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RESEARCH ARTICLE

Biocatalyzed synthesis of antidiabetic drugs: A review

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ABSTRACT

The biocatalyzed production of building blocks for synthesizing drugs is a very attractive research field, because of the sustainability introduced in a synthetic schedule when chemical steps are substituted by biocatalyzed protocols. In this article, we will show how different antidiabetic drugs, for treating diabetes mellitus Type 1 and Type 2, can be more efficiently and effectively synthetized with the help of different types of biocatalysts. The huge overall drug market for these drugs, as well as the great number of people suffering from diabetes (the prevalence of all types of diabetes is growing), makes this topic attractive enough to focus on more efficient synthetic protocols for preparing antidiabetic drugs. Examples covering biocatalyzed synthesis of insulin analogues, sensitizers (PPAR agonists), secretagogues (GLP-1 analogues, GPR119 agonists) and enzyme inhibitors (α-glucosidase inhibitors, DPP4-inhibitors, SGLT-2 inhibitors and 11β-HSD1 inhibitors) will be presented.

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Introduction

Diabetes mellitus (DM) is a disorder of metabolic homeostasis, showing hyperglycaemia and altered lipid metabolism caused by dysfunction of pancreatic islets, which do not produce enough insulin (a hormone that regulates blood sugar or glucose), or rather caused when the body cannot effectively use the insulin it produces (World Health Organization 2016). According to this, there are three main types of DM (American Diabetes Association 2014; Ramachandran et al. 2017); thus, Type 1 DM results from the inherent pancreas's failure to produce enough insulin, being this type formerly known as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". Conversely, Type 2 DM is caused by insulin resistance, and it was previously referred to as "non-insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". Finally, gestational diabetes is the third main form and occurs when pregnant women without a previous history of diabetes develop high blood sugar levels.

DM is one of the most common chronic conditions in nearly all countries. In 2014, the International Diabetes Federation (IDF) assessed that 8.2% of adults between 20 and 79 (around 387 million people) were living with diabetes, an increase compared to 382 million people in 2013 (Fernandes et al. 2016). Clearly, the prevalence of all types of diabetes is growing, particularly Type 2 DM; this can be seen from 2014 data (422 million (World Health Organization 2016; Zhou et al. 2016)) or 2015 (415 million, 1 out of 11 adults (International Diabetes Federation 2015)), while the number of people affected by DM is estimated to increase up to 439 million people in 2030, reaching 592 million people by 2035 (World Health Organization 2016) and 642 in 2040, which means 1 out of 10 adults (International Diabetes Federation 2015; Ali et al. 2017).

Obviously, DM is a major cause of morbidity and mortality in many countries, although 80% of people with diabetes live in low- and middle-income countries (International Diabetes Federation 2015; World Health Organization 2016); globally, it caused around 5.0 million deaths in 2013 (IDF Diabetes Atlas Group 2015) and a similar number in 2015 (International Diabetes Federation 2015). Considering global costs of DM, it has been reported that at least $612 billion were spent globally on diabetes in 2014, representing 11% of all global health expenditures. This represents an increase of 12% compared to the data published in 2013 ($548 billion), due to increases in the total number of people with diabetes (Fernandes et al. 2016). In 2015, 12% of global health expenditure (around $673 billion) was dedicated to diabetes treatment and related

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complications, and the majority of countries spent between 5% and 20% of their total health expenditure on DM (International Diabetes Federation 2015; Williams 2016).

Therefore, the world market for diabetes drugs is really huge, $35.6 billion in 2012 (Visiongain 2013), growing up to 51.1 billion in 2015 (Global Market Insight, Inc. 2016), and it is estimated to reach $55.3 billion in 2017 (Visiongain 2013) and more than double, $116.1 billion, by 2023 (Global Market Insight, Inc. 2016). While Type 1 DM can only be treated with insulin or synthetic insulin analogues (Freeland and Farber 2016; Zaykov et al. 2016), for the treatment of Type 2 DM either insulin or other types of mostly oral drugs, either as a single API or a combination of them, can be used (Freeland and Farber 2015; Kokil et al. 2015; Gaitonde et al. 2016)

Taking into consideration all of these data, it is certainly understandable that the development of efficient and sustainable methodologies for synthesizing antidiabetic drugs is highly desirable. For this purpose, the use of biocatalyzed protocols is increasingly becoming recognized as a very important part inside Green Chemistry (Malhotra et al. 2015; Sheldon 2016), because those synthetic routes mediated by enzymes or cells are generally conducted under mild reaction conditions, at ambient temperature and can use water as reaction medium in many cases (Hoyos et al. 2013); moreover, their high selectivity avoids the need of functional group activation and protection/deprotection steps usually required in traditional organic synthesis. Thus, biocatalysis provides processes which are shorter, produce less waste (generally measured using E-factor value, ratio between the kilograms of waste to the kilograms of desired product (Sheldon 2017)) and reduce manufacturing costs and environmental impact. These features are even more significant in drug synthesis, because it is well known that Pharma Industry produces a great amount of waste, so that implementing biocatalyzed protocols is increasingly being employed (Hoyos et al. 2014; Patel 2016a, 2016b, 2016c).

To focus this article, we will comment some examples illustrating the use of chemoenzymatic protocols for the sustainable synthesis of antidiabetic drugs. For this purpose, first we will mention some cases in which biocatalysis has been useful for the preparation of insulin analogues (treatment of DM Type 1 and 2), and subsequently we will focus in the chemoenzymatic synthesis of drugs specially designed for the treatment of Type 2 DM.

Insulin and insulin analogues

Semisynthetic human insulin was commercially developed in the 1970s by Novo Nordisk A/S, starting from porcine insulin, by substituting the B-30 alanine residue of porcine insulin with a threonine residue (Markussen 1981; Andresen and Balschmidt 1982), as shown in Figure 1.

For these biotransformations, five steps were required (Barfoed 1987): insulin was first extracted from frozen porcine pancreas glands. In a second step, of the purified porcine insulin was converted into human insulin in a medium that contains only a small amount of water and trypsin and a large quantity of organic solvent and threonine ester. Subsequently, trypsin hydrolyzed insulin at Lys_{B29}-Ala_{B30}, while at the same time catalyzed the reverse reaction in which the threonine ester displaced alanine from position B30 in the insulin molecule. This transpeptidation of porcine insulin to human insulin was optimized to 97% yield using soluble trypsin (Morihara et al. 1979). This was followed by chromatographic purification to reduce measurable levels of proinsulin and remove the other reagents, pharmaceutical formulation and distribution into the market. Finally, the product was formulated and then filled under sterile conditions, packaged, and distributed. Transpeptidation was also catalyzed by immobilized trypsin, although the yield was lower (80%) (Ueno and Morihara 1989). Another protease, from *Achromobacter lyticus*, was also found to be completely specific in the hydrolysis of Lys_{B29}-Ala_{B30} and the subsequent condensation con H-Thr-OBu^t

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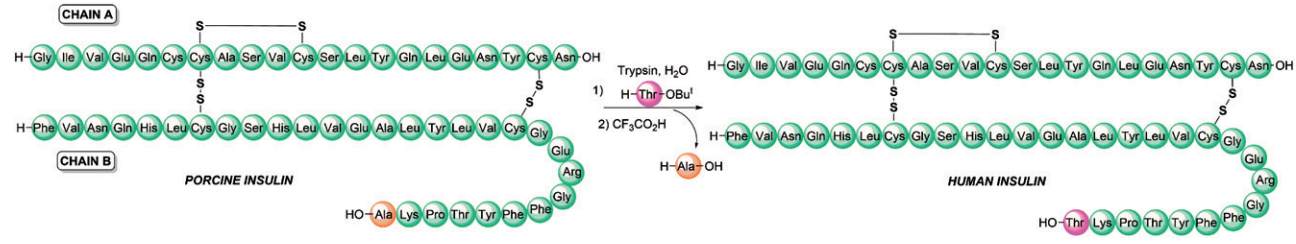

Figure 1. Semisynthesis of human insulin from porcine insulin by trypsin-catalyzed transpeptidation.

(Morihara et al. 1980; Morihara and Ueno 1991). Also, the use of carboxypeptidase A for the same procedure was described (Andresen et al. 1983).

Human insulin was the first animal protein to be made in bacteria in a sequence identical to that of the human pancreatic peptide, as early as 1978 by Genentech (lab scale) and Eli Lilly and Co. (scale-up) (Johnson 1983), working together to achieve the expression of recombinant human insulin in *Escherichia coli* K-12 using genes for the insulin A and B chains; thus, each insulin chain was produced as a β-galactosidase fusion protein in separate fermentations using *E. coli* cells transformed with plasmids containing either the A or B insulin peptide sequence. The intracellular products, once removed from the inclusion bodies, were chemically cleavage by CNBr at the Met residue between the β-gal and the A or B chains, purified, suffered an oxidative sulfitolysis and chemically linked to afford crude insulin. A final purification process led to the first production of recombinant human insulin, approved by drug regulatory agencies in 1982 (Ladisch and Kohlmann 1992).

After this pioneering work, some other strategies were developed using recombinant microorganisms that produce intact proinsulin instead of the A or B chains separately. For instance, Novo used *Saccharomyces cerevisiae* to secrete insulin as a single-chain insulin precursor, in which amino acid 30 of the B chain of insulin was connected to amino acid 1 of the A chain by a peptide (Chain C), as shown in Figure 2. Two enzymatic cleavages, the first one catalyzed by trypsin leading to the removal of most of the C chain, and the second one catalyzed by caboxypeptidase to delete two Arg residues from BThr30, yield the human insulin (Thim et al. 1986; Ladisch and Kohlmann 1992).

Since those innovative methods, many other protocols based on genetic engineering have been developed, and nowadays recombinant human insulin is mainly produced either in *E. coli* or *S. cerevisiae*, although several other alternate yeast strains have been explored for insulin production, as well as mammalian cells, transgenic animals or plant expression systems have been also employed as a host for large-scale production of recombinant insulin (Walsh 2005; Baeshen et al. 2014).

On the other hand, by using recombinant DNA technology different insulin analogues have been synthesized. This term refers to an altered form of insulin, different from any occurring in nature, still available to the human body for performing the same action as human insulin in terms of glycaemic control, but displaying improved ADME (absorption, distribution, metabolism, and excretion) characteristics (Zaykov et al. 2016). Officially, the U.S. Food and Drug Administration (FDA) refers to these as "insulin receptor ligands", although they are more commonly named as insulin analogues, which can be classified into two main classes:

a. those that are more readily absorbed from the injection site and therefore act faster than natural insulin injected subcutaneously, intended to supply the bolus level of insulin needed at mealtime (prandial insulin)

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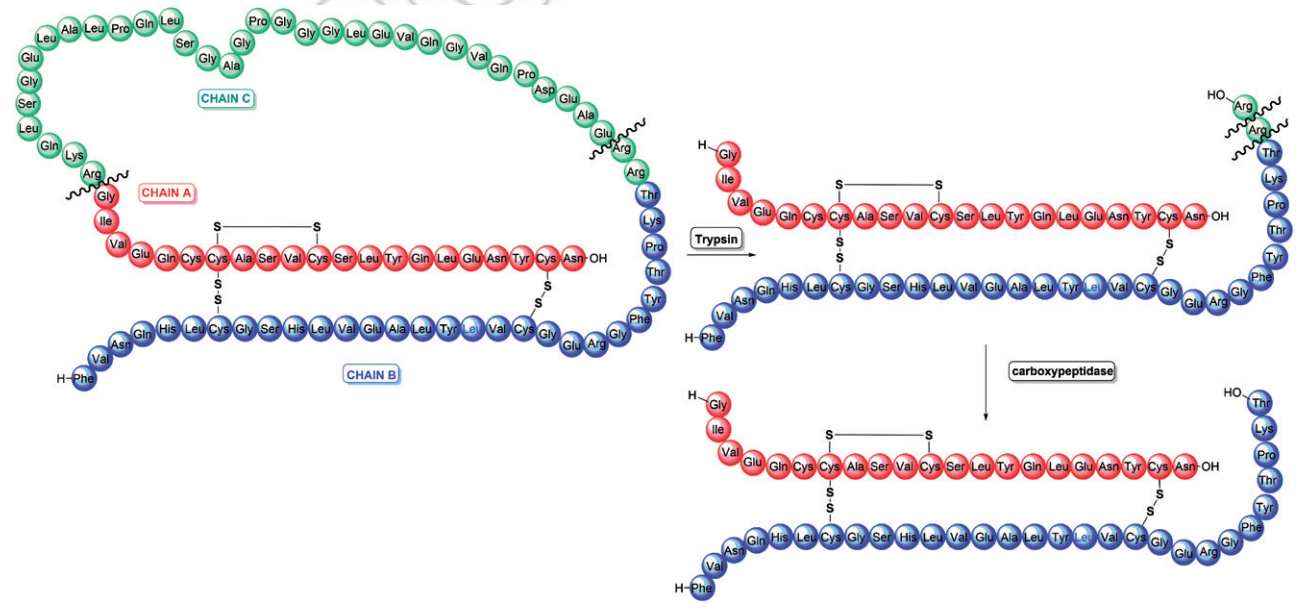

Figure 2. Preparation of human insulin from a precursor.

b. those that are released slowly over a period of between 8 and 24 h, intended to supply the basal level of insulin during the day and particularly at night time (basal insulin).

Among fast-action analogues, Eli Lilly and Co. developed and marketed in 1996 Humalog, the first rapid-acting insulin analogue (insulin lispro rDNA). This analogue was engineered through recombinant DNA technology, so that the penultimate lysine and proline residues on the C-terminal end of the B-chain ($ProB_{28}LysB_{29}$) were reversed (Figure 3). This modification did not alter the insulin receptor binding, but blocked the formation of insulin dimers and hexamers (Howey et al. 1994; Torlone et al. 1994), therefore allowing larger amounts of active monomeric insulin to be available for postprandial (after meal) injections (Anderson et al. 1997). The second entry in this class, insulin aspart (Novolog, Novo Nordisk), was first marketed in 2000 and utilizes an Asp at position B28 (Brange et al. 1988; Home et al. 1998, 2000). The most recent rapid-acting analogue, insulin glulisine (Apidra, Sanofi), was marketed in 2006 and is based on replacements of $LysB_{29}$ with Glu and of $AsnB_3$ with Lys (Becker et al. 2005; Dreyer et al. 2005; Becker and Frick 2008).

Considering those analogues intended to control basal insulin levels, insulin glargine (Lantus, Sanofi),

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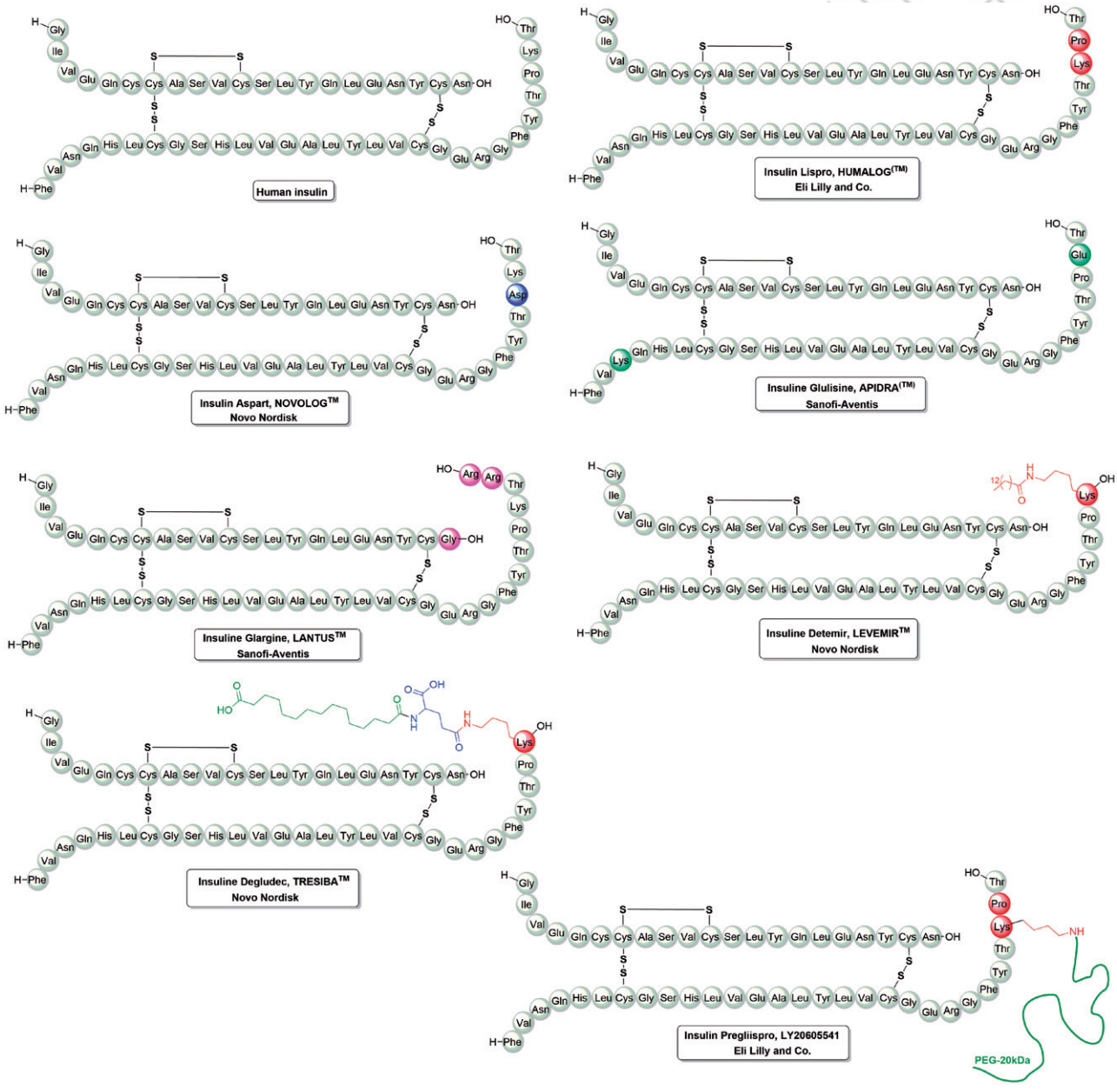

Figure 3. Some insulin analogues.

launched in 2001, uses a shift in isoelectric point (achieved through two additional Arg residues at positions B_{31} and B_{32}) in order to dramatically lower solubility at physiological pH, rendering the insulin far less soluble at the injection site. This results in an extended time–action profile as the analogue slowly re-solubilizes (Rosenstock et al. 2001). Additionally, a Gly residue is introduced at position A_{21} to maintain chemical stability in the aqueous, acidic formulation. Another different tactic consists in attaching a long-chain fatty acid with the purpose of slowing adsorption and facilitating extended plasma circulation through non-covalent albumin binding. This strategy was reported by Eli Lilly with insulin lipidated at LysB29 with palmitic acid (W99-S-32) (Myers et al. 1995) and by Novo Nordisk with LysB29-myristyl desB30-insulin, launched in 2006 under the name of insulin detemir (Levemir) (Havelund et al. 2004; Hermansen et al. 2006). Very recently, two new basal insulin analogues completed Phase III trials (Pettus et al. 2016): insulin degludec (Tresiba, Novo Nordisk), a des-B30 human insulin that is uniquely fatty-acylated at LysB29 with hexadecanedioic acid via gamma-L-glutamyl spacer (Wang et al. 2012; Gough et al. 2013), which have been recently approved by FDA in the USA, and insulin peglispro (Ly2605541), derived by covalent attachment of a linear 20 kD polyethylene glycol (PEG) polymer to the LysB28 side-chain in insulin lispro (Henry et al. 2014).

