Biocatalyzed Synthesis of Statins: A Sustainable Strategy for the Preparation of Valuable Drugs

Pilar Hoyos [1], Vittorio Pace [2] and Andrés R. Alcántara [1,*]

[1] Department of Chemistry in Pharmaceutical Sciences, Faculty of Pharmacy, Complutense University of Madrid, Campus de Moncloa, E-28040 Madrid, Spain; phoyosvi@ucm.es
[2] Department of Pharmaceutical Chemistry, Faculty of Life Sciences, Althanstrasse 14, A-1090 Vienna, Austria; vittorio.pace@univie.ac.at
* Correspondence: andalcan@ucm.es; Tel.: +34-91-394-1823

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Abstract: Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are the largest selling class of drugs prescribed for the pharmacological treatment of hypercholesterolemia and dyslipidaemia. Statins also possess other therapeutic effects, called pleiotropic, because the blockade of the conversion of HMG-CoA to (*R*)-mevalonate produces a concomitant inhibition of the biosynthesis of numerous isoprenoid metabolites (e.g., geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP)). Thus, the prenylation of several cell signalling proteins (small GTPase family members: Ras, Rac, and Rho) is hampered, so that these molecular switches, controlling multiple pathways and cell functions (maintenance of cell shape, motility, factor secretion, differentiation, and proliferation) are regulated, leading to beneficial effects in cardiovascular health, regulation of the immune system, anti-inflammatory and immunosuppressive properties, prevention and treatment of sepsis, treatment of autoimmune diseases, osteoporosis, kidney and neurological disorders, or even in cancer therapy. Thus, there is a growing interest in developing more sustainable protocols for preparation of statins, and the introduction of biocatalyzed steps into the synthetic pathways is highly advantageous—synthetic routes are conducted under mild reaction conditions, at ambient temperature, and can use water as a reaction medium in many cases. Furthermore, their high selectivity avoids the need for functional group activation and protection/deprotection steps usually required in traditional organic synthesis. Therefore, biocatalysis provides shorter processes, produces less waste, and reduces manufacturing costs and environmental impact. In this review, we will comment on the pleiotropic effects of statins and will illustrate some biotransformations nowadays implemented for statin synthesis.

