the downstream. Additionally, the process requires harsh and eco-unfriendly reagents, generating wastes.

Due to these drawbacks in the chemical oxidation of pyrmetazole, a first biocatalytic approach to esomeprazole at large scale was developed in 2018 by Codexis Inc. [66]. The selected biocatalyst was the cyclohexane monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CHMO). This enzyme is one of the most versatile BVMOs [67], enabling the oxidation of linear and cyclic ketones as well as sulfoxidations, but its use is hampered because of its sensitivity to temperature and organic solvents. There was thus an opportunity for genetic design and upgrade of the enzyme, to adapt it to the *real* industrial synthetic conditions.

Initial studies indicated that wild-type CHMO could catalyze the oxidation of pyrmetazole to esomeprazole, albeit at low conversion. Directed evolution was performed to tailor a CHMO mutant with proper enzymatic productivity, high enantio- and chemoselectivity (no formation of the sulfone as a byproduct). Thousands of variants were generated over 19 rounds of evolution. After combining the beneficial mutations in subsequent rounds, a CHMO variant with an impressively improved activity of 140,000-fold over the wild-type enzyme was obtained. Subsequently, other critical oxidation parameters were assessed, namely, the oxygen supply,

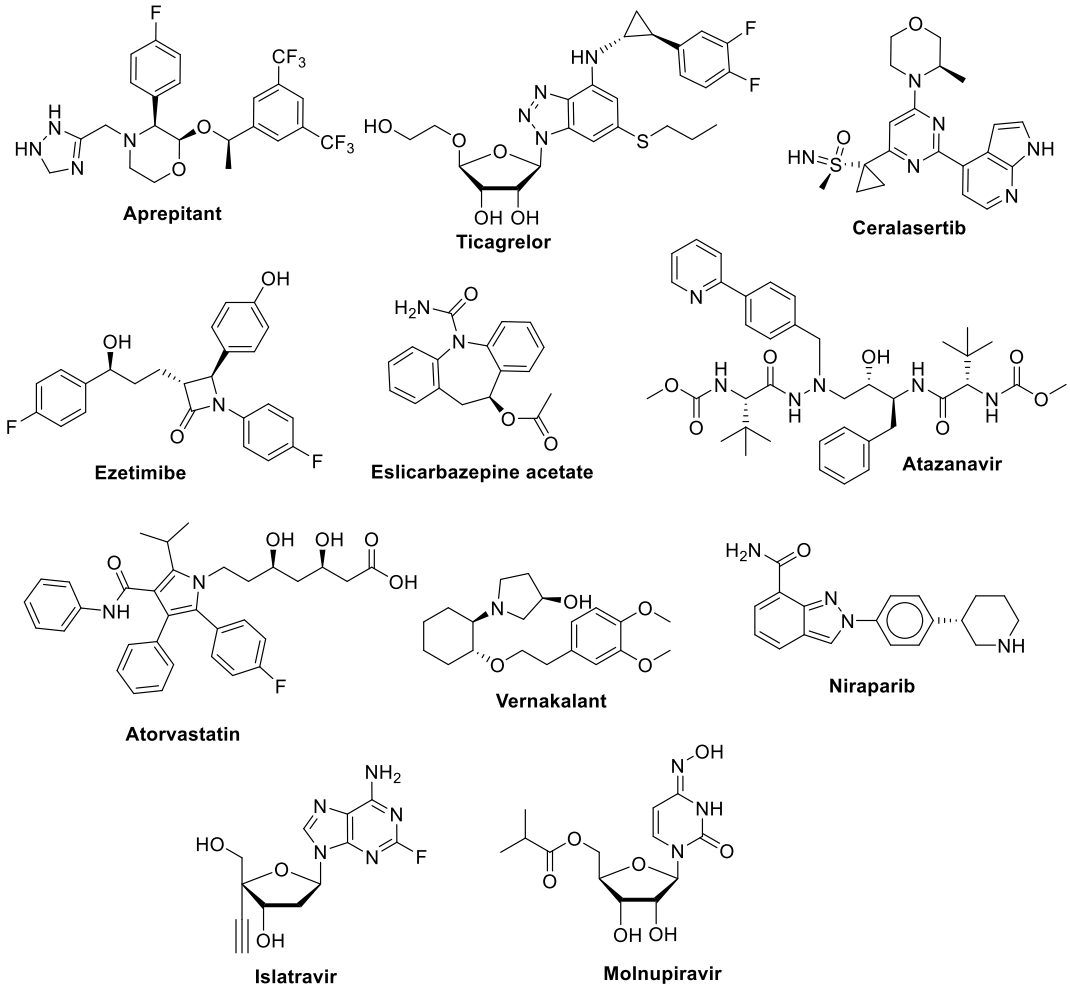

Figure 5. Some interesting examples of APIs manufactured by enzymes and mentioned in the text. Apart from these cases, many other examples can be found in recent comprehensive reviews [12–19].