Generally speaking, the use of synthetic biology for creating cell factories is the methodology used at industrial scale in the preparation of these insulin analogues (Baeshen, Baeshen et al. 2014; Sanchez-Garcia et al. 2016). For sure, the improvement of metabolic pathways for increasing their production can be classified as sequential (or cascade) biocatalytical processes, and not many examples of protease modifications of precursors leading to insulin analogues, which can be *sensu stricto* considered biotransformations, can be found in literature (Bogsnes et al. 2003; Habermann and Zocher 2008). Anyway, ample research is being carried out in the development of new analogues of insulin (Freeland and Farber 2016; Pettus et al. 2016; Zaykov et al. 2016; Sherr et al. 2017) and new and faster formulations (Sherr et al. 2017), because there is little to no alternatives to brand-named analogue insulin and non-analogue human alternatives for treating both types of DM in low- and middle-income countries (Kaplan and Beall 2017).

Other antidiabetic drugs

Inside this category we will include:

a. drugs that increase the sensitivity of target organs to insulin, called sensitizers
b. agents that increase the amount of insulin secreted by the pancreas, known as secretagogues
c. agents that decrease the rate at which glucose is absorbed from the gastrointestinal tract.
d. enzyme inhibitors

We will show now different examples of biocatalyzed synthesis of some of these compounds. The use of biocatalysts is especially adequate for the stereoselective synthesis of those drugs possessing at least one stereogenic centre.

Sensitizers

Drugs belonging to this category are biguanides (such as metformin, **1**), thiazolidinediones (TZDs, also named glitazones, **2**), and glitazares **3**), shown in Figure 4. The first class is represented by metformin, the first-line medication for the treatment of Type 2 DM (Maruthur et al. 2016; Wise 2016), which is synthetized only by classical chemical methods, so that we will mention the other two classes, glitazones and glitazars.

Peroxisome proliferator-activated receptors (PPAR) agonists

The PPARs are ligand-dependent transcription factors. The three mammalian PPARs (α, β/δ and γ)

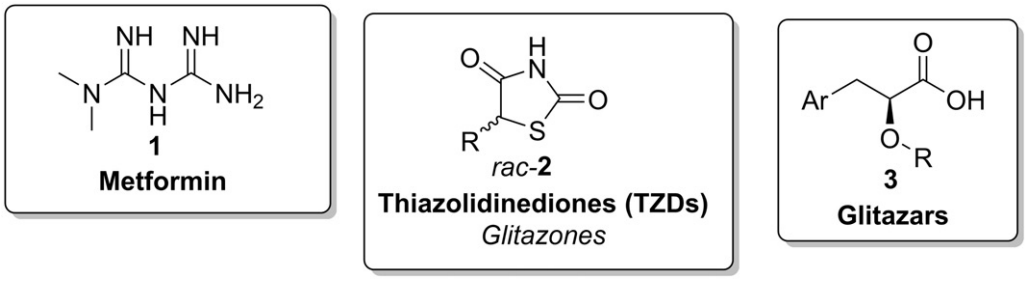

Figure 4. General structure of insulin sensitizers.

(Nevin et al. 2011; Wright et al. 2014) are crucial regulators of fatty acid and lipoprotein metabolism, glucose homeostasis, cellular proliferation/differentiation and the immune response. Therefore, PPARs are key targets in the treatment of metabolic disorders such as insulin resistance and Type 2 DM; furthermore, PPARs are also involved in chronic inflammatory diseases such as atherosclerosis, arthritis, chronic pulmonary inflammation, pancreatitis, inflammatory bowel disease, psoriasis, blood pressure regulation, neuroinflammation, nerve-cell protection, inflammatory pain reduction, and hypothalamic control of metabolism (Menendez-Gutierrez et al. 2012). However, PPAR-γ, which is expressed in adipose tissue, lower intestine, and cells involved in immunity, is the most extensively investigated PPAR, while PPAR-δ, which regulates several metabolic processes, has also been investigated for the development of new drugs for treating DM (Wright et al. 2014).

There are three marketed TZDs, acting as PPAR-γ agonist: pioglitazone (**2a**, Actos™ or Glustin™, Takeda Pharmas USA and Eli Lilly), rosiglitazone (**2b**, Avandia™, GlaxoSmithKline), and lobeglitazone (**2c**, Duvie™, Chong Kun Dang), whose chemical structures are shown in Figure 5. Common for the chemical structure of TZDs compounds is the chiral centre at the C-5 of the ring, prone to racemization at physiological pH (Welch et al. 2003; Rippley et al. 2007; Jamali et al. 2008), as also shown in Figure 5. Consequently, in animal models, the individual enantiomers and racemates of glitazones appear to show equivalent activity as antidiabetic agents, so that most synthetic methodologies are conducted following conventional chemical steps (Ortiz and Sansinenea 2011).

However, Parks et al. (1998), through the *in vitro* analysis of rosiglitazone enantiomers in a PPARγ-binding assay, suggested that the (*S*)-isomer is the main responsible for the antidiabetic activity, and a similar behaviour of the (*S*)-eutomer was described for rivoglitazone, another glitazone which failed to reach the drug market (Izumi et al. 2013). On the other hand, pioglitazone is currently undergoing clinical trials for treatment of Alzheimer's disease (AD): when mice were dosed with racaemic pioglitazone, the concentration of (*R*)-(+)-pioglitazone was 46.6% higher than that of (*S*)-(−)-pioglitazone in brain tissue and 67.7% lower than that of (*S*)-pioglitazone in plasma, and dosing mice with pure (*R*)-pioglitazone led to a 76% increase in brain exposure levels compared to those from an equivalent dose of racaemic pioglitazone (Chang et al. 2015). Furthermore, pure (*R*)-pioglitazone was also shown to have comparable amyloid-lowering capabilities to the racaemic pioglitazone in an *in vitro* AD model, so that dosing with (*R*)-pioglitazone instead of the racaemic mixture may result in higher levels of brain exposure to

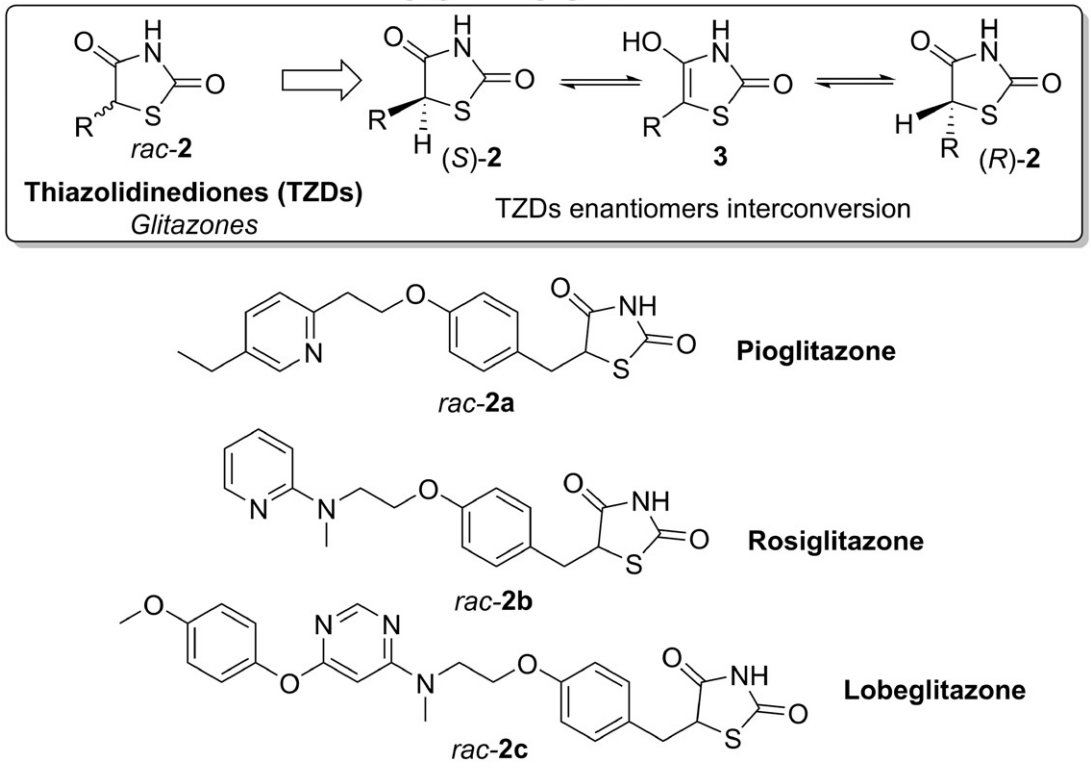

Figure 5. Chemical structure of glitazones.

pioglitazone, thus potentially improving the development of pioglitazone treatment of AD.

Thus, it is possible to find in literature some examples of the resolution of both enantiomer of glitazones, either through their derivatization to diastereoisomers (Sohda et al. 1984; Gahafu et al. 2010; van Niel et al. 2011a, 2011b) or by preparative chiral HPLC (Calixto and Bonato 2013). On the other hand, the first example of stereoselective biocatalytic synthesis was described by researchers at SmithKline Beecham by means of the bioreduction of the precursor benzylidine compound **4** (Figure 6), catalyzed by whole cells from red yeast *Rhodotorula rubra* CBS 6469 (Cantello et al. 1994).

These authors observed that the bioreduction proceeded at basic pH values with a high degree of stereoselectivity, but that the product was undergoing racemization, the rate of racemization being slower than the rate of product formation under these conditions. Thus, the biotransformation was carried out under acidic pH conditions. Over a 4 h reaction at pH 3.75, the product was found to be of >98% enantiomeric purity. These authors also described the use of alginate-entrapped immobilized cells for performing the bioreduction (Cantello et al. 1994), which was further scaled-up by some other scientists of the same company (Heath et al. 1997).

On the other hand, PPAR-α agonists serve as cellular receptor for fibrates, a class of drugs used in the treatment of dyslipidaemia, and also used for the treatment of vascular complications associated with Type 2 DM (Verges 2004; Steiner 2007). These fibrates possess a common chemical structure (2-methyl-2-aryloxypropionic acids or esters), not displaying any chiral centre. In an attempt to obtain an enantiopure PPAR-α agonist, Astra Zeneca synthesized AZD 4619 (Figure 7, (*S*)-**6**), an α agonist, by means of an enzymatic dynamic kinetic resolution (DKR) of the corresponding racaemic thioester, using an organic base to promote the racemization (Brown et al. 2006), as shown in Figure 7.

The thioester *rac*-**5** was resolved with *Pseudomonas cepacia* lipase in the presence of a tert-amine base, trioctylamine. The desired acid (*S*)-**6** is stable, and residual (*R*)-thioester was racemized by deprotonation and reprotonation catalyzed by the organic base, which cannot make a similar undesired racemization step of (*S*)-**6**, because the α protons of the carboxylate product are not acidic enough to be deprotonated by tert-amine bases. Although this process was scaled to grams, AZD 4619 was discontinued because of hepatotoxicity problems detected in Phase I (Thulin et al. 2008). Similarly, the resolution of the racaemic alpha-chloro thioester intermediate **7** has been described using the same strategy, shown in Figure 8 (Dow et al. 2012). Remarkably, the use of a protease instead of a lipase allowed the synthesis of the antipode (*R*)-**8**, although with a lower ee (90% with Savinase versus 98% with lipase).

Glitazars (Figure 9) are dual PPAR α/γ agonists that improve the lipid profile and exert an antidiabetic action, similar to a combination of a fibrate and a thiazolidinedione, so that they are considered as "two drugs in one" (Wilding 2012).

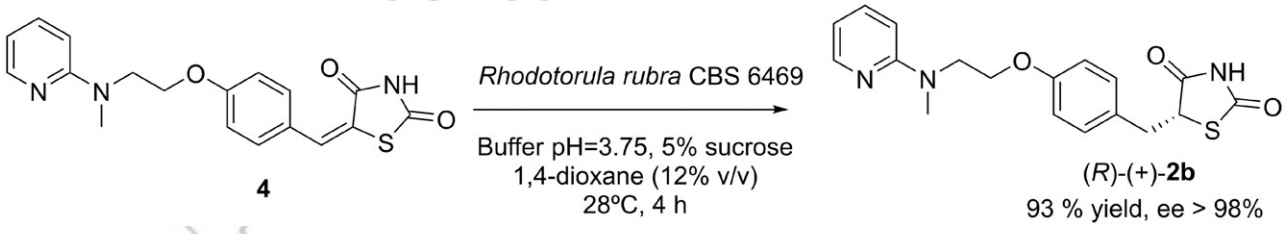

Figure 6. Biocatalyzed synthesis of (*R*)-(+)-rosiglitazone.

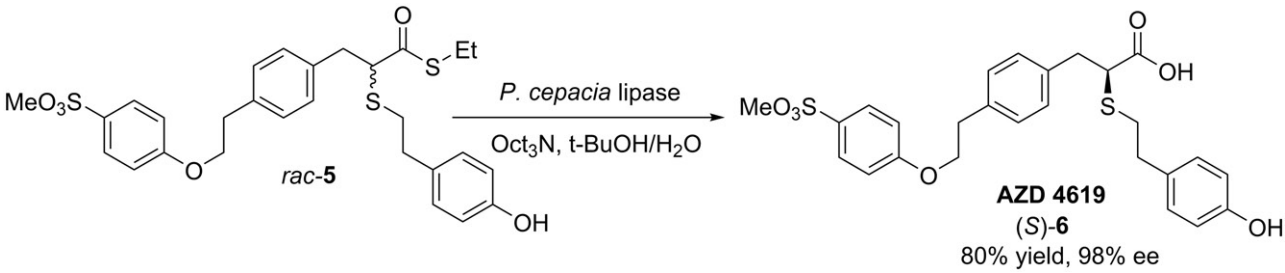

Figure 7. DKR process to synthesize AZD 4619.

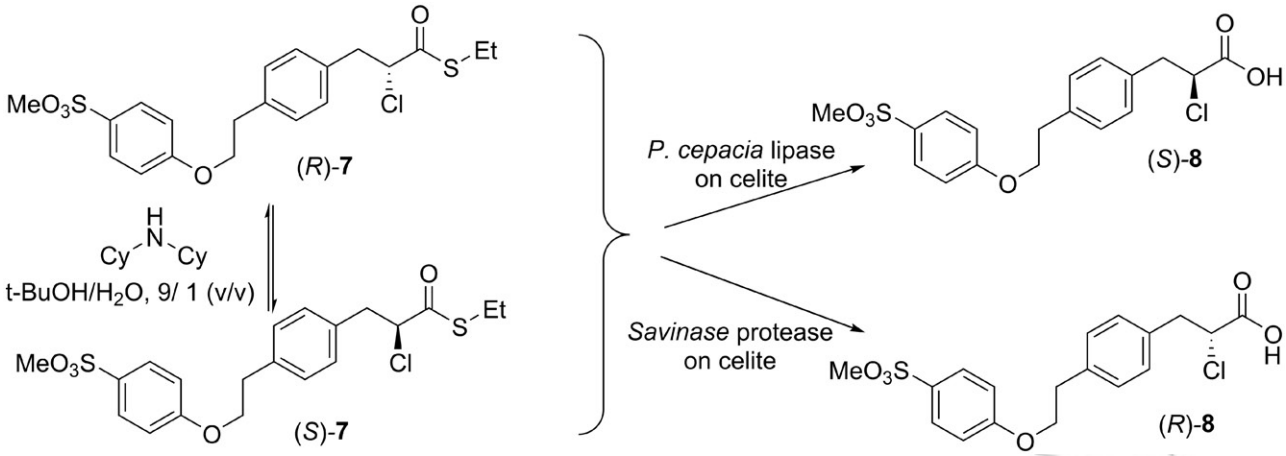

Figure 8. DKR process to synthesize a precursor of AZD 4619.

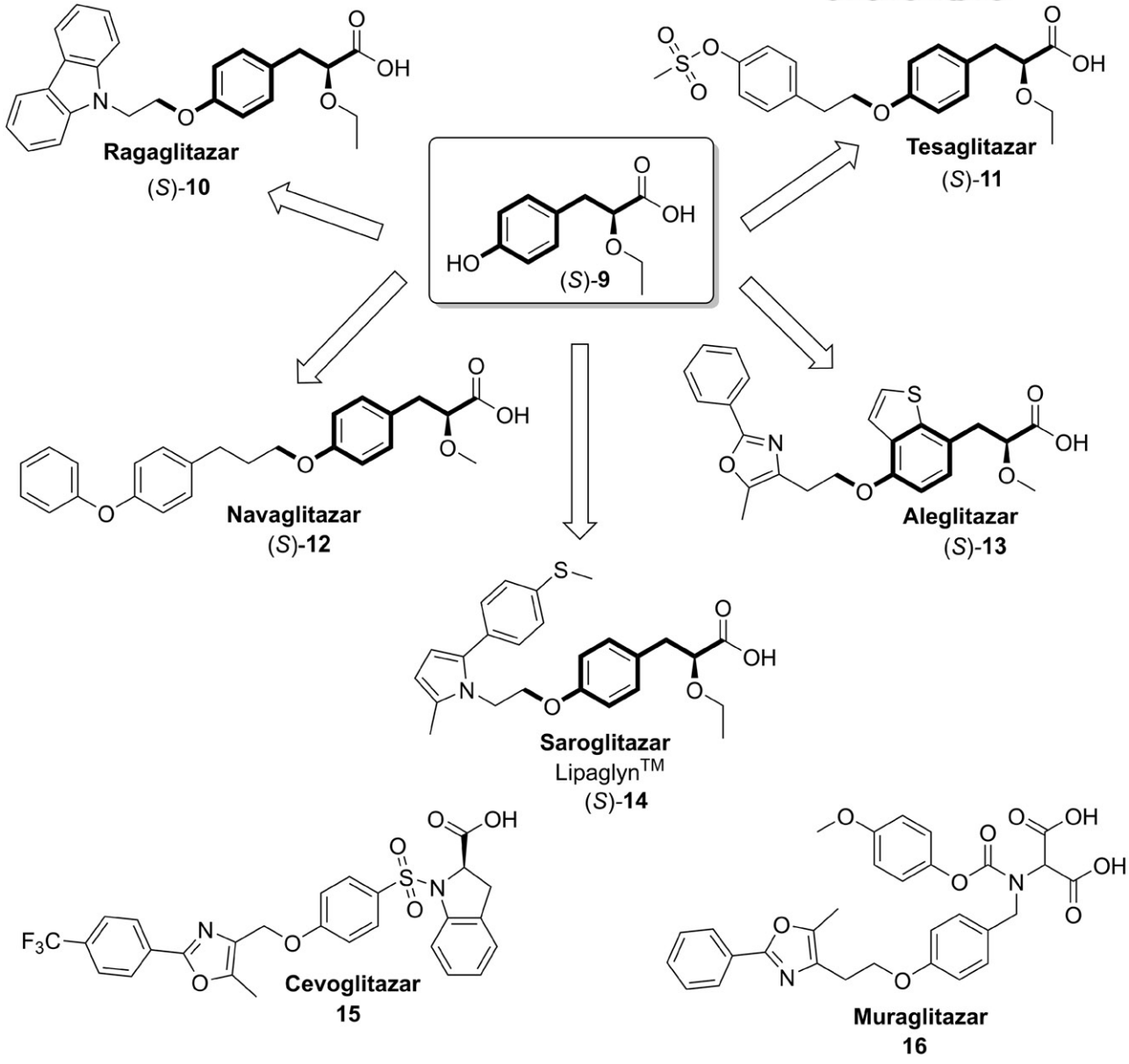

Figure 9. Some glitazars.