Keywords: biocatalysis; biotransformations; statins; pleiotropic effects

1. Introduction

It is very well known that raised cholesterol levels increase the risks of heart disease and stroke. Globally, a third of ischaemic heart disease is attributable to high cholesterol and, according to the World Health Organization, raised cholesterol is estimated to cause 2.6 million deaths (4.5% of total) and 29.7 million disability adjusted life years (DALYS) [1]. In this sense, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, commonly known as statins (Figure 1), are the largest selling class of drugs prescribed for the pharmacological treatment of hypercholesterolemia and dyslipidaemia [2,3], and it has been also reported that since their introduction in 1987, the lives of millions of people have been extended through statin therapy and, more importantly, quality of life has been drastically improved [4].
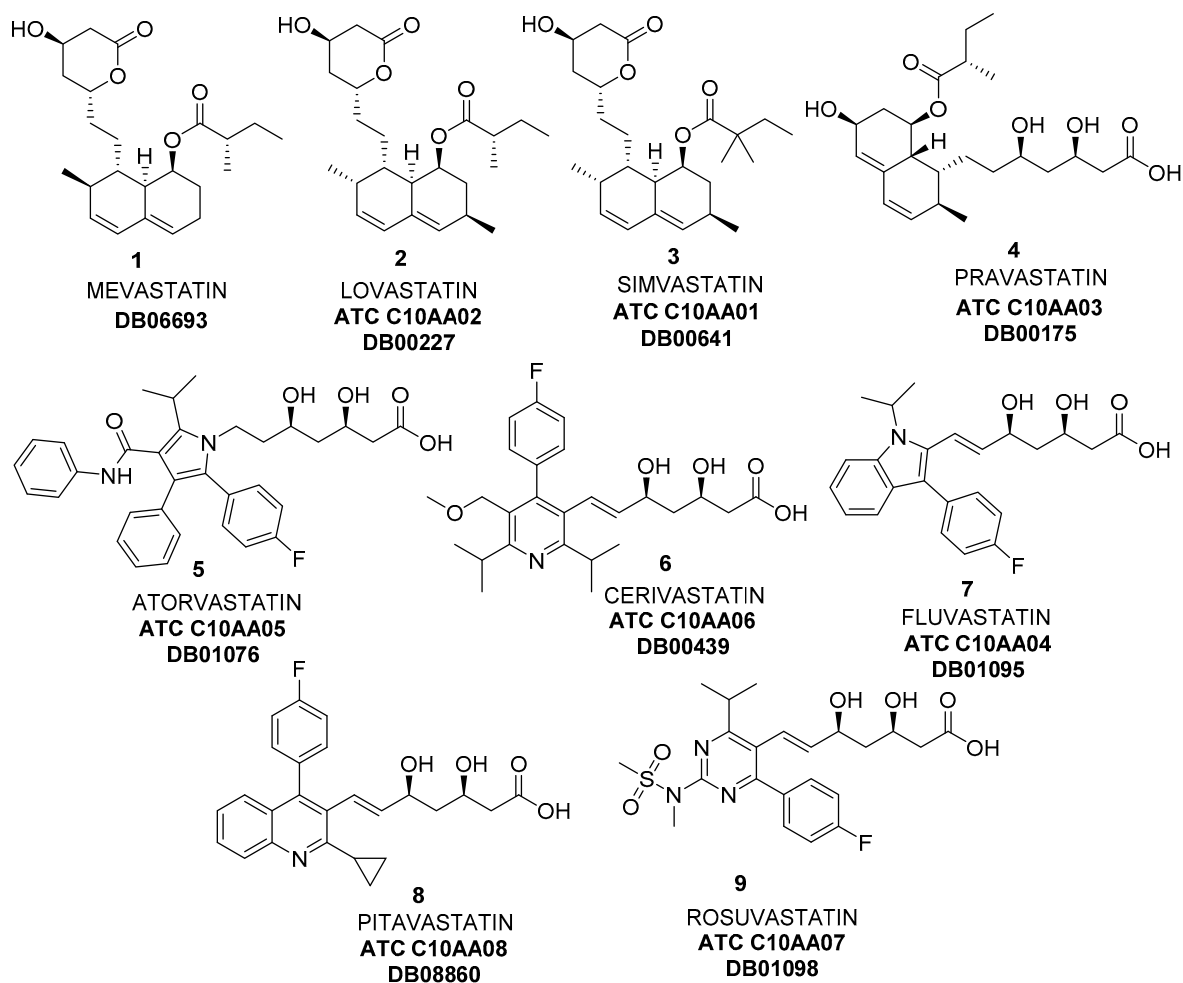
Figure 1. Some Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.

Since the discovery of the first statins from natural sources, mevastatin (Figure 1, **1**, also named compactin, from the fungi *Penicillium citrinum* [5] and *Penicillium brevicompactum* [6]), lovastatin (Figure 1, **2**, Mevinolin, found in *Aspergillus terreus* [7] and food such as oyster mushrooms [8] or red yeast rice [9]), simvastatin (Figure 1, **3**, Mevacor, also isolated from *Aspergillus terreus* [10]), and pravastatin (Figure 1, **4**, initially known as CS-514, originally identified in the bacterium *Nocardia autotrophica* [11]), synthetic more potent compounds (Figure 1, **5–9**), also known as superstatins, were introduced into the drug market [12,13]. As can be observed, the common structure of these compounds is formed by a central core of different heterocyclic aromatic rings containing nitrogen, and a lateral chain derived from (3*R*,5*R*)-3,5-dihydroxyheptanoic acid.