substrate concentration, the addition of catalase to remove the hydrogen peroxide formed in the oxidation, and the presence of *iso*propanol and a KRED for cofactor regeneration. Thus, the reaction was carried out at 30-gram scale (50 g L^{-1} pyrmetazole) in phosphate buffer pH 9.0 containing 4% v/v IPA, the BVMO, KRED CDX-019, catalase and $NADP^+$ to recover the final product after extraction with isobutyl methyl ketone with 99% purity in 87% isolated yield and 99.9% *ee*. Results were promising compared to the Kagan procedure in terms of productivity, cost and environmental impact, but were still insufficient to develop an effective large-scale process.

CHMO was further optimized into a proper pyrmetazole monooxygenase (AcPSMO) by a structure-based protein-engineering approach [68]. AcPSMO-biocatalyzed oxidation has been recently conducted at pilot-plant scale (300 L) for the preparative synthesis of omeprazole [69]. Under these conditions, the formate dehydrogenase (FDH)/sodium formate system was selected for cofactor regeneration. The oxidation of formate to CO_2 was irreversible, with no need of removing acetone or using high IPA concentrations (required when employing KRED). Methanol (5% v/v) was selected as co-solvent for substrate solubilization. Both AcPSMO and FDH retained almost all their activity and methanol can be removed at low temperature, something to consider given that omeprazole is unstable at temperatures of >40°C. The oxygen transfer rate was improved by increasing the volumetric transfer coefficient and the saturation concentration of dissolved oxygen employing pressurized air instead of pure oxygen. The pH of the reaction media was optimized to pH 8.0 to ensure the best enzyme activity and NADPH stability. The biooxidation was carried out starting from 600 g of pyrmetazole and once completed, NaOH was added to yield the omeprazole sodium salt, which was recovered from the crude mixture by membrane filtration, resulting in a great reduction of the ethyl acetate employed during downstream. After drying, 380 g of esomeprazole (58% yield) was obtained with a 99.1% purity and more than 99% enantiomeric excess. This procedure represents the first scaling up with AcPSMO – the first green-by-design system for esomeprazole –, which still requires further optimization in its activity.

2.3. Selected examples of transaminases in the preparation of APIs

Amines global market was over USD 17 billion in 2020, and it is projected to be worth USD 24 Billion by 2030, registering a compound annual growth rate (CAGR) of 7.35% between

2022 and 2030 [70]. Amines are useful compounds as chemical intermediates in the synthesis of fine chemicals, agrochemicals, polymers, dyes and pigments, emulsifiers, plasticizing agents, and, to a greater extent, APIs. Among the plethora of APIs currently available, it was estimated that around 40–45% of them possess an α-chiral amine group inside their structure [71]. Substantial progress in the chemical synthesis of enantiopure amines have been reported in the last years [72–74]. Likewise, the use of biocatalysis for the preparation of these compounds is gaining importance, as a consequence of the great variety of enzymatic strategies than can be followed, leading to high yields of final products with excellent selectivity (either chemo-, regio-, and/or stereoselectivity) under mild and sustainable reaction conditions [75,76].

There are different enzymatic approaches which can be used for the preparation of (mainly primary) α-chiral amines [75]. Among them, transaminases (TAs, also called aminotransferases) are probably the most used strategy for the preparation of enantiopure amines [77,78]. Transaminases use pyridoxal-phosphate (PLP) as cofactor. These PLP-dependent enzymes catalyze the reversible transfer of an amine moiety from an amine donor to a ketone acceptor, so that it is possible to use TAs in both senses, that is, to catalyze either the asymmetric reduction of a prochiral ketone (keto to amine) or the kinetic resolution of a racemic amine (amine to keto) [78–81].

With respect to APIs, the seminal paper from Savile and coworkers [82,83], in a joint cooperation between researchers from Solvias, Codexis and Merck, illustrates the TA-catalyzed synthesis of the oral antidiabetic drug sitagliptin (*R*)-**22** (commercialized by Merck & Co. as its phosphate salt under the trade name of Januvia®, **23**). In that paper, a homologue of an (*R*)-selective TA from *Arthrobacter* sp. (ATA-117) was used for the direct asymmetric synthesis of chiral amine sitagliptin from its corresponding prochiral ketone, prositagliptin **20** (Figure 4).