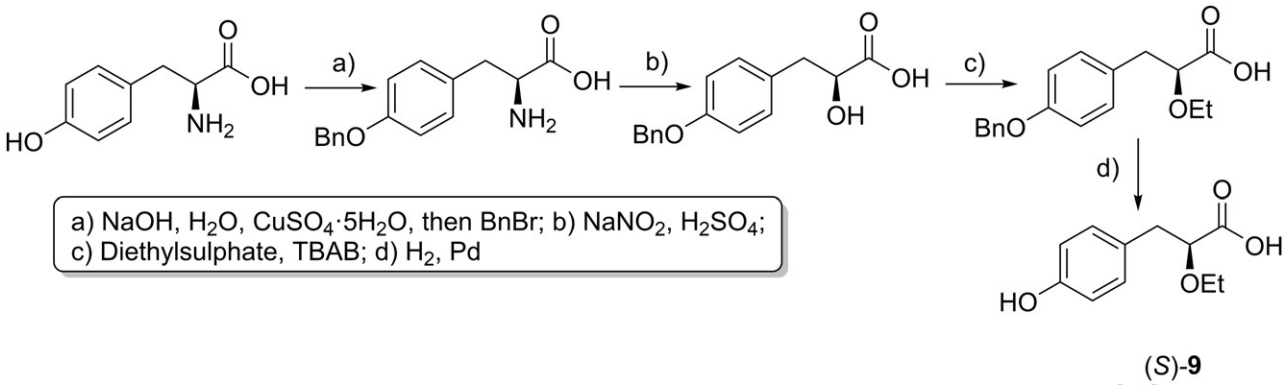

Figure 10. Chemical synthesis of (*S*)-2-ethoxy-3-(4-hydroxyphenyl)propanoic acid ((*S*)-**9**) starting form *L*-tyrosine.

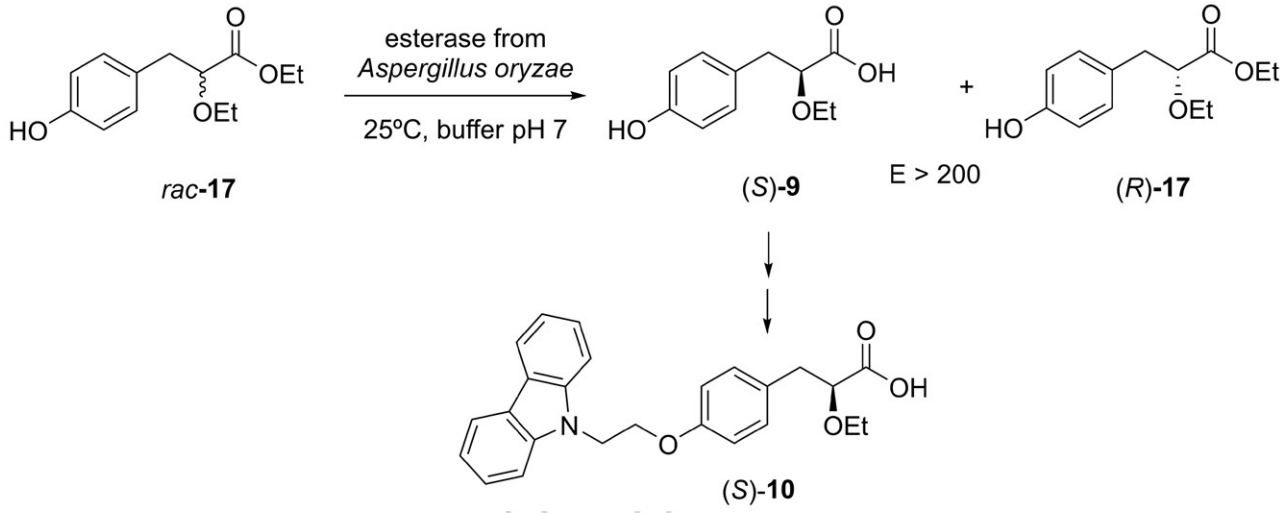

Figure 11. Enzymatic kinetic resolution of *rac*-**17** by an enantioselective hydrolysis.

Ragaglitazar (Figures 9 and 10) was discontinued by Novo Nordisk and Dr. Reddy's Laboratories because of its adverse effects after detecting urinary bladder tumour in mice. After different clinical trials, in May 2006 the two glitazars most advanced in development at that time, muraglitazar (**16**, Pargluva™, developed by Brystol Myers Squibb) and tesaglitazar (**11**, Galida™, Astra Zeneca) were discontinued. In fact, **16** was associated with an increased incidence of heart failure, while ragaglitazar **10** was associated with decreased glomerular filtration (Conlon 2006). Some other newer glitazars are aleglitazar **13**, from Hoffmann-La Roche (Wilding 2012), which has been discontinued in July 2013 after Phase III trials, and cevoglitazar **15** (Chen et al. 2010; LBM-642, from Novartis AG), which failed to pass Phase I. Very recently, in June 2013, the Indian company Zydus Cadila has presented Lipaglyn™ (Saroglitazar **14**), the first glitazar to be approved in the world, accepted for launch in India by the Drug Controller General of India (DCGI) for the treatment of diabetic dyslipidaemia or hypertriglyceridaemia in patients with Type II DM not controlled by statins alone (Agrawal 2014; Sharma et al. 2015; Dwivedi et al. 2015b).

As can be seen, the structure of (*S*)-2-ethoxy-3-(4-hydroxyphenyl)propanoic acid ((*S*)-**9**, shown in bold font in Figure 9) is common feature for many glitazars; its synthesis has been described starting from L-tyrosine (Dwivedi et al. 2014), in a rather dull 4-step methodology depicted in Figure 10.

Therefore, establishing an alternative greener methodology using biocatalyzed protocols would be highly desirable. In fact, in the synthesis of ragaglitazar (Figure 11), the key intermediate (*S*)-**9** was obtained through a very mild enantioselective hydrolysis of the racaemic ethyl ester **17**, catalyzed by an esterase from *Aspergillus oryzae*; this process was run on a 44 kg pilot

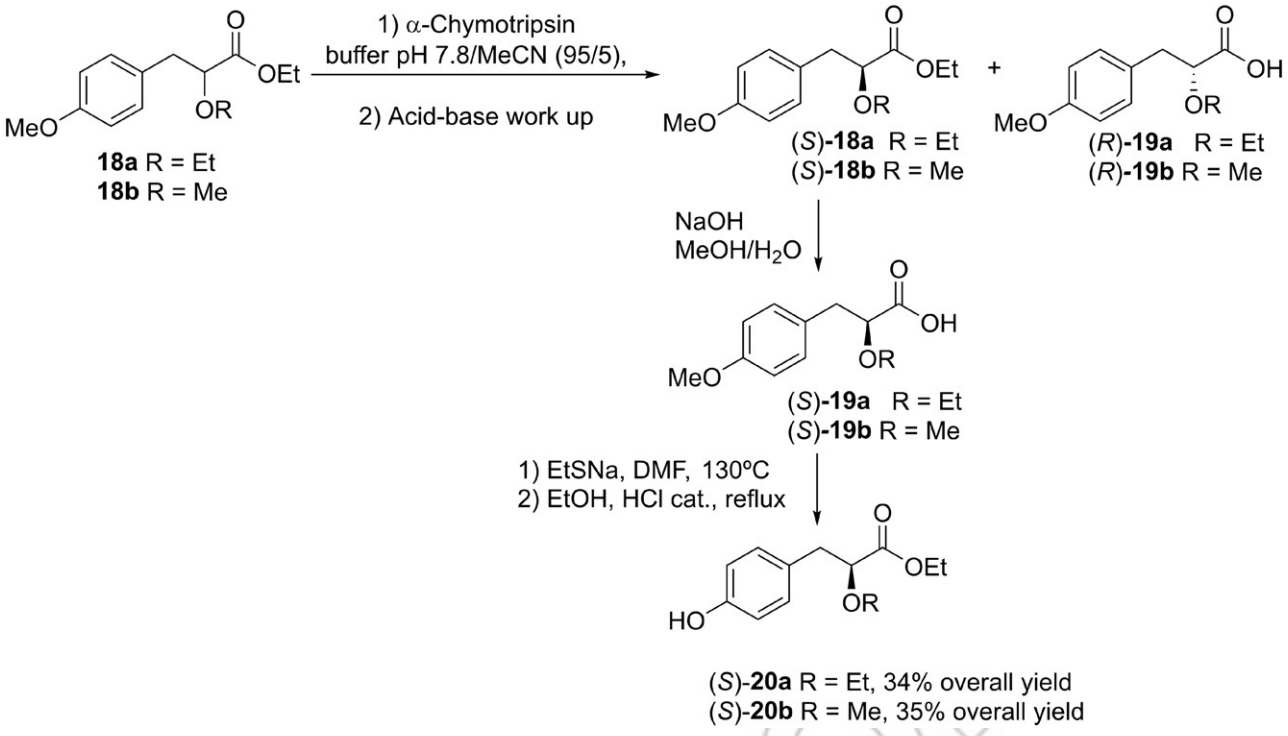

Figure 12. Enzymatic kinetic resolution of *rac*-**18a** and **18b** by an enantioselective hydrolysis.

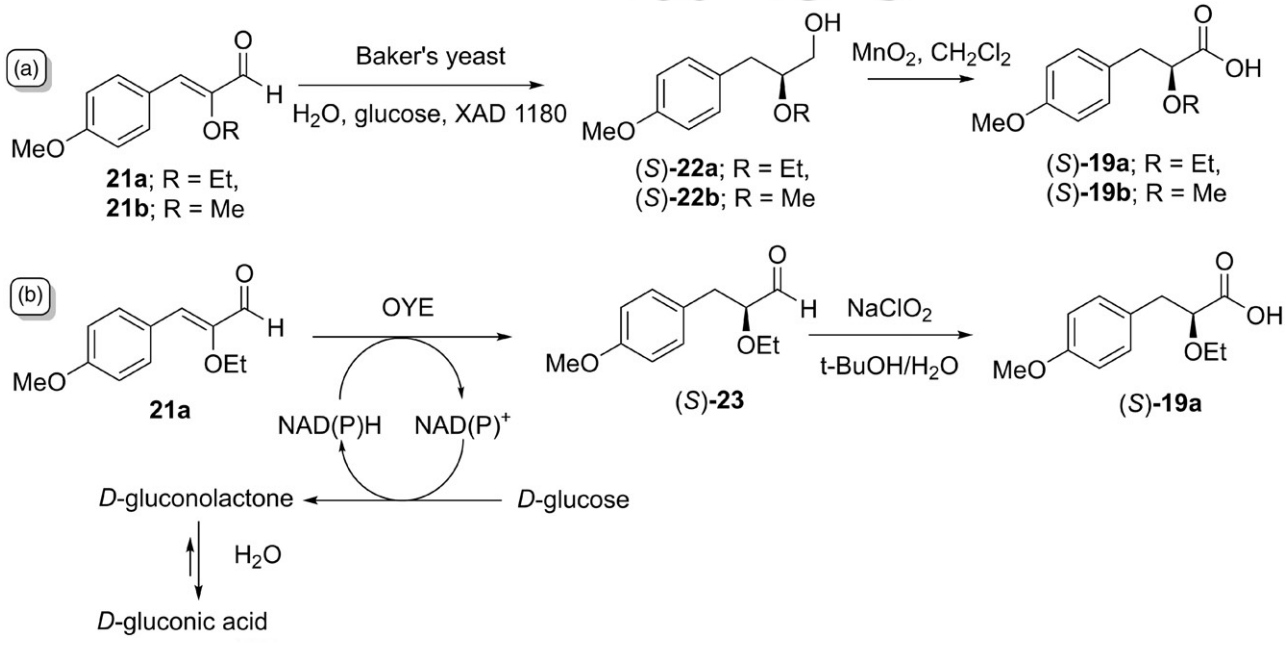

Figure 13. Preparation of enantiopure ethyl-(*S*)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP) (*S*)-**19a** by bioreduction.

scale, to produce enantiopure ragaglitazar (*S*)-**10** in 43-48% yields with ee values between 98.8% and 99.6% (Deussen et al. 2003).

On the other hand, Brenna and coworkers have reported two different biocatalytic approaches to obtain another enantiopure precursor for the preparation of glitazars. In a first strategy, the preparation of (*S*)-**20a** or (*S*)-**20b** was initiated by an α-chymotrypsin-catalyzed hydrolysis of racaemic **18a** or **18b**, and subsequent work up to finally obtain (*S*)-**20a** or (*S*)-**20b** with moderate overall yields (Brenna et al. 2009a) (Figure 12).

Due to the lower yield obtained, this research group decided to change to a reductase-catalyzed strategy, depicted in Figure 13(a), finally leading to the corresponding compounds (*S*)-**19** (after chemical

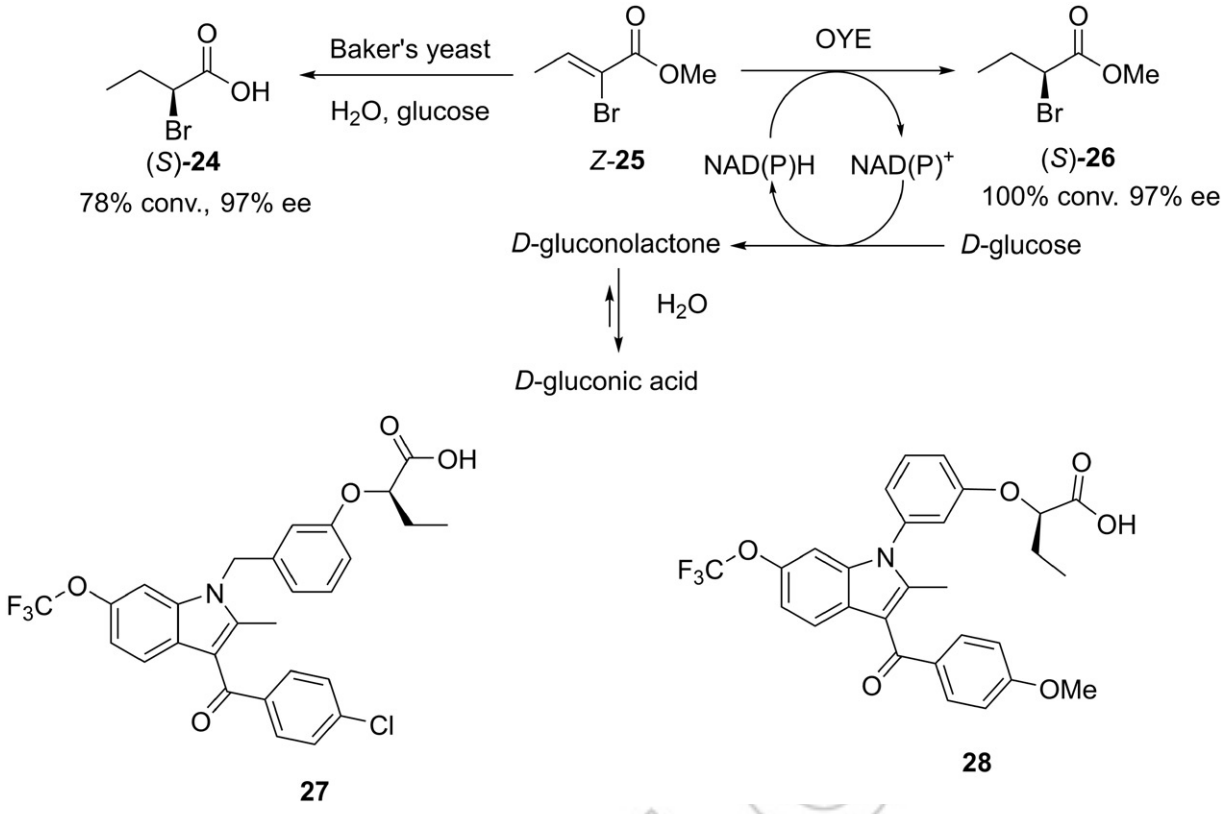

Figure 14. Preparation of enantiopure methyl (*S*)-2-bromobutyrate (*S*)-**26** and (*S*)-2-bromobutyric acid (*S*)-**24** by bioreduction.

oxidation of (*S*)-22) in good yield of 78% and an excellent ee of 99%, using baker's yeast and an *in situ* substrate feeding product removal (SFPR) technique (Brenna et al. 2009b). Nevertheless, it suffers from (i) an extremely low productivity (0.39 g L^{-1} d^{-1}), (ii) a nonquantitative conversion, (iii) a complex purification process based on the chemoselective oxidation of the byproduct, the undesired allylic alcohol, and (iv) a useless and counterproductive reduction of the carbonyl group (by alcohol dehydrogenases present in baker's yeast) since the final target is an ester. Therefore, Brenna and coworkers improved the methodology (Figure 13(b)) by using a genetically engineered enereductase (Old Yellow enzyme, OYE) from *S. cerevisiae* expressed in *E. coli*, and glucose dehydrogenase for cofactor regeneration; thus, it was possible to improve the productivity (up to 55.6 g L^{-1} d^{-1}, 74% yield, 98% ee), at gram scale (Bechtold et al. 2012) in the preparation of the desired ethyl-(*S*)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP) (*S*)-**19a**, ultimately obtained through chemical oxidation of intermediate aldehyde (*S*)-23.

Using this same cloned OYE, a similar strategy (Figure 14) has been developed by the same group (Brenna et al. 2012) for the preparation of enantiopure methyl 2-bromobutyrate (*S*)-**26** (100% conversion, 97% ee) starting from the corresponding *Z*-α,β-insaturated ester **25**. By using baker's yeast, it was possible to prepare the corresponding (*S*)-2-bromobutyric acid (*S*)-24 (78% conversion, 97% ee) starting from the same substrate. These enantiopure molecules can be useful as chiral building blocks in the preparation of compounds such as **27** (Liu et al. 2009) or **28** (Acton et al. 2009), 3-acyl-1-(phenyl or benzyl)indolecarboxylic acids which were also tested as PPAR-γ modulators (Pirat et al. 2012).