The economic impact of statins on the drug market is enormous. For instance, simvastatin was originally developed by Merck under the brand name Zocor™; in 2005, Zocor™ was Merck's best-selling drug and the second-largest selling statin in the world (more than US\$3 million only in USA, according to different reports [14–21]). In 2006, Zocor™ went off patent, and the annual sales drastically dropped; anyhow, from that moment, generic simvastatin became the most-prescribed statin in the world between 2010 and 2015 [19,20,22]. On the other hand, atorvastatin (Figure 1, ATC (Anatomical Therapeutic Chemical) classification system, according to World Health Organization) Code C10AA05, DrugBank Code DB01076, **5**) is the greatest blockbuster drug in pharmaceutical history, and the best known representative of superstatins, receiving this name because of its pronounced ability to reduce low-density lipoprotein cholesterol levels and increase high-density lipoprotein cholesterol compared with other existing agents [13]. It was first synthesized in 1985 by Bruce Roth of Parke-Davis Warner-Lambert Company (now Pfizer), which commercialized it under the name of

Lipitor™. Since it was approved in 1996, sales have exceeded US$125 billion, and the drug has topped the list of best-selling branded pharmaceuticals in the world for nearly a decade. When Pfizer's patent on Lipitor™ expired in USA by the end of 2011 and in Europe in mid-2012, generic atorvastatin from other companies became available, and it is still being widely sold (US$2.16 billion in sales, standing as the year's fourth-best-selling cardiovascular drug, with analysts predicting sales of US$1.85 billion in 2024 [23]). Finally, rosuvastatin (Figure 1, **9**, ATC Code C10AA07, DrugBank Code DB01098) was marketed as calcium salt in 2003 by AstraZeneca under the name of Crestor™. Like atorvastatin, rosuvastatin is also a superstatin; the initial patent on rosuvastatin synthesis (purely chemical) was developed by Shionogi Research Laboratories [24] and later sold to AstraZeneca. This patent expired in June 2016, but anyhow, it still can be considered a blockbuster drug, by looking at the great volume of sales of Crestor™ (around U$2.7 billion in 2017, and US$727 million for the first half of 2018 [23]). A recent study [25] points toward global sales of statins of US$1 trillion by 2020, thus pharmaceutical companies are still interested in developing new synthetic strategies for putting these drugs on the market.

Thus, it is undoubtedly clear that the statin market involves a huge amount of money. Furthermore, the importance of this type of drug is even higher because of their new therapeutic uses that are recently becoming more and more recognized, which will be commented on in Section 2. Finally, as the absolute configuration of statins plays a crucial role in the activity of these compounds, the enormous potential of an enantioselective biocatalytic process for the sustainable synthesis of chiral building blocks involved in statin preparative procedures will further be commented on in Section 3.

2. New Therapeutic Effects of Statins

As mentioned before, these drugs act by reversibly and competitively inhibiting the bioreduction of *S*-3-hydroxy-3-methylglutaryl-coa (HMG-CoA), the rate-limiting step of the mevalonate pathway in cholesterol biosynthesis (Figure 2), because of the chemical similitude with mevalonyl-CoA, the intermediate obtained after the first reduction of HMG-CoA. Furthermore, there is extensive recent evidence suggesting that statins are more than simple lipid-lowering drugs [3,26]; in fact, a large amount of up-to-date experimental data have confirmed that statins may exert many different potentially beneficial therapeutic effects, by several mechanisms not essentially related to cholesterol metabolism. These so-called pleiotropic effects [27] could be attributed to their ability to prevent the conversion of HMG-CoA to *R*-mevalonate, which results in the concomitant inhibition of the downstream biosynthesis of cholesterol, as well as of numerous isoprenoid metabolites, such as geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP), as shown in Figure 2.

These molecules are well-known key intermediates for prenylation of several cell signalling proteins (such as small GTPase family members: Ras, Rac, Rho, Rab), which act as molecular switches controlling multiple pathways and cell functions (maintenance of cell shape, motility, factor secretion, differentiation, and proliferation), so that they can be inhibited by statin treatment [28]. For instance, when Ras and Rho isoprenylation is inhibited, there is a concurrent accumulation of inactive forms of both proteins in cytoplasm and an inhibition of these signalling molecules [29]. Certainly, it has been reported that small G-proteins like Rho and Rac influence endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) availability [30]. Rho negatively regulates eNOS expression, while Rac activates nicotinamide dinucleotide phosphate (NADPH)-oxidase and the correspondent superoxide production, which in turn inactivates NO. If statins block both Rho and Rac GTPase