Initially, the wild-type enzyme could not convert **20** due to the steric constrictions caused by its bulky fluorinated ring, which impeded its recognition in the small site of the enzyme-binding site (wild-type TAs only accepted small methyl or ethyl groups in that cavity [84]). Two different solutions could be proposed; on the one hand, *rational design*, which requires making a logical guess about which amino acids in an enzyme must be altered to achieve the desired enzymatic improvement, and making the amino acids replacement by site-directed mutagenesis of the corresponding gene [85,86]. On the other hand, *directed evolution*, an already-mentioned genetic improvement (see section 2.2) [22–24,87], which, worth to be mentioned, granted F. Arnold the Chemistry Nobel Prize in 2018 [88]. In this particular case, for searching a TA suitable to be used for the transamination of **20**, a hybrid of the two mentioned approaches was used. Firstly, computational rational modeling was employed to map the enzyme's binding pocket, identifying the two regions (small and large [84]) needed to be adjusted for the substrate fitting, and designing a simplified truncated substrate, for which the enzyme already presents a limited reactivity. After a few rounds of mutations, once the first problematic area of the active site had been widened, it was the moment to switch to the real substrate structure to modify the second area. This strategy, called *substrate-walking* [82], was employed using a truncated analogue of **20** without the fluorinated aromatic ring. Once a first S223P mutant (large site) was found to convert the truncated substrate, subsequent rational mutations were conducted to accommodate both the large- and small-binding pockets of the active site, enabling the recognition of substrate **20** inside the mutant enzyme. Then, directed evolution was used to improve the operational conditions. In this way, the optimized process employed a mutant enzyme containing 27 individual mutations compared to the original enzyme (after 11 rounds of evolution), capable to work in 50% DMSO (helping the substrate solubilization without affecting enzyme stability) in buffer pH 10, to convert up to 200 g L^{-1} of **20** at 45°C (enhanced thermostability) with 92% yield (>99.95% ee). This enzymatic process (Figure 4, right) provided sitagliptin (*R*)-**22** with a 10 − 13% increase in overall yield compared to the chemical methodology using a chiral rhodium catalyst (Figure 4, left), a 53% increase in productivity and 19% reduction in total waste, removing any need of using toxic heavy metals or very expensive high pressure specialized hydrogenation devices [82].

Considering the time scale of the enzymatic synthesis, the directed evolution of the transaminase took one year [82,83]. As Januvia® was already on the market (since 2006, protected by different patents [89–91]) when the biocatalytic synthesis was ready for implementation [92], a refile with regulatory agencies was required [93]. In any case, sitagliptin patents protection is about to end (July 2022 for sitagliptin, November 2026 for the phosphate salt [94]) and the enzymatic procedure, easily scalable [95,96], was demonstrated on a 45 kg scale [92]. Some further studies from the same group showed how the immobilization of the engineered enzyme on a polymeric resin (SEPABEADS EXE120) displayed excellent performance (activity and stability) under the optimized process conditions, allowing multiple rounds of enzyme reuse without any detectable loss of activity [97]. A subsequent study showed the 3D structure of several mutants of this transaminase confirmed the presence of an adaptable loop next to the active site as a new target for the rational design to change the substrate specificity of TAs [98].

Following similar methodologies, many other APIs have been prepared at (semi)industrial scale using TAs [78], such as Vernakalant or Niraparib (see Figure 5).

3. Outlook

The selected examples discussed in this article have illustrated how biocatalysis has become an important ally to perform efficient synthetic reactions for the production of APIs while maintaining mild conditions and sustainability. Apart from the examples mentioned and/or discussed so far, many other APIs are currently being manufactured with enzymes, clearly emphasizing the potential of biocatalysis as a key enabling technology for the future (Figure 5).

Advantageous to the setup of biocatalytic strategies is the versatility of the enzymes, able to perform reactions both in aqueous media and in non-conventional systems, like organic or neoteric solvents. This creates synergies in multi-step synthetic processes because the same solvent can be used

throughout a complete route, eliminating the need of tedious and waste-generating downstream units. As noted in this article, enzymes found in Nature do not often display the required activity and stability for an industrial reaction. However, genetic tools allow the biocatalyst upgrading, with some remarkable examples of enzyme improvement and adaptation to the desired industrial process (see previous sections). Furthermore, current developments assure an even brighter future for biocatalytic reactions. Some emerging examples are the setup of solvent-free processes, in which substrates are the reaction media. This creates highly efficient strategies with the highest substrate loading possible. Examples involve the use of oxidoreductases [99–101], and more recently aldoxime dehydratases to afford (chiral) nitriles from aldoximes in a cyanide-free fashion [102,103]. In most of these cases whole cells are used, to protect the enzymes from the somewhat aggressive solventless media. Likewise, novel new-to-nature catalytic transformations are being created [104], based on computational tools. These new reactions have the potential to become future synthetic paths for APIs. Finally, the option of employing multi-step catalytic processes leads to highly integrated processes, with significantly reduced wastes and costs. A recent, paradigmatic example is the synthesis of the antiviral islatravir (see Figure 5) by Merck, in which nine engineered enzymes were combined in one-pot reaction [104,105]. Furthermore, another important aspect is the speed at which biocatalytic transformations may be established. The time-to-market has been reduced significantly over the last years, and now it is possible to setup efficient routes involving enzymes in a matter of months. As an outstanding example, the recently introduced molnupiravir (Figure 5), an antiviral against SARS-COV2 developed by Pfizer, is synthetized through a multi-step cascade biocatalytic process, starting from readily available substrates [11,106].