Secretagogues

These are drugs that increase insulin output from the pancreas, and they can be divided in two main types: sulfonylureas and non-sulfonylureas secretagogues. As long as most of sulfonylureas do not possess any chiral centre in their structures, their syntheses are carried out following classical chemical methodologies.

There are different types of non-sulfonylureas secretagogues. For instance, meglitinides, which bind to an ATP-dependent K^+ (KATP) channel on the cell membrane of pancreatic beta cells in a similar manner to sulfonylureas (Proks et al. 2002), although with weaker binding affinity and faster dissociation (Dornhorst 2001). The only described example in which a biocatalyzed protocol is employed in the synthesis of this type of compounds is the preparation of (*S*)-(+)-3-

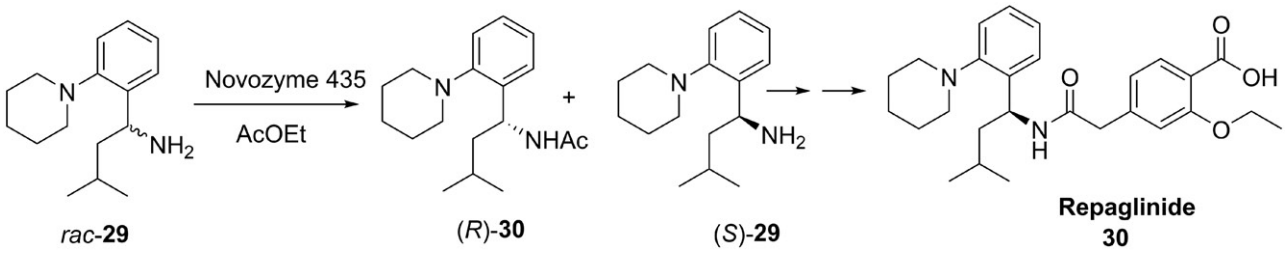

Figure 15. Preparation of enantiopure (*S*)-(+)-3-methyl-1-(2-(1-piperidinyl)phenyl)butylamine (*S*)-**29**.

methyl-1-(2-(1-piperidinyl)phenyl)butylamine (*S*)-**29** through enzymatic catalysis, as described in Figure 15 (Zhao et al. 2010).

This reaction was carried out in AcOEt, acting as solvent and acyl donor, and the enzyme used was Novozyme 435, a commercial preparation of immobilized lipase from *Candida antarctica*. The yields were not very high, although the use of an immobilized biocatalyst allows its recycling to afford pure (*S*)-**29** and the subsequent synthesis of repaglinide **31** (Prandin™, Novo Nordisk) (Scott 2012).

GLP-1 analogues

The main role of pancreatic β cells, to synthesize and secrete insulin, is somewhat modulated by a group of heterotrimeric G proteins, which are the immediate downstream targets of diverse G protein-coupled receptors (GPCRs). Hence, different GPCRs expressed by pancreatic β cells regulate insulin secretion, and therefore these compounds, acting as insulin secretagogues, are potential therapeutic targets for treating Type 2 DM (Ahren 2009; Lovshin and Drucker 2009). One of them is the receptor for the glucagon-like peptide-1 (GLP-1R), which binds to and is activated by glucagon-like peptide-1 (GLP-1), a 30 amino acid residue peptide, originated from preproglucagon, synthesized in the L-cells in the distal ileum, in the pancreas and in the brain. GLP-1 is member of the incretin hormone family, a term that refers to the observation that orally administered glucose results in a larger increase in plasma insulin levels and insulin-dependent decrease in blood glucose concentration when compared to the same amount of glucose given intravenously (Rondas et al. 2013). Then, injectable GLP-1 mimetics (synthetic polypeptides such as exenatide (Amylin Pharmaceuticals, Byetta™/Bydureon™), liraglutide (Novo Nordisk, Victoza™, Saxenda™), lixisenatide (Sanofi, Lyxumia™), albiglutide (GSK, Tanzeum™), dulaglutide (Eli Lilly, Trulicity™), taspoglutide (phase III halted Sept 2010) or semaglutide are used in the treatment Type 2 DM, displaying an advantage over older insulin secretagogues, such as sulfonylureas or meglitinides, due to their lower risk of causing hypoglycaemia (Garber 2012).

Like so, (*S*)-2-amino-3-(6-o-tolylpyridin-3-yl)propanoic acid (*S*)-**32**, Figure 16, is a key intermediate needed for synthesis of GLP-1 mimics or GLP-1 receptor modulators: its synthesis has been described using three different enzymatic procedures (Chen et al. 2011); in the first one, depicted in Figure 16, (*S*)-**32** was prepared (gram scale) in 68% solution yield and 54% isolated yield (100% ee), starting from racaemic **32** using a recombinant (*R*)-amino acid oxidase from *Trigonopsis variabilis*, cloned and overexpressed in *E. coli* and then immobilized on Celite, and an (*S*)-amino acid dehydrogenase from *Sporosarcina ureae*. The cofactor NADH required for the reductive amination reaction was regenerated using formate and formate dehydrogenase (FDH).

In a second strategy (Figure 17), (*S*)-**32** could be prepared in 73% isolated yield with 99.9% ee from racaemic amino acid using the same initial enzyme, (*R*)-amino acid oxidase from *T. variabilis* expressed in *E. coli*, but now in combination with an (*S*)-aminotransferase (purified from a soil organism identified as *Burkholderia* sp., also cloned and expressed in *E. coli*), and using (*S*)-aspartate as amino donor. This procedure had the advantage that both enzymes could be added at the start of reaction in a one-pot system, and several batches containing 9.11 g (15 g of the monosulfate monohydrate) of *rac*-**32** were run in a 2-L reactor at 30 °C 22 h, to produce 85% yield (73% after crystallization, ee 99.9%). Hence, the reaction was scaled up with 607 g (1 kg of the monosulfate monohydrate) of *rac*-**32** to give a 66% isolated yield of (*S*)-**32** as the monosulfate monohydrate (ee 99.9%).

Finally, a cyclic deracemization of *rac*-**32** by (*R*)-selective oxidation was developed using Celite-immobilized (*R*)-amino acid oxidase, in combination with chemical imine reduction using the borane-ammonia complex (Figure 18). Before the imine **34** (bounded to the enzyme as initial product of the oxidase reaction) gets hydrolyzed to the keto acid **31**, the

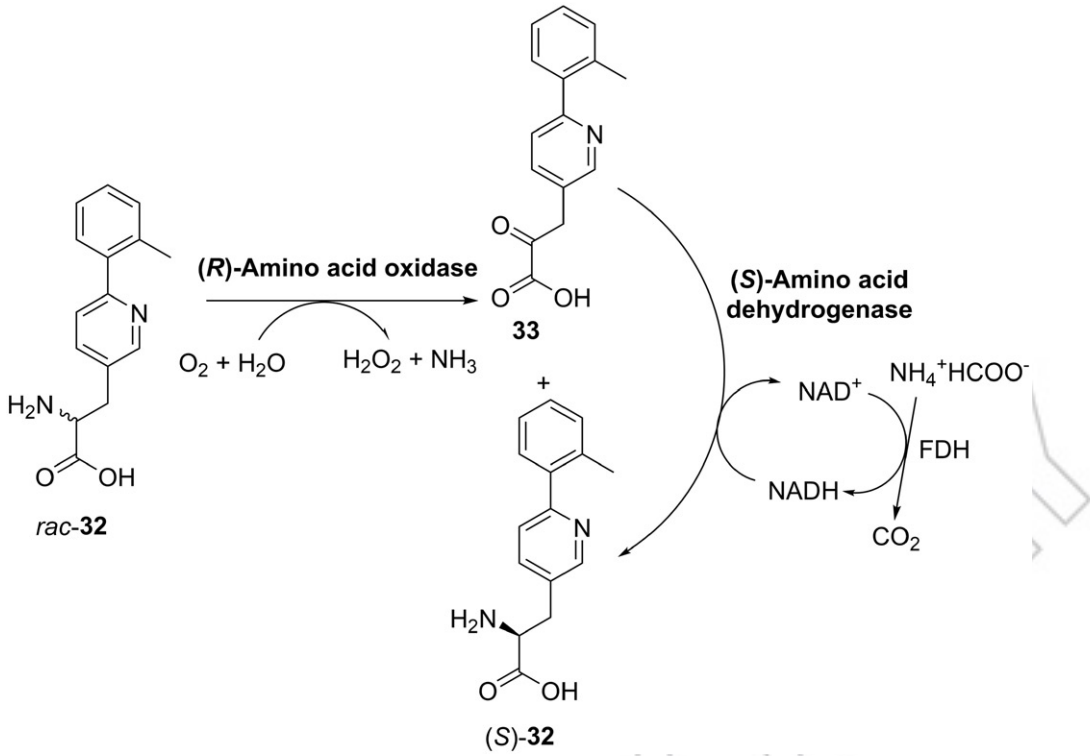

Figure 16. Synthesis of (*S*)-**32** using a (*R*)-amino acid oxidase and an (*S*)-amino acid dehydrogenase.

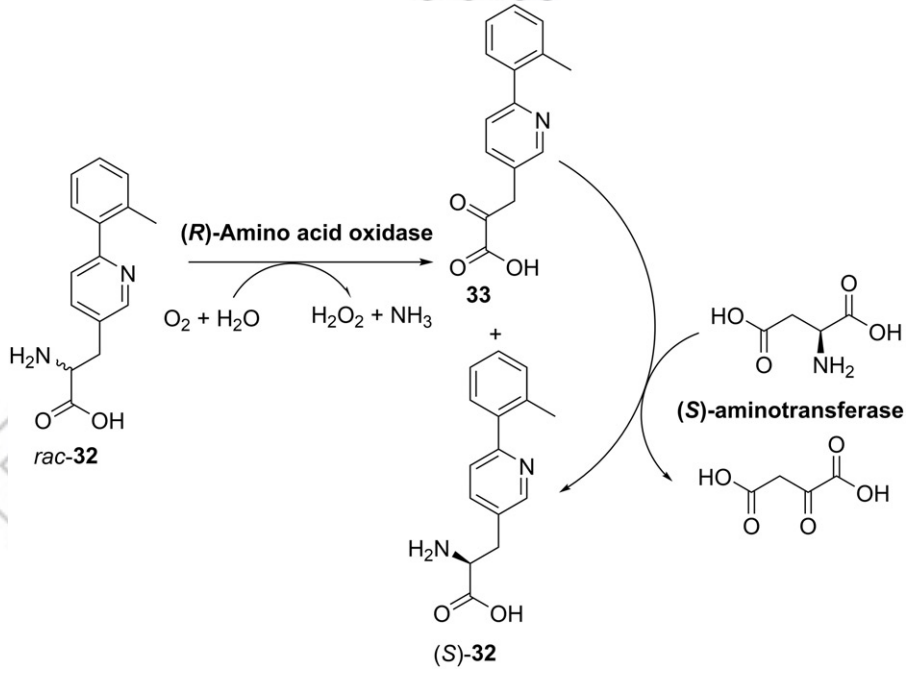

Figure 17. Synthesis of (*S*)-**32** using a (*R*)-amino acid oxidase and an (*S*)-aminotransferase.

borane-ammonia reduces it to regenerate *rac*-**32** in a dynamic process. Through this strategy, a maximum yield of 76–79%, was obtained at pH 6.0–7.0, with ee values reaching >99.9% at pH 6–8 using 10 equiv. of the borane–ammonia complex.

GPR119 agonists

The GPCR 119 (GPR119, also named "glucose-dependent insulinotropic receptor") is a recent potential target for the development of oral antidiabetic drugs (Overton et al. 2008; Ritter et al. 2016). In fact, more

than 20 pharmaceutical companies have been developing GPR119 agonists, but many clinical candidates have been discontinued for different reasons not always explained (Buzard et al. 2012; Ritter et al. 2016). For instance, Bristol-Myers Squibb, after disclosing some pyridone, pyridazone, benzothiazole, dihydrobenzofuran, bicyclic pyrimidines and piperidinyl sulfone GPR119 agonists (Wacker et al. 2014; Ye et al. 2014), is working now on some new

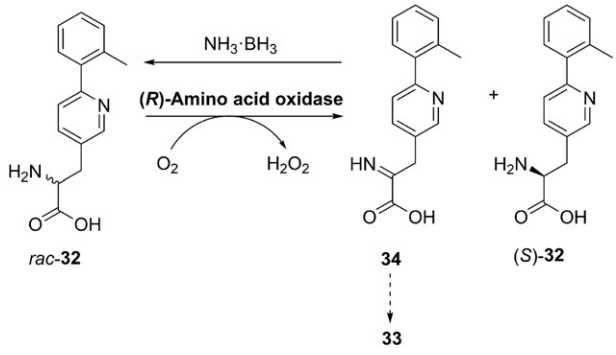

Figure 18. Synthesis of (*S*)-**32** using an (*R*)-amino acid oxidase combined with a chemical racemization protocol.

pyrimidinylpiperidinyloxypyridone analogues **39** (Figure 19) possessing chirality (Broekema et al. 2013), and for this purpose, an scalable synthesis of enantiomers of *N*-substituted 3-hydroxypyrrolidin-2-ones have been recently reported (Singh et al. 2015), as shown in Figure 19.

Thus, lipase PS 30 from *P. cepacia* immobilized on polypropylene catalyzed the enantioselective esterification of racaemic-1-(2-fluoro-4-iodophenyl)-3-hydroxypyrrolidin-2-one **35** with succinic anhydride and 2-methyltetrahydrofuran at 4 °C, leading to (*S*)-**35** in high enantiomeric excess >99% and yield ~40%, after an easy separation from (*R*)-**36** (Singh et al. 2015). Following the initial experiments, it was possible to scale-up to 50 g/L of substrate; in this process, after 8.5 h, immobilized enzyme was removed by simple filtration, and (*S*)-**35** was isolated in ~40% yield and ee >99%. 2-Methyltetrahydrofuran, a green biosolvent (Pace et al. 2012, 2014) served as a reaction medium and a solvent to extract the desired compound from the reaction mixture, eliminating the use of chromatography. On the other hand, Novozyme 435 (*C. antarctica* lipase B) was employed for the resolution of

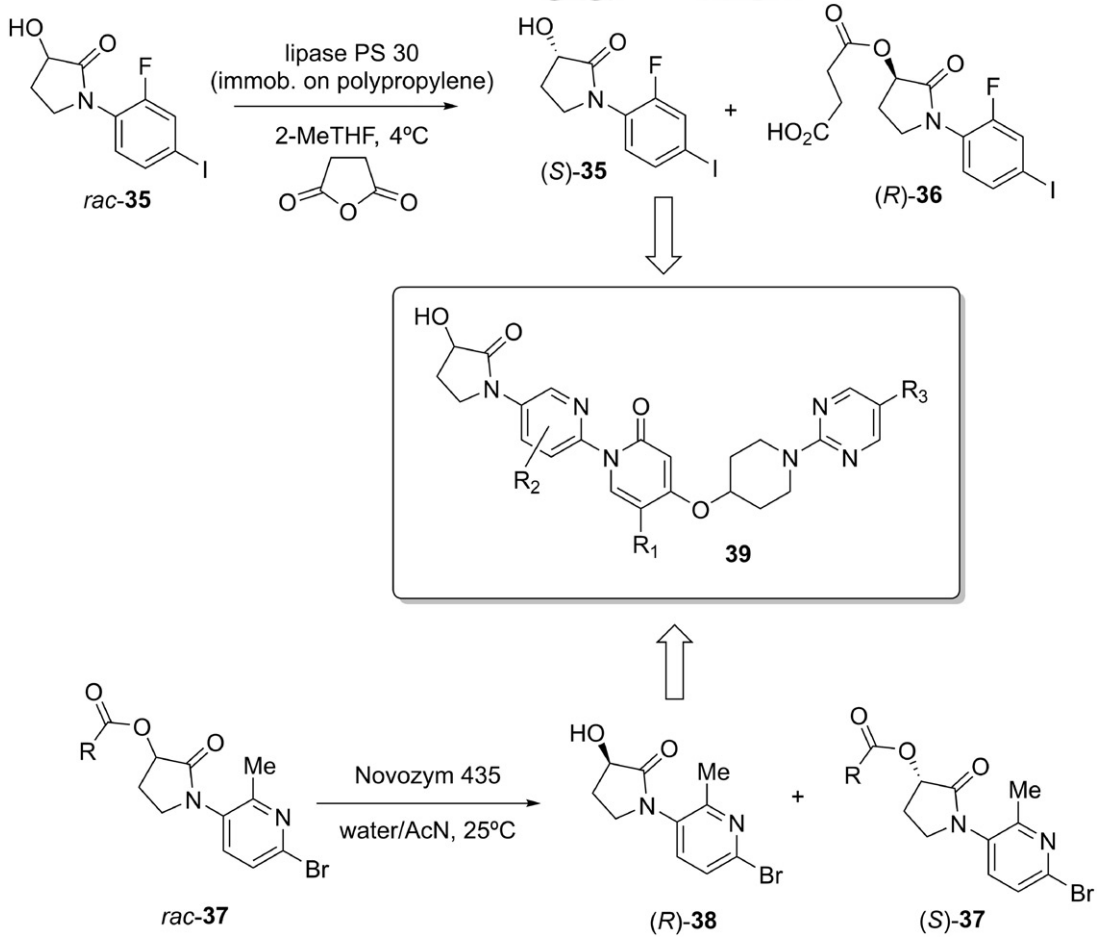

Figure 19. Enzymatic resolution of *N*-substituted 3-hydroxypyrrolidin-2-ones.

racaemic acetate **37** for obtaining the desired alcohol (*R*)-**38** in ~37% isolated yield and high enantiomeric excess (ee >99.4%). This process was scaled up to kg scale (two successive campaigns (4.1 kg, ee >99.4% and 5.5 kg, ee >99.5%), and the major disadvantage of a kinetic resolution (using only 50% of starting material) was overcome by recycling the undesired enantiomer into the desired enantiomer via Mitsunobu inversion (probed at gram scale) (Singh et al. 2015).

Enzyme inhibitors

The inhibition of enzymes involved in metabolic pathways is undoubtedly one of the most active areas inside Medicinal Chemistry (Harriman et al. 2010; Copeland 2013). There are many enzymatic processes which inhibition could lead to a beneficial effect on patients suffering from diabetes; in fact, two main types of enzyme inhibitors, such as α-glucosidase inhibitors and dipeptidyl peptidase-4 (DPP-4) inhibitors have already been commercialized and frequently prescribed, while some others are still under evaluation at different (pre)clinical stages. In the following sections, we will describe both types, showing how biocatalysis can help in the synthesis of their chemical structures.

α-Glucosidase inhibitors

It is well known that inhibitors of intestinal α-glucosidase enzymes promote a delay in the absorption of sugars because of the retard in the final steps of carbohydrate digestion, so that they are useful for reducing postprandial hyperglycaemia in DM (Derosa and Maffioli 2012; Campo et al. 2013). These α-glucosidase inhibitors act as glycomimetics, because they bear a certain grade of resemblance to the natural carbohydrates, but the differential part of their structure promotes a blockade of enzymatic action (Ernst and Magnani 2009). The use of iminosugars (N atom replacing O) (Winchester 2009; Horne et al. 2011), thiosugars (S instead of O) (Witczak and Culhane 2005) or carbasugars (ethereal bridge substituted by a methylene) (Mayato et al. 2012) as glycomimetics is a well-developed strategy. More specifically, iminosugars mimics transition state (oxocarbenium) of glycosidases mechanism, due to the nitrogen protonation at physiological pH values (Caines et al. 2007; Winchester 2009). Recently, Alcántara and coworkers published a review covering chemo-enzymatic protocols for synthesizing this type of glycomimetics (Alcántara et al. 2014). These authors described the biocatalyzed synthesis of different iminocyclitols, such as the polyhydroxylated piperidine 1-deoxynojirimycin (DNJ, Figure 20, **40**), precursor of the commercialized Miglitol **41** (Glyset™, Pfizer) or Miglustat **42** (Zavesca™, Actelion). Other type of iminocyclitols described in that review by Alcántara and coworkers are polyhydroxylated pyrrolidines, such as 2,5-dideoxy-2,5-imino-D-mannitol **43**, commonly known as DMDP, found in many plants and microorganisms, have been studied for their antihyperglycaemic properties (Horne et al. 2011). A representative member of polyhydroxylates indolizidine **44** is the toxic alkaloid castanospermine, a potent inhibitor of lysosomal α-glucosidase (Lahiri et al. 2013). DNJ, DMDP, and castanospermine also inhibit glycoprotein-processing enzymes to varying degrees (Asano et al. 2000). Casuarine **45** is an example of a pyrrolizidines, which are also isolated from plants and have been used in the treatment of breast cancer, diabetes, and bacterial infections (Wardrop and Waidyarachchi 2010). Finally, Calystegine A_3 **46** belongs to nortropane-type alkaloids possessing glycosidase inhibitory activity (Asano et al. 2000).

The chemoenzymatic preparation of another type of α-glucosidase inhibitors, aminocyclitols, are also described in the above-mentioned review (Alcántara et al. 2014), illustrating biocatalyzed protocols for synthesizing Voglibose **47** (Voglib™, marketed by Mascot Health Series), or Acarbose **48**, generic sold in Europe and China as Glucobay™ (Bayer AG), in North America as Precose™ (Bayer Pharmaceuticals), and in Canada as Prandase™ (Bayer AG).

DPP-4 inhibitors

DPP-4, also known as adenosine deaminase complexing protein 2 or CD26 (cluster of differentiation 26) is a homodimer protein consisting of 766 amino acids with cytoplasmic, transmembrane, and extracellular regions, playing a pivotal role in glucose metabolism, because it is responsible for the degradation of the GLP-1 incretins previously mentioned. In fact, DPP-4 is a serine exodipeptidase highly specific in recognizing peptide substrates with proline or alanine in the last position (P1) prior to the scissile amide bond of the N-terminal of incretins (Deacon and Holst 2013).

Thus, DPP4 inhibitors are a pharmacological class of glucose-lowering agents that open up new perspectives for Type 2 DM treatment because of their unique mechanism of action (Scheen 2012; Deacon and Holst 2013; Mize and Salehi 2013). Furthermore, recently the cardioprotective effects of these compounds have been described (Dai et al. 2013; Wang et al. 2013; Juillerat-Jeanneret 2014), so that this type of drugs, called generically gliptins and shown in Figure 21, are becoming increasingly more studied

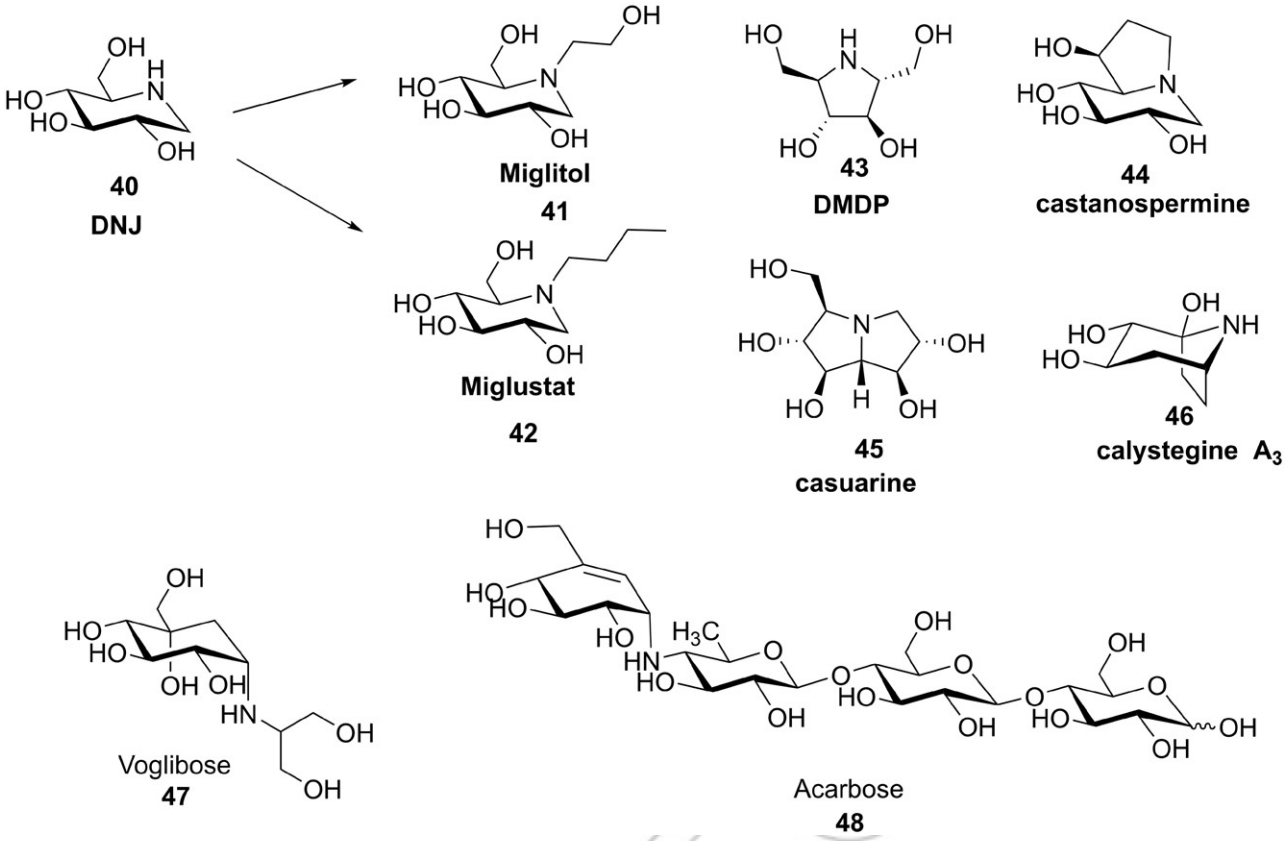

Figure 20. Some inhibitors of α-glucosidases.

(Mittermayer et al. 2015; Cahn et al. 2016; Doggrell and Dimmitt 2016; Thomas et al. 2016), as they are commonly used as second-line therapy for diabetes in high-income regions (Cahn et al. 2016).

Although in many cases, purely chemical syntheses have been described for the preparation of gliptins, there are some very attractive examples of biocatalyzed protocols for preparing the homochiral building blocks required for their synthesis; in some cases, the chirality comes directly from commercially available proline, which is transformed either into (*S*)-pyrrolidine 2-carbonitrile, as for the preparation of anagliptin **56** (Kato et al. 2011), or into the corresponding proline amide, as required for the synthesis of Vildagliptin **50** (Pellegatti and Sedelmeier 2015). In other cases, chirality comes from other compounds, and these examples will be commented in the following paragraphs.

Sitagliptin. Sitagliptin (Figure 21, **49**) (sold under the trade name Januvia™ by Merck Sharp & Dhome) was the first marketed oral antihyperglycaemic drug belonging to the gliptin family (Aroda et al. 2012). Sitagliptin can be used either alone or combined with metformin or thiazolidinedione, another oral antihyperglycaemic agents in the treatment of Type 2 DM, already commented before (Kim et al. 2005). Sitagliptin is the most widely sold DPP-4 inhibitors in the USA and worldwide, reaching sales of US$6358 million in 2014 with an expected rise to 7525 in 2020. Sitagliptin was the second leading antidiabetic product in 2014, after insulin glargine, and is predicted to be the leading product by 2020 (Cahn et al. 2016; Fernandes et al. 2016).

The first chemical synthesis of sitagliptin (Hansen et al. 2009) involved in asymmetric hydrogenation of an enamine **65** using a rhodium-based chiral catalyst (Rh[Josiphos]) at high pressure (Figure 22); nevertheless, this process is not stereoselective enough (97% ee), and the final product is contaminated with rhodium, so that different additional purification steps are required. Some other chemical syntheses have been recently reviewed by Davies et al. (2015).

Nevertheless, an enzymatic process has substantially improved the efficiency of sitagliptin manufacturing (Savile et al. 2010a, 2010b); in fact, using an engineered transaminase, developed at Codexis by rational design, a biocatalyst with broad applicability towards the synthesis of chiral amines was obtained. Under optimal conditions, the best variant converted 200 g/L prositagliptin ketone **64** (Figure 22) to sitagliptin **49** with a 92% yield and an enantiomeric excess higher that 99%, by using 6 g/L enzyme in 50% dimethyl

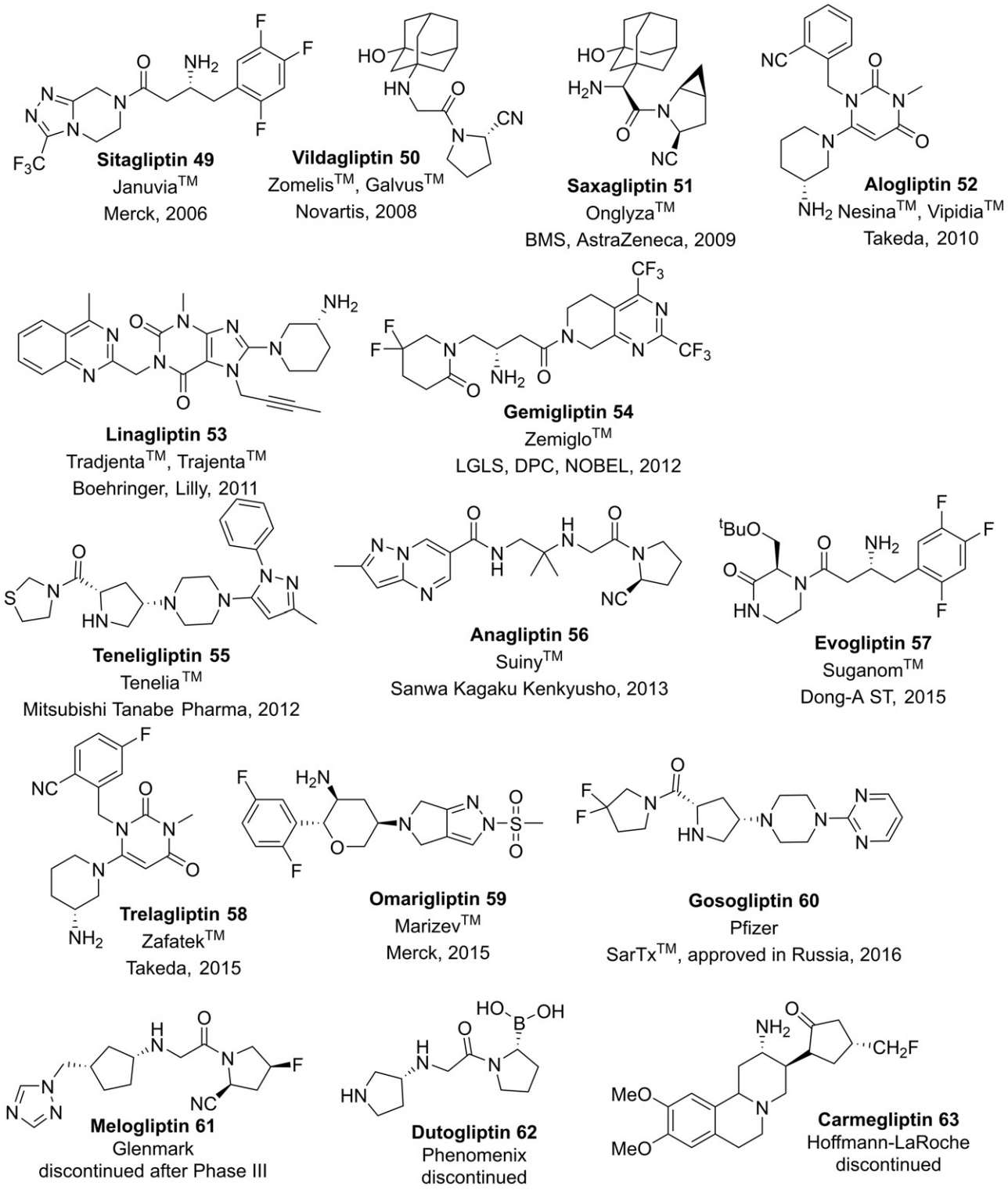

Figure 21. Chemical structure of several DPP4 inhibitors registered for clinical uses (**49**–**60**) and some other discontinued ones (**61**–**63**).

sulfoxide. The biocatalytic process provides sitagliptin with a 10–13% increase in overall yield compared to the chemical process, a 53% increase in productivity (kg/L per day), a 19% reduction in total waste, the elimination of all heavy metals, and a reduction in total manufacturing cost. Furthermore, the enzymatic reaction is run in multipurpose vessels, so that specialized high pressure hydrogenation equipment is no longer needed. Full details of this process, which obtained the Presidential Green Chemistry Challenge

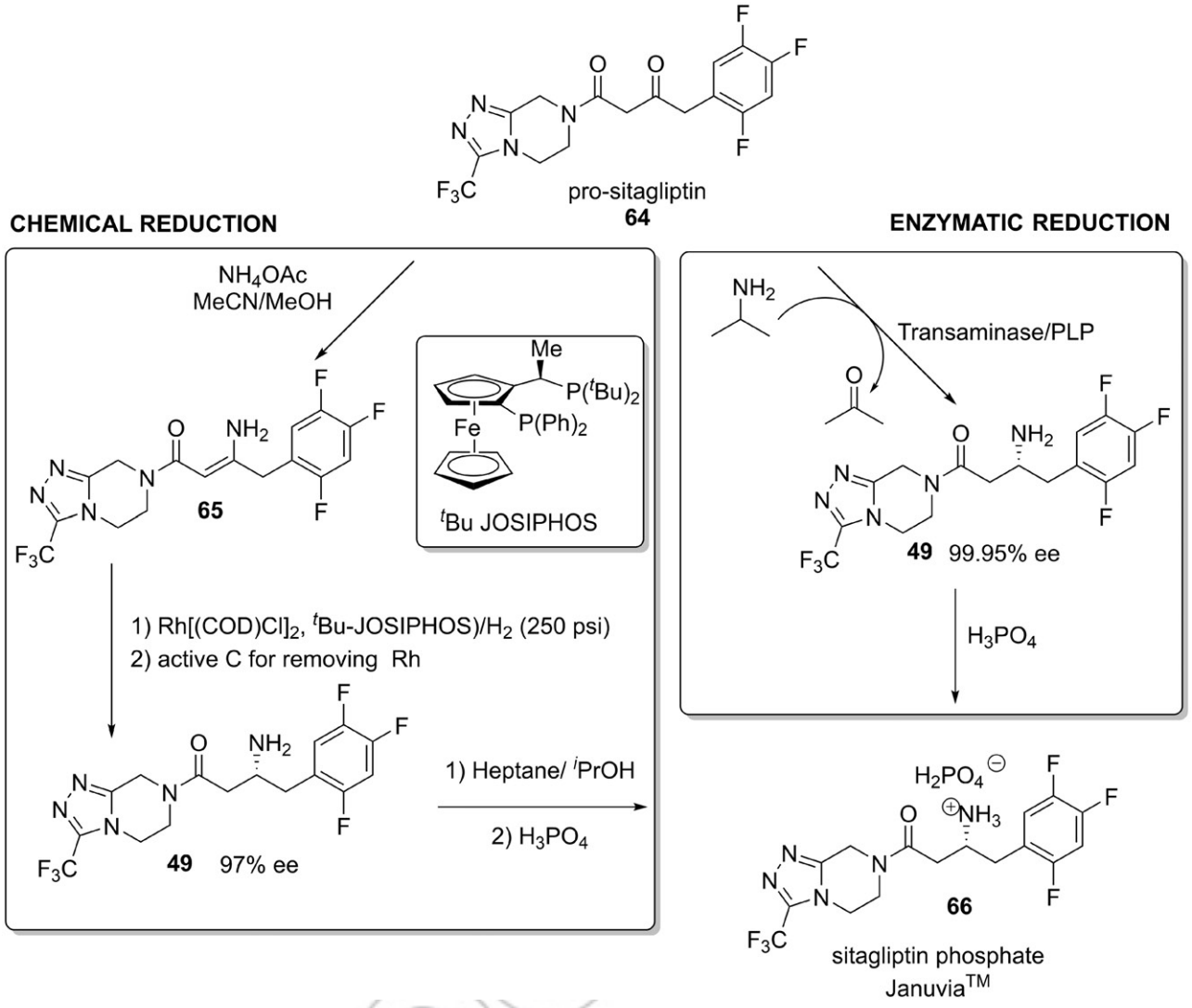

Figure 22. Chemical versus biocatalyzed synthesis of Januvia™, sitagliptin phosphate **66**.

Award (Greener Reaction Conditions Award) from the U.S. Environmental Protection Agency (EPA) in 2010 (http://www.epa.gov/greenchemistry/pubs/pgcc/past.html), can be found in literature (Moore et al. 2012; Willies et al. 2012; Busto et al. 2016). Since this innovative approach of biocatalyzed synthesis of sitagliptin using transaminases, other similar examples have been described (Hou et al. 2016; Wei et al. 2016).

Saxagliptin. Saxagliptin (Onglyza™, **51**) is another inhibitor of DPP-4 developed by Bristol-Myers Squibb (Kania et al. 2011). This compound inhibits DDP-4 by covalent bonding to the catalytic serine presented in DDP-4 active site (Aroda et al. 2012). Its synthesis (Savage et al. 2009) requires (*S*)-*N*-Boc-3-hydroxyadamantylglycine **69** as a key chiral intermediate. For its preparation, a process for conversion of the keto acid **67** to the corresponding amino acid **68** using (*S*)-amino acid dehydrogenases was developed, as depicted in Figure 23. A modified form of a recombinant phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* as well as in *E. coli* was used for this process development and scale-up. NAD^+ produced during the reaction was recycled to NADH using FDH cloned and overexpressed in *E. coli*. The modified phenylalanine dehydrogenase contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus (Hanson et al. 2007). The production of multikilogram batches was originally carried out with extracts of *P. pastoris* expressing the modified phenylalanine dehydrogenase from *T. intermedius* and endogenous FDH. The reductive amination process was further scaled up using a preparation of the two enzymes, FDH and phenylalanine dehydrogenase, expressed in a single recombinant *E. coli*. The amino acid **68** was directly protected as its Boc derivative **69** without isolation to afford the intermediate.