While, in the past, enzyme catalysis did not use to be the first choice when pharmaceutical synthetic routes were designed, the tremendous development in all fields related to biocatalysis – from biological areas to organic synthesis and scale-up – have changed this vision. An increasing number of enzymatic processes are added to industrial systems, due to their superiority in terms of efficiency, simplicity, and sustainability. Nowadays, biocatalysis in pharmaceutical chemistry has started to be for real.

4. Expert opinion

Although the first examples of using enzymes as catalysts to perform synthetic reactions appeared a century ago, the field remained largely unexplored until the 80s, when different enzymes applied in organic solvents were reported. The realization that biocatalysis could be performed not only in aqueous media – the inherent place of enzymes in Nature – but also in non-aqueous systems stimulated the research and the implementation of biocatalytic synthetic processes. However, in those early decades, the availability of enzymes was scarce, and only limited to a bunch of commercial ones, often presented in the form of crude (impure) powders with reproducibility problems, and with poor selectivity for the desired substrate. This far-from-ideal situation slowly changed from the 90s, when molecular biology and especially directed evolution techniques emerged, enabling the expression and tailoring of biocatalysts, and their adaptation to a desired synthetic reaction. More recently, emerging fields like metagenomics – the ability to express and screen non-culturable genetic material, giving access to many unknown biocatalysts – bioinformatics and artificial intelligence have established the basis of modern biocatalysis. Nowadays, it is not the desired process which is adapted to the properties of the biocatalyst; conversely, it is the enzyme which is particularly designed for the specific synthetic conditions and entire production pipeline. Therefore, a broad range of useful enzymes can be expressed, produced, and designed for industrial purposes. Currently, industrial biocatalytic steps are fully integrated into synthetic routes. In particular, for pharmaceutical chemistry, the use of enzymes creates powerful options for the chirality generation, under mild reaction conditions and in media that can be adapted to the entire process route (e.g. use of aqueous or non-conventional systems). In recent years, two new aspects have started to be incorporated; on the one hand, the increasing implementation of flow-based continuous strategies, which enable resource optimization and generation of less wastes by using immobilized enzymes (or whole cells), allowing processes running for days or even weeks. On the other hand, the setup of multi-step (bio)catalytic processes, combining more than one enzyme in the same reactor. This approach reduces the waste generation significantly, since less downstream units are needed, and substrates and intermediates are used properly without losses among reactions.

It has been recently stated that we are living now in the Golden Age of Biocatalysis. In fact, what started as an academic curiosity has become a mature field with useful applications and contributing significantly to the Green Chemistry Postulates. There is still, however, room for a broader penetration of biocatalysis in chemical industries. This must start from education programs providing the future leaders with the knowledge to look at enzymes as a powerful solution in hands. We are confident that during the next decade we will witness the implementation of many more enzymatic steps in pharmaceutical chemistry, with tailored enzymes performing complex reactions in an exquisite, selective manner. It will involve not only known reactions, or naturally occurring enzyme reactivities, but also new-to-nature transformations, as some examples have been already reported. Artificial Intelligence and modern screening technologies will facilitate the identification of the best enzyme variants. Likewise, the use of more benign solvents (e.g. bio-based derivatives, deep eutectic solvents) will become more common in synthetic procedures, replacing more hazardous alternatives. The possibility of adapting enzymes to perform reactions in those media will become an important asset to combine sustainability and process efficiency in the future. Biocatalysis is inherently bound to Green Chemistry and will be one of the research paths for future chemical industries.

We sincerely hope that we have managed to answer the questions that we addressed to ourselves when this article was being prepared: Are enzymes being used in the

pharmaceutical industry? If so, what are the incentives for using enzymes in the pharmaceutical industry? What is the relevance of biocatalysis and its advantages over traditional chemical synthesis? While organic chemistry will stand as a discipline in the future, enzymes may complement it significantly. The selected examples of industrial biocatalysis that have been showcased in this paper – covering lipases, oxidoreductases, and transaminases – pinpoint the advantages and the motivation to implement enzymatic reactions, and to integrate them in more complex synthetic pipelines.

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ORCID

Andrés R. Alcántara http://orcid.org/0000-0003-4856-4000

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