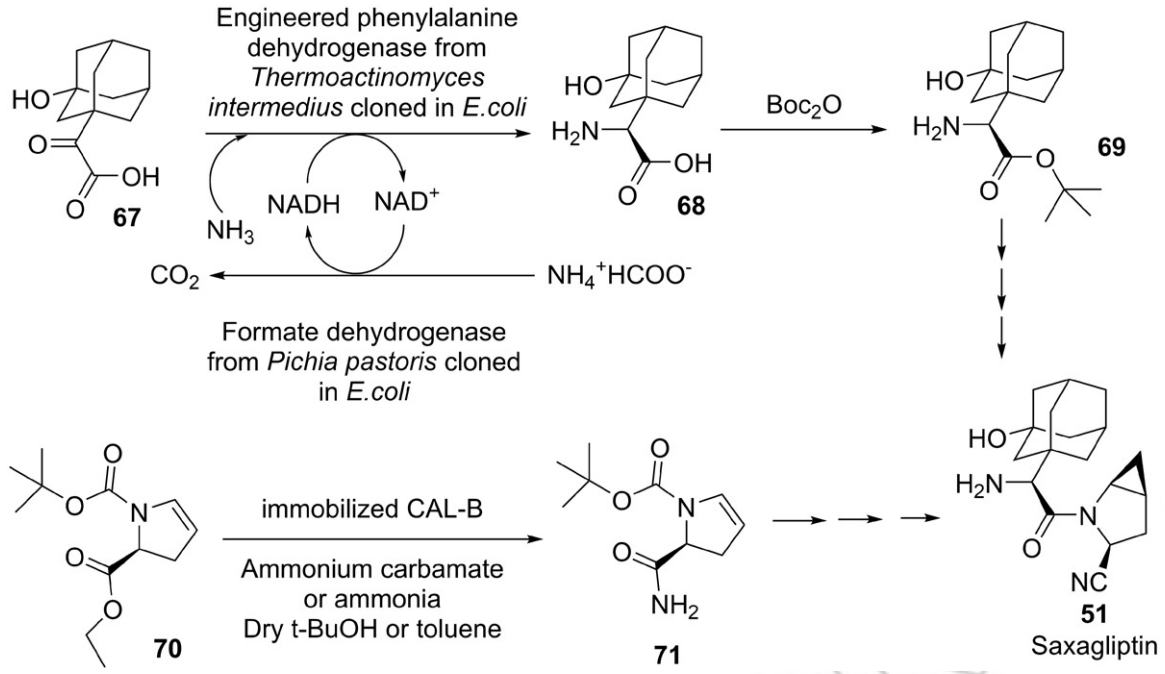

Figure 23. Enzymatic preparation of two intermediates in the synthesis of saxagliptin **51**.

Yields before isolation were close to 98% with 100% ee. This process has now been used to prepare several hundred kilograms of **69** to support the development and manufacturing of saxagliptin.

Also (*S*)-5-aminocarbonyl-4,5-dihydro-1*H*-pyrrole-1-carboxylic acid,1-(1,1-dimethylethyl)-ester **70** is required in the synthetic scheme for obtaining saxagliptin. Direct chemical ammonolysis was hindered by reaction conditions, which resulted in unacceptable levels of amide racemization and side-product formation, whereas milder two-step hydrolysis condensation protocols using coupling agents such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), were compromised by reduced overall yields (Kunishima et al. 2001). To address this issue, a biocatalytic procedure was developed, based upon the CAL-B-mediated ammonolysis of **68** with ammonium carbamate to furnish amide **71** without racemization and with low levels of side-product formation (Gill and Patel 2006). Experiments utilized process stream ester feed, which consisted of ~22% w/v (0.91 M) of the ester in toluene. Since the latter precluded the use of free ammonia due to its low solubility in toluene, solid ammonium carbamate was employed. Reactions were performed using a mixture of neat process feed, ammonium carbamate (71 g/L, 2 mol equiv. of ammonia), and biocatalyst (25 g/L) and shaken at 400 rpm, 50 °C. Under these conditions, CAL-B provided optically pure amide **71** with yields of 69%, together with 21% of side-products (by HPLC). The inclusion of drying agents such as calcium chloride gave significant improvement (79% amide and 13% side-products), as well as the use of sodalime and ascarite, respectively, at 200 g/L in the reaction headspace (increase in amide yield to 84 and 95%), this presumably by way of adsorption of carbon dioxide liberated from the decomposition of ammonium carbamate. A further increase in yield to 98% was attained via the combined use of 100 g/L of calcium chloride and 200 g/L of ascarite. A prep-scale reaction with the process ester feed was used. So, in the optimized process, **70** (220 g/L) was reacted with 90 g/L (1.25 mol equiv.) of ammonium carbamate, 33 g/L (15% w/w of ester input) of CAL-B, 110 g/L calcium chloride, and 216 g/L of ascarite (in the headspace) and run at 50 °C for 3 days. Complete conversion of ester was achieved, with the formation of 96% (182 g/L) of **71** and 4% of side-products; finally, after workup, 98% potency amide in >99.9% ee was isolated in 81% yield (Gill and Patel 2006).

Aloglitin, linagliptin and trelagliptin. As can be seen in Figure 24, three gliptins, alogliptin **52**, linagiptin **53** and trelagliptin **58** shared a common moiety, (*R*)-piperidin-3-amine (*R*)-**74**, in their chemical structures, which apropos is the only chiral centre present in these drugs. Therefore, this enantiopure amine is required in the chemical synthesis of **52** (Feng et al. 2007a, 2007b; Ludescher et al. 2010), **53** (Eckhardt et al. 2007) and **58** (Zhang et al. 2011). Although there are different chemical methods to produce optically pure (*R*)-**74**, the use of transaminases allows very clean

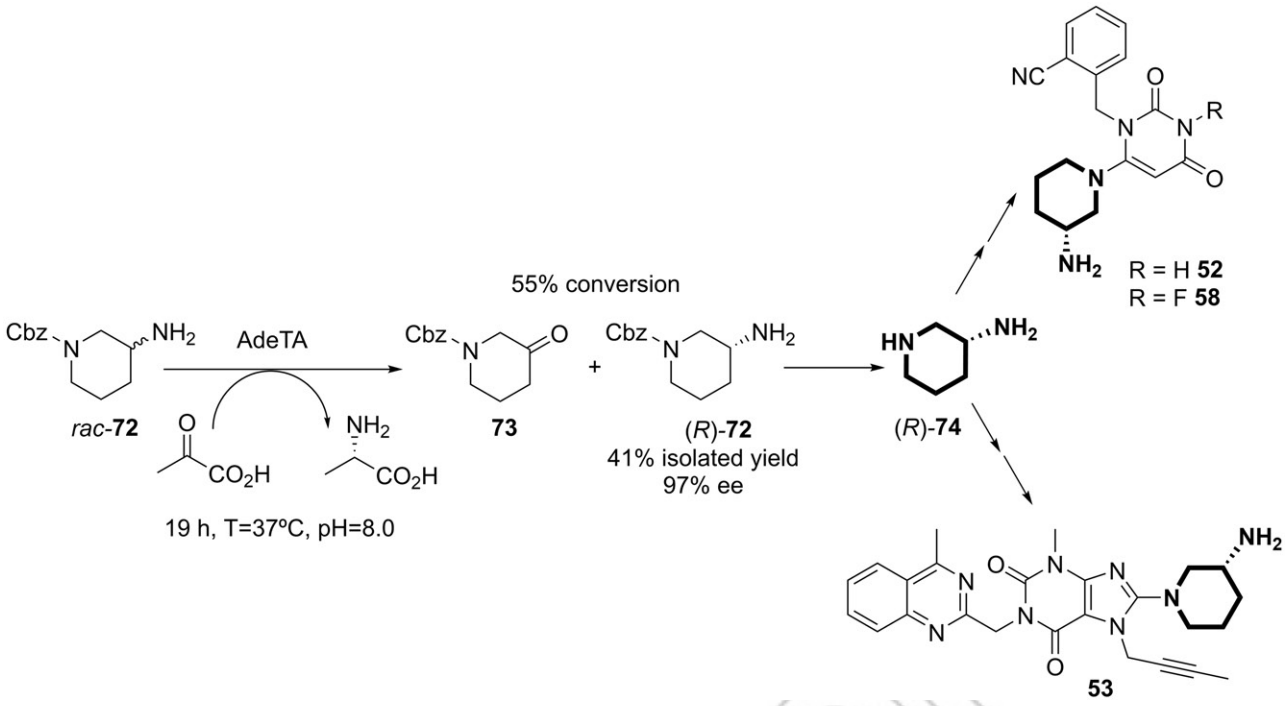

Figure 24. Enzymatic transaminase-catalyzed kinetic resolution of **72** to produce enantiopure (*R*)-**74**.

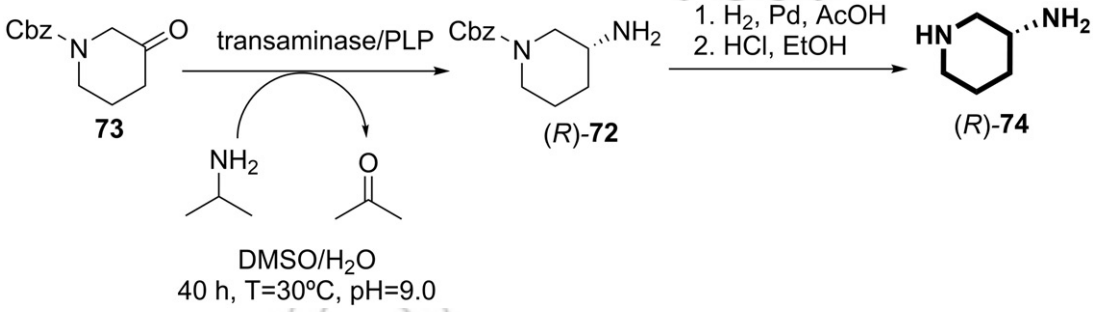

Figure 25. Enzymatic preparation of (*R*)-**74**, through a transaminase-catalyzed asymmetric synthesis.

methodologies. Thus, Hoehne et al. (2008) described the kinetic resolution of racaemic N-protected 3-aminopiperidine **72**, as shown in Figure 24.

In this process, the ω-transaminase of *Alcaligenes denitrificans* (AdeTA) was the selected catalyst for the kinetic resolution of **72**, which was coupled to the simultaneous transformation of pyruvic acid into L-Ala, and the desired amine (*R*)-**72** was obtained with 41% isolated yield and 97% ee. This transaminase was also tested in the synthesis of homologous N-protected 3-aminopyrrolidine, through an analogous kinetic resolution. Anyhow, the drawback of these resolutions is their inherent limited yield (max. 50%).

On the other hand, enantiopure (*R*)-**74** can be synthesized using a transaminase-catalyzed asymmetric synthesis (not limited to 50% yield), as depicted in Figure 25. In this process, the use of a transaminase and isopropilamine as sacrificial substrate allowed the production of (*R*)-**74** starting from Cbz-protected pyrimidine-3-one **73**, and a subsequent deprotection of the correspondent amine (*R*)-**72** allowed a good yield (up to 92%) of (*R*)-**74** (Yang et al. 2014). Furthermore, similar results regarding yield and optical purity could be obtained starting from Boc- or Bn-protected pyrimidine-3-one.

Recently, a similar approach has been proposed employing a recombinant transaminase from *Mycobacterium vanbaalenii*, both as isolated enzyme and whole cells (and also in their immobilized forms), leading to good yield and optical purity (Luo et al. 2016).

Teneligliptin and gosogliptin. Teneligliptin **55** is a DPP4-inhibitor initially developed by Mitsubishi Tanabe Pharma under the name of Tenelia™ (recently available also in Argentina (Teneglucon™) and India

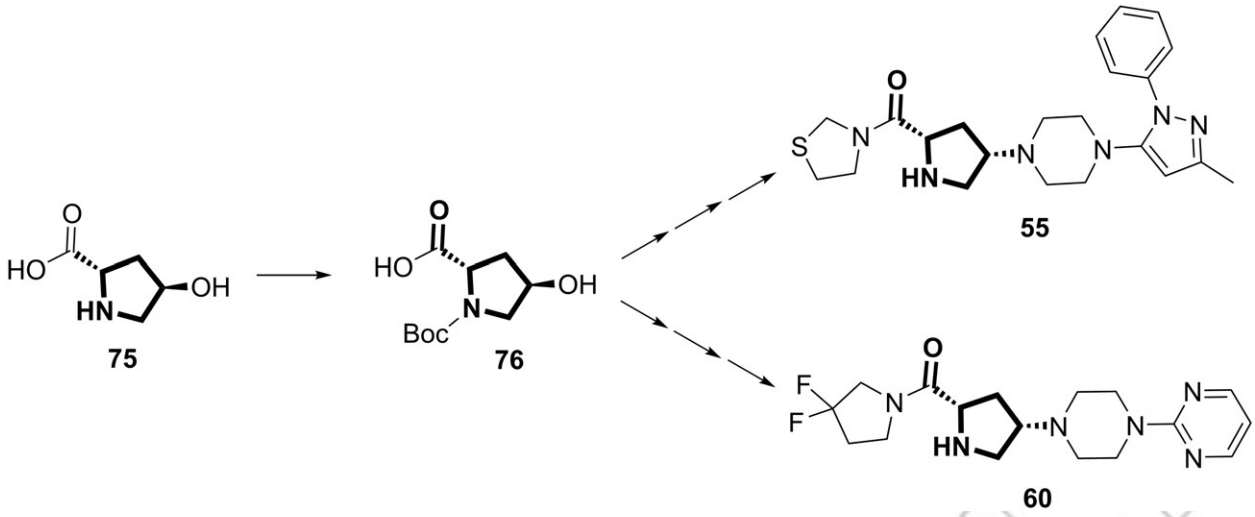

Figure 26. Schematic synthesis of teneligliptin **55** and gosogliptin **60**.

(Tenepure™; Teneza™) at relatively affordable price, with an unique structure characterized by five consecutive rings, explaining its powerful activity and its extremely long half-life (24.2 h), with resulting DPP-4 inhibition throughout the day (Scott 2015; Pujadas et al. 2016; Sharma et al. 2016). Gosogliptin **60** was developed by Pfizer, but it was discontinued in 2012 in Phase II trials; on June 2012 exclusive rights were granted to SatRx LLC, a Russian company, for further development, and it was launched in Russian market in 2016 under the trade name SatRx™.

As can be seen in Figure 26, teneligliptin **55** and gosogliptin **60** share a somehow similar structure, in which the chirality in the molecule is introduced by using enantiopure N-Boc-*trans*-4-hydroxy-L-proline (**76**, Figure 26), as described for teneligliptin (Yoshida et al. 2012; Dwivedi et al. 2015a) and gosogliptin (Lafrance and Caron 2012).

4-(*R*)-Hydroxyproline (Hyp, **75**) is a non-proteinogenic amino acid present in collagen, and which abundance among the residues in animal proteins is very high, around 4%, a value calculated from the abundance of collagen amongst animal proteins (1/3) and that of Hyp within collagen (~38% × 1/3). There are different biocatalyzed methodologies for the synthesis of **75**; the most obvious one requires the employ of prolyl 4-hydroxylase (P4H, E.C. 1.14.11.2, also named procollagen-proline 4-dioxygenase), an 2-oxoacid dioxygenase requiring 2-oxoglutaric acid and molecular oxygen as cosubstrates (Gorres and Raines 2010), as depicted in Figure 27. Nevertheless, although there are many references for this procedure at lab scale (Hara et al. 2014; Yi et al. 2014; Chen et al. 2015; Pozzolini et al. 2015), its technical application is limited, because these 2-oxoacid dioxygenases

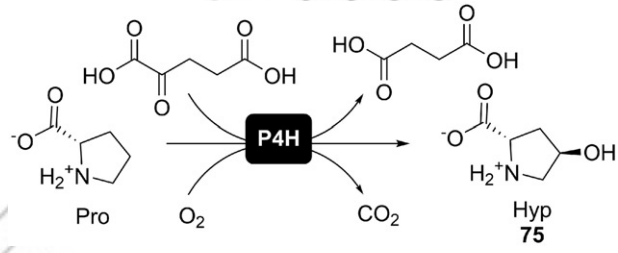

Figure 27. Synthesis of Hyp **75** catalyzed by P4H.

are usually difficult to process (Huttel 2013; Wu et al. 2016).

Another chemoenzymatic methodology for producing Hyp starts from racaemic ethyl 2-Boc-amino-4-pentenoate **78**, obtained from the corresponding malonate derivative **77**, as shown in Figure 28 (Krishnamurthy et al. 2014).

Racaemic **78** is resolved by enantioselective hydrolysis catalyzed by subtilisin, leading to acid (*S*)-**79**, which is subsequently converted into the bencylic ester and epoxidized, to furnish diastereomers **80** (69% yield, 57:43 (2*S*,4*R*):(2*S*,4*S*), as detected by NMR). Amine deprotection with HCl (4M in dioxane) for 2.5 h quantitatively produced the hydrochloride **81** (not isolated) as a white solid upon solvent evaporation. Subsequently, **81** was dissolved in DMF (4.6 mmol in 30 mL DMF), and two equivalents of Et_3N were added to neutralize HCl. After 72 h, TLC results showed the disappearance of **81**, and 1^H NMR analysis of the evaporated reaction mixture showed the absence of characteristic methylene epoxide signals, strongly suggest that the nucleophilic ring opening of the **81** occurred intramolecularly, possibly to produce both *cis*- and *trans*-diastereomers of L-hydroxyproline benzyl ester **82**. Nonetheless, these products were not isolated, and this reaction mixture was re-dissolved in

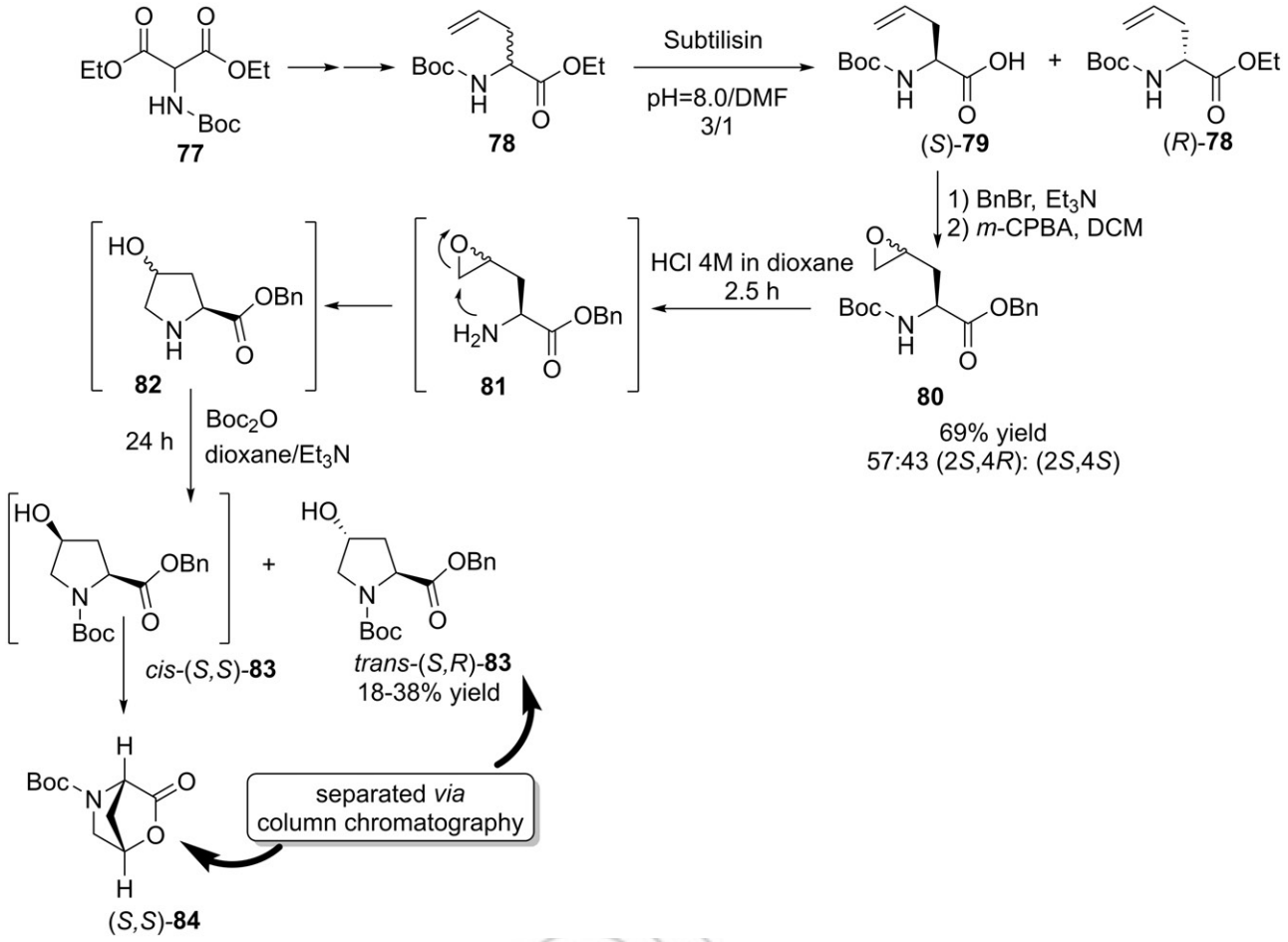

Figure 28. Chemoenzymatic synthesis of Hyp ester **83**.

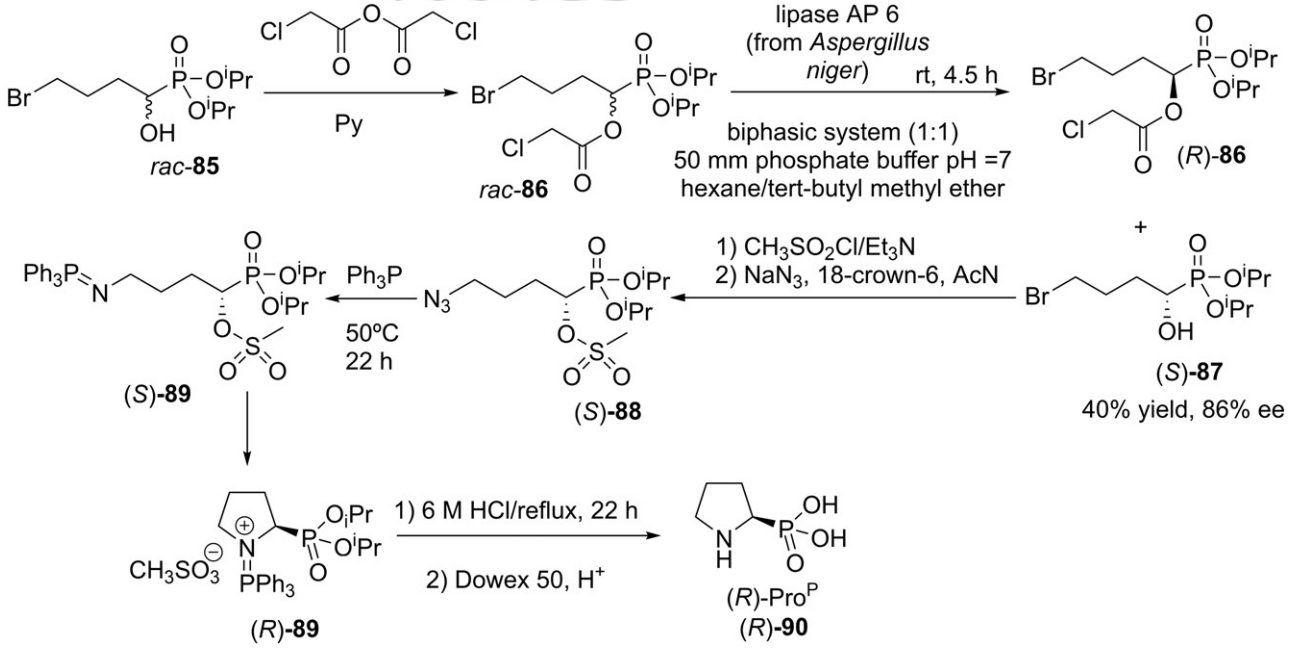

Figure 29. Chemoenzymatic synthesis of (*R*)-Pro^{P} **91**.

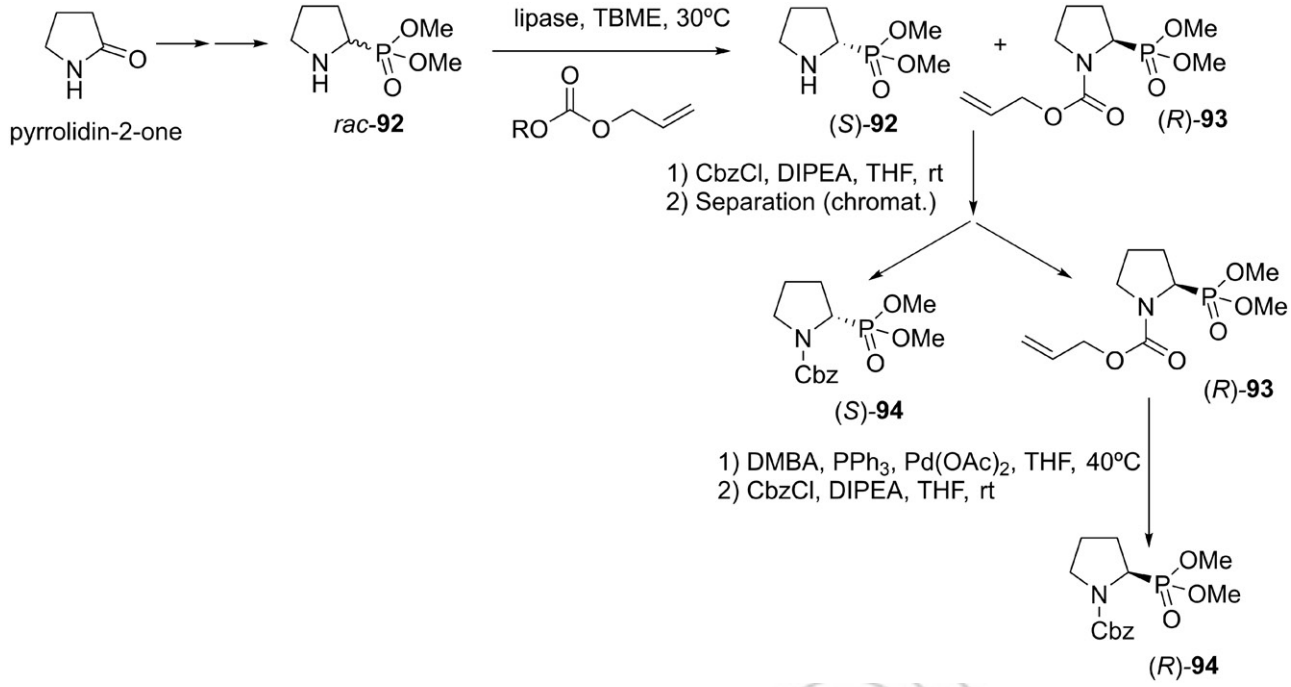

Figure 30. Enantioselective preparation of both enantiomers of benzyl-2-(dimethoxyphosphoryl)pyrrolidine-1-carboxylate **94**.

dioxane and reacted with a small excess of Boc_2O using Et_3N to re-protect the secondary amino groups, and to furnish after 24 h both diastereomers N-Boc-*cis*-L-hydroxyproline benzyl ester (*cis*-(*S*,*S*)-**83**) and N-Boc-*trans*-L-hydroxyproline benzyl ester (*trans*-(*S*,*R*)-**83**), as a yellow oil. Column chromatography (silica gel, hexane–50% EtOAc (v/v) as eluent) allowed the separation of L-*cis*-hydroxyproline lactone (*S*,*S*)-**84** (obtained via intramolecular cyclization, white solid, 29–42%) and the desired Hyp ester *cis*-(*S*,*S*)-**83** (yellowish oil, 18–38%).

Other DPP4 inhibitors: phosphoproline containing dipeptides. Dipeptides containing phosphoproline (Pro^P, the phosphonic counterpart of proline) are known to inhibit several serine DPP4 proteases, as well as other serine proteases (Boduszek et al. 1994; Moonen et al. 2004; Mucha et al. 2011). For preparing these enantiopure dipeptides, it is thus mandatory to prepare homochiral Pro^P, which chemical asymmetric synthesis (Katritzky et al. 1999; Davis et al. 2004; Ma et al. 2011; Ordoñez et al. 2015) or chemical resolution by diastereomers preparation (Kaboudin et al. 2013) have been described in literature.

The development of biocatalyzed protocols for the preparation of enantiopure Pro^P is a very recent research area, and not many cases have been described; in fact, (*R*)-Pro^P was obtained by kinetic resolution of δ-bromo-α-(chloroacetoxy)butylphosphonate **86** mediated by a lipase from *Aspergillus niger*, as depicted in Figure 29 (Wuggenig et al. 2011).

Subsequently, the α-hydroxyphosphonate (*S*)-**87** (obtained in 40% yield and 86% ee) was mesylated in 93% yield and then selectively converted (NaN_3/18-crown-6/MeCN/24 h/reflux) into the monoazide (*S*)-**88** in 91% yield; subsequently, a Staudinger reaction with Ph_3P in DMF furnished the intermediate iminophosphorane (*S*)-**89**, which cyclized to the protected L-phosphaproline (*R*)-**90**, and after deprotection and purification yielded (*R*)-Pro^P **91**.

Recently, a kinetic resolution of racaemic Pro^P has been described (Arizpe et al. 2015), using lipases for the enantioselective synthesis of carbamates, as depicted in Figure 30.

The starting racaemic dimethyl pyrrolidin-2-ylphosphonate **92** was chemically derived from pyrrolidin-2-one. Different lipases were tested to check the best option for enantioselective alkoxycarbonylation with several carbonates; best results were obtained using allyl 3-methoxyphenylcarbonate and *C. antarctica* lipase type A (CAL-A), which catalyzed the allyloxycarbonylation of *rac*-**92** to yield the unreacted substrate (*S*)-**92** with 90% ee and the allyl carbamate (*R*)-**93** with 20% ee (Figure 30) after 92 h of reaction. Nevertheless, separation of these compounds by silica-gel column chromatography was not possible, because of the lability of **92**, so that the crude obtained from the enzymatic reaction was treated with benzyl

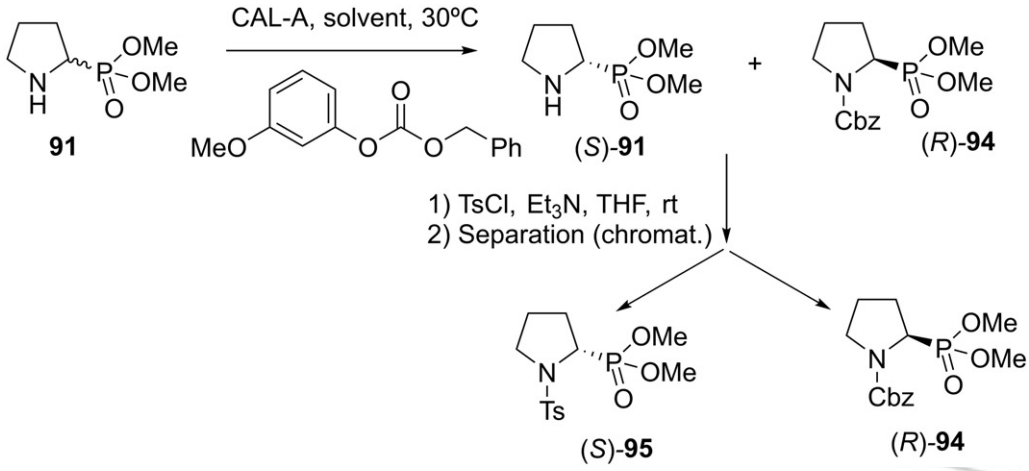

Figure 31. Enantioselective preparation of carbamates of Pro^P dimethyl esters.

chloroformate to give a mixture of optically active carbamates (*S*)-**94** and (*R*)-**93**, which could be separated, and finally (*R*)-**93** was converted into (*R*)-**94**. In all cases, the enantiomers could be separated by chiral chromatography (Arizpe et al. 2015). These same authors described a somehow simpler protocol by using benzyl 3-methoxyphenyl carbonate for catalyzing the carbamoylation (after 84 h, 82% of (*R*)-**94** and 94% ee of (*S*)-**92**), and then an easier separation by a previous tosilation of non-converted (*S*)-**92** (Figure 31).

Sodium–glucose co-transporter 2 (SGLT2) inhibitors: gliflozins

Sodium–glucose co-transporters or sodium–glucose-linked transporter (SGLTs) play an important role in the intake and elimination of glucose. SGLTs are located in the intestinal mucosa (enterocytes) of the small intestine (SGLT1), and the proximal tubule of the nephron (SGLT2 in proximal convoluted tubule, SGLT1 in proximal straight tubule). SGLT2 is the main responsible for reabsorption of glucose in kidney; thus, inhibition of SGLT2 would lead to a very low or even null glucose reabsorption and an increased glycosuria, highly desirable for patients suffering Type 2 DM (Madaan et al. 2016; Solini 2016).

There are several SGLT2 inhibitors, called generically gliflozins, already marketed (Figure 32): dapagliflozin **96**, canagliflozin **97**, empagliflozin **98**, ipragliflozin **99**, tofogliflozin **100**, and luseogliflozin **101** are approved in different countries, and the prodrug remogliflozin etabonate **102** is under study for commercialization (Madaan et al. 2016).

The chemical structures of dapagliflozin **96**, canagliflozin **97**, empagliflozin **98** and ipragliflozin **99** present a C-glycosidic linkage between the glucose moiety and the aglycon; luseogliflozin **101** also possess the C-glycosidic bond, but now between the aglycon and the corresponding 5-thio-D-glucopiranose. Remogliflozin etabonate **102** is the only member of the family possessing the classical O-linkage, while tofogliflozin **100** is a spiranic compound, so that C- and O-linkages are simultaneously presented.

Any biocatalytic method for creating the C-glycoside would demand the use of Leloir C-glycosiltransferases (C-GTs) (Gutmann and Nidetzky 2013), but this possibility has not been developed for gliflozins, as far as we know. Nevertheless, empagliflozin **98** does contain a chiral fragment in its structure, (*S*)-tetrahydrofuran-3-ol (*S*)-**103**, required for the synthesis of the drug (Wang et al. 2014), and different biotransformations can be found in literature for producing both enantiomers of **103**, as shown in Figure 33. The first strategy is based on a kinetic resolution of the corresponding racaemic alcohol, but this is not that trivial because of the small differences in the size of both groups attached to the carbinol moiety: in fact, Baumann and coworkers did not find any measurable enantioselectivity in the hydrolysis of different racaemic tetrahydrofuran-3-yl esters after testing more than 100 commercial hydrolases (Baumann et al. 2000). Using enzymes modified by mutations of amino acids it became possible to increase the enantioselectivity in the hydrolysis, but only up to a moderate value (enantiomeric ratio $E = 10$, compared to $E = 4.3$ with the wild-type enzyme) using the D31T/L93F double mutant of an esterase from *Bacillus stearothermophilus* (Nobili et al. 2013).

Similarly, the bioreduction of the corresponding ketone dihydrofuran-3(2*H*)-one **105** did not lead to high enantioselectivity values, once again because of the similar size of both methylene groups around the carbonyl moiety; in fact, Sun et al. (2016) tested this

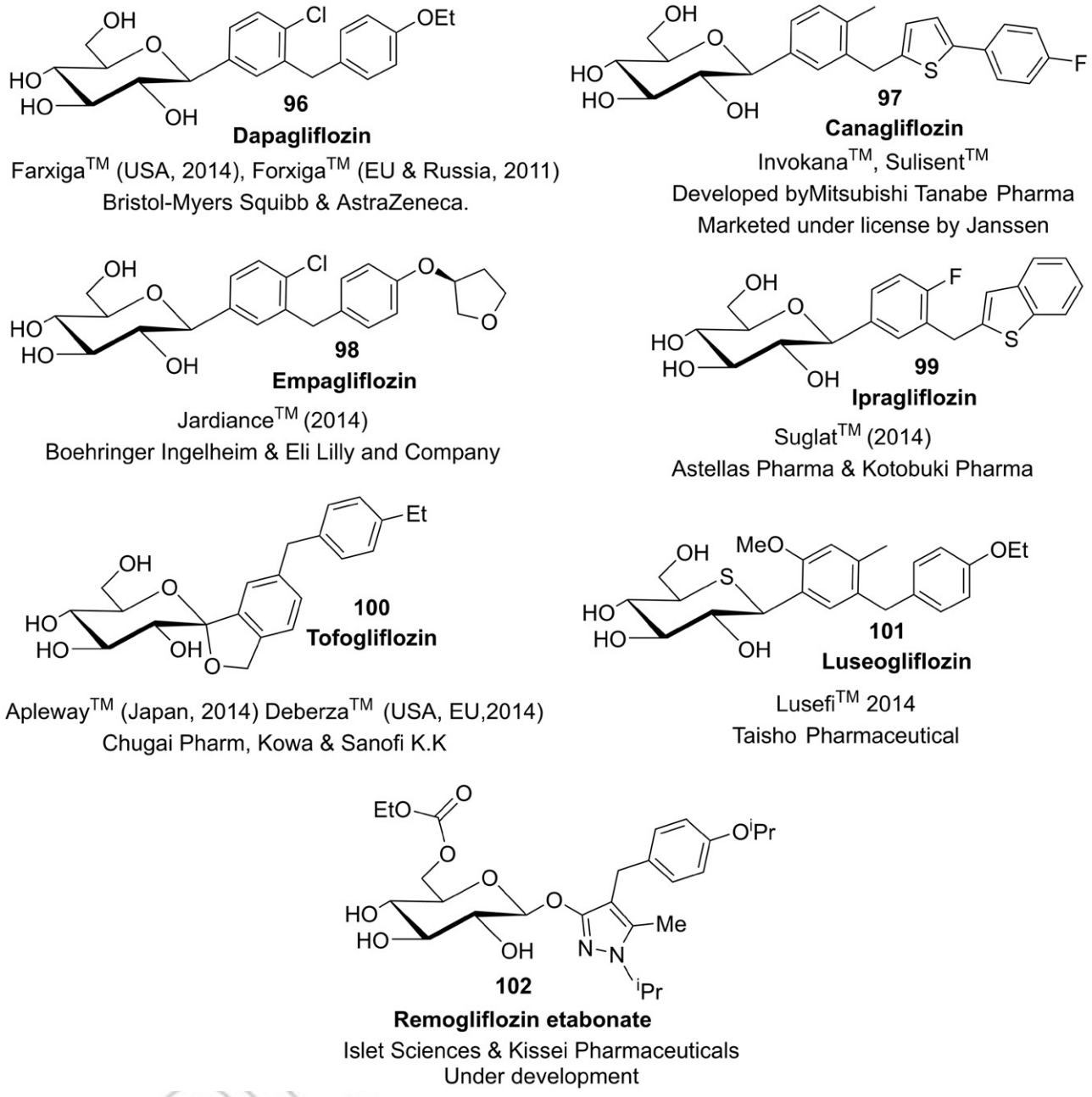

Figure 32. Some marketed gliflozins.

bioreduction using two commercial ADH kits (47 enzymes), describing only a 22% ee for the best (*R*)-selective enzyme and 91% ee for the best (*S*)-selective one, but under suboptimal conversions. So, this group decided to modify the ADH from *Thermoethanolicus brockii* (TbSADH, slightly (*R*)-selective (23% ee) at full conversion) by genetic engineering, using triple-code saturation mutagenesis (TCSM), obtaining highly (*R*)- and (*S*)-selective variants (62–94% ee for (*S*)-selective, 95%—99% ee for (*R*)-slective) with minimal screening at semipreparative scale (Sun et al. 2016).

An indirect biocatalyzed methodology for producing (*S*)-**103** was described by Pienaar et al. (2008), using a chemoenzymatic approach also presented in Figure 33. Hence, the racaemic epoxide **106** was opened using whole cells of *Yarrowia lipolytica* containing epoxide hydrolase activity, and the non-converted substrate (*S*)-**106** (20% yield, 97.8% ee) was chemically opened leading to halohydrin (*S*)-**108**, which upon a lipase-catalyzed hydrolysis and acid catalysis furnished (*S*)-**103** with moderate yields (79%), not altering the good enantioselectivity obtained in the preparation of starting (*S*)-**106**.

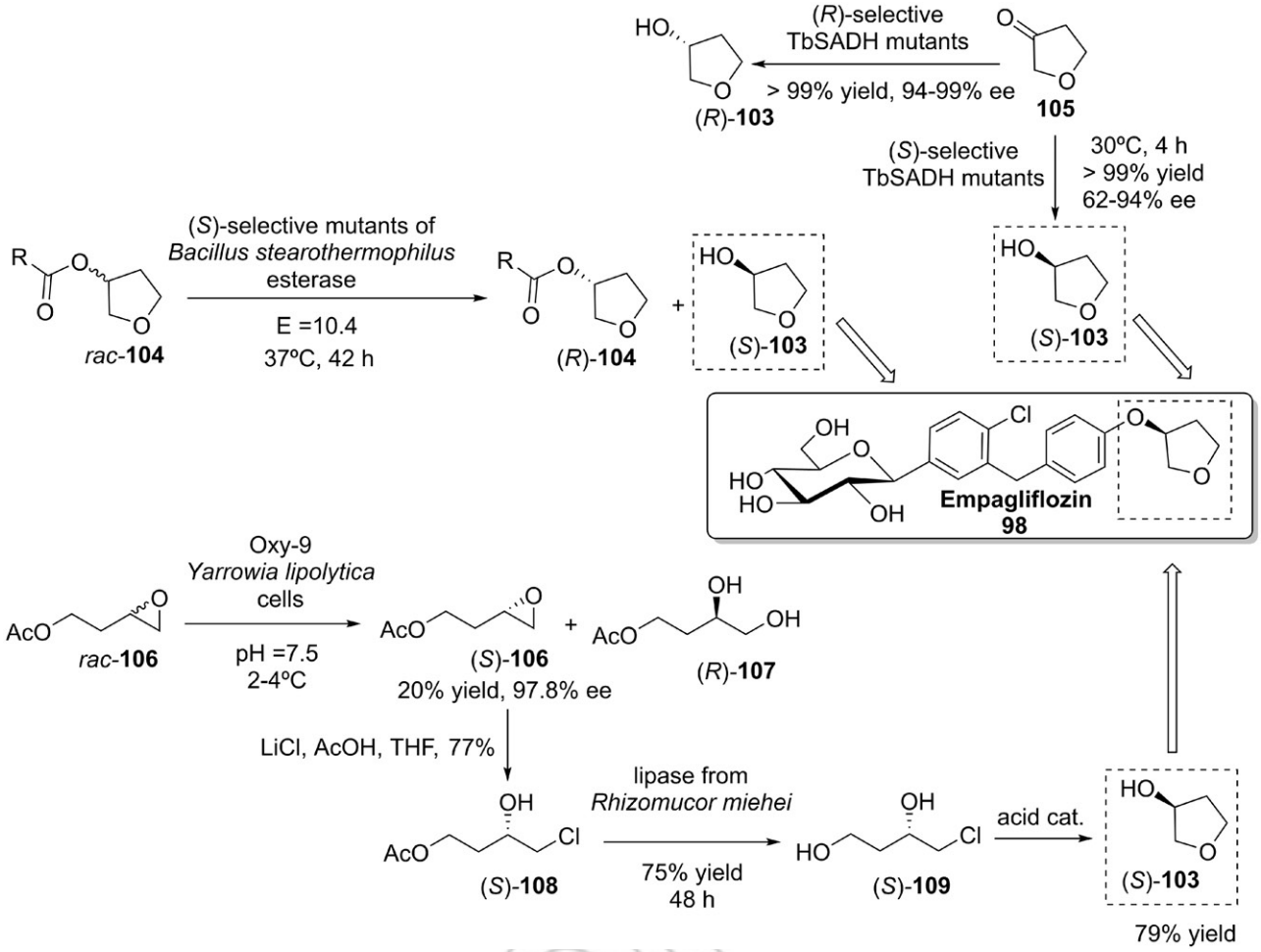

Figure 33. Some biocatalyzed methods for obtaining (*S*)-tetrahydrofuran-3-ol (*S*)-**103**.

11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1) inhibitors

An increased abnormal concentration of glucocorticoids (GCs) may lead to its precipitation, with the concomitant aggravation of truncal obesity, insulin resistance, hepatic triacylglycerol accumulation, hyperglycaemia, hypertension and dyslipidaemia. This is known as metabolic syndrome (Grundy et al. 2004), and it represents a major risk factor for Type 2 DM and cardiovascular disease. Thus, interventions to reduce GC action can prevent and reverse these effects. One of the most important enzymes involved in GCs activity is 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), which converts cortisone to cortisol, the primary GC in humans, mostly in liver and adipose tissue, so that the inhibition of this enzyme could reduce cortisol production within these tissues without substantially affecting circulating cortisol (Anderson and Walker 2013). This is the reason why 11β-HSD1 has been proposed as an innovative therapeutic target for the treatment of Type 2 DM (Bailey et al. 2016; Bailey 2017). There are many pharmaceutical companies working very actively in this area, developing many different chemical structures displaying inhibition of 11β-HSD1 (Scott et al. 2014); many of these chemical structures present stereogenic centres, and thus they could be synthesized with the help of biocatalysis. We will show only some examples covering this field, being the first one oxazolone **112** (Figure 34) reported by Biovitrum, having reasonable potency tested *in vitro* (Sutin et al. 2007). Preparation of the chiral amine (*S*)-**111**, required for synthesizing **112**, has been recently described by Martinez-Montero and coworkers, by means of a transamination of the corresponding ketone **110**, in high yield (95% conversion) and stereoselectivity (>99% ee) (Martinez-Montero et al. 2017).

In another example, monoester **114**, required for preparing the 11β-HSD1 inhibitor **115** (Peddi et al. 2010), has been prepared starting from diester **113**, by using a lipase catalyzed mono-hydrolysis, not continuing to the diacid (Guo et al. 2014). Thus, about 100 kg of **114** were prepared in 78% yield by hydrolysis of **113** with a commercially available lipase from *Burkholderia cepacia*: a more efficient enzymatic

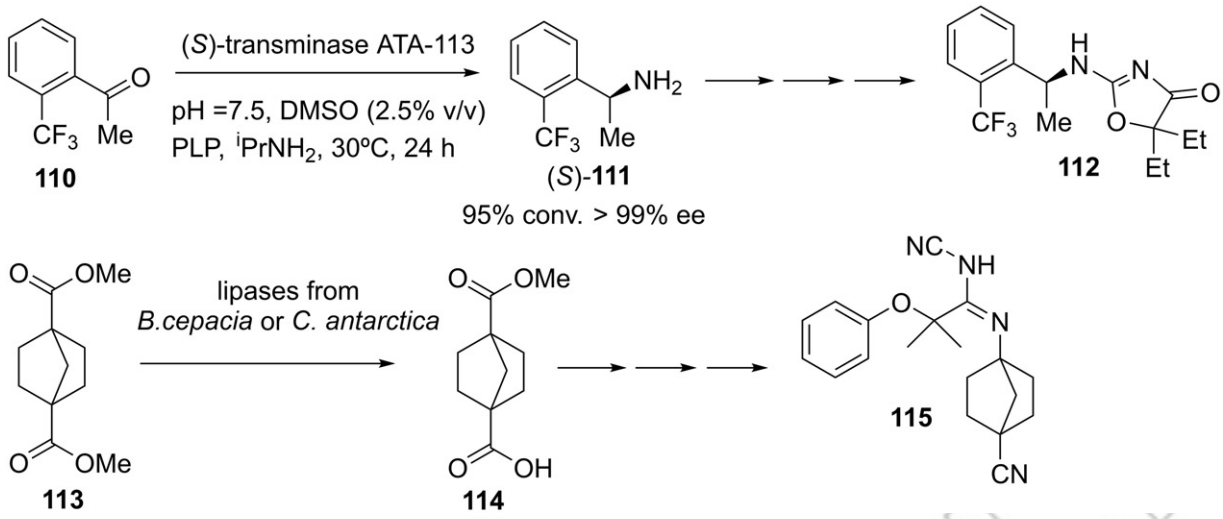

Figure 34. Biocatalytic procedures for preparing some 11β-HSD1 inhibitors.

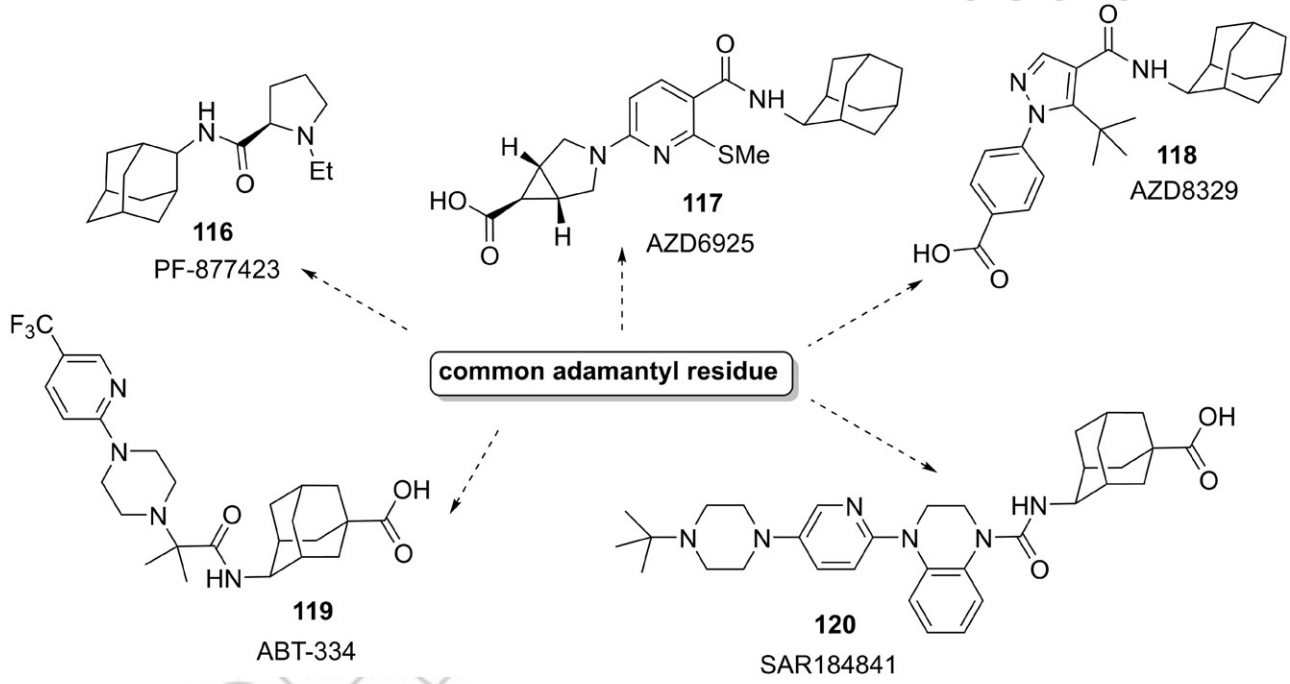

Figure 35. Some 11β-HSD1 inhibitors possessing an adamantyl moiety in their structures.

process was developed for the hydrolysis of **113** that gave the monoester **114** in 82% yield using significantly lower amounts of the commercially available immobilized lipase B from *C. antarctica*.

As we commented before, there are many pharmaceutical companies working very actively in the preparation of 11β-HSD1 inhibitors; many of the candidates present an adamantyl moiety in its structure, as shown in Figure 35. In fact, compounds such as **116**, from Pfizer (Cheng et al. 2010); **117** (Scott et al. 2012a) and **118** (Scott et al. 2012b), from AstraZeneca; **119** (Becker et al. 2008; An et al. 2013) from Abbott, or **120** (Venier et al. 2011), developed by Sanofi, are at different levels of clinic assays.

Microbial oxidation of adamantane **121** (Figure 36) is a good alternative for obtaining hydroxylated derivatives, because chemical methods require harsh oxidants, are poorly selective and are prone to overoxidation; subsequently, these hydroxylated derivatives can be chemically converted in other structures required for the preparation of those drug candidates presented before.

Biohidroxylation of **121** catalyzed by *Streptomyces griseoplanus* was described by Mitsukura and

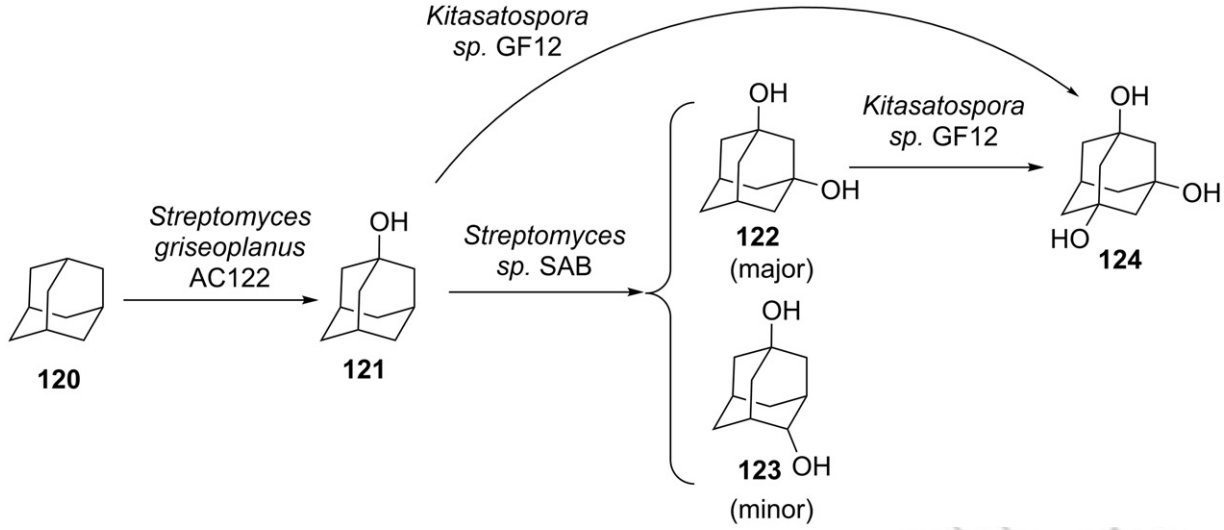

Figure 36. Microbial hydroxylation of adamantane and derivatives.

coworkers in 2006: among 470 strains tested, *S. griseoplanus* was highly regioselective to give 1-adamantanol **122** in 32% molar conversion yield after 72-h cultivation in the presence of 3% (v/v) Tween 60. This same group also described the production of 1,3-adamantanediol **123** by a regioselective monohydroxylation of **122** using *Streptomyces* sp. SA8, producing 5.9 g L^{-1} of **123** starting from 6.2 g L^{-1} of **122** in culture broth after 120 h at 25 °C (Mitsukura et al. 2010). Using resting cells, 2.3 g L^{-1} of **122** was produced after 96 h of incubation at a 69% conversion rate. In both cases, 1,4-adamantanediol **124** was formed as a byproduct at a rate of about 15%; this strain SA8 was also able to hydroxylate 2-adamantanol and 2-methyl-2-adamantanol. Similarly, washed cells (62 mg) of *Kitasatospora* sp. GF12 in 4 mL buffer (pH 7) were used for catalyzing the regioselective hydroxylation of 60 mM **123** to 30.9 mM 1,3,5-adamantanetriol **125** over 120 h at 24 °C (Mitsukura et al. 2012), adding glycerol (400 mM) to the reaction mixture to recycle the intracellular NADH/NADPH. The same cells also catalyzed the hydroxylation of 10 mM of **122** directly to **125** (3.6 mM).

Conclusions

We have presented in this review some examples of how biocatalysis can help in the development of drugs possessing antidiabetic activity. Our objective has been to illustrate how the employ of biocatalysts, enzymes (wild-type or genetically modified, soluble or immobilized) or whole cells (in any state) points towards a definitive improve in the sustainability of the synthetic process, because of the excellent biocatalytic precision (chemoselectivity, regioselectivity, stereoselectively). The focus has been centred in the different types of drugs actually being used, and we have not considered the effect of combined drugs (a very common therapeutic strategy), because this is out of the aim of this revision. According to the market volume of these types of drugs, we can foresee a clear increase in the use of versatile biocatalyzed protocols for the production of antidiabetic drugs.

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Disclosure statement

The author reports no declarations of interest.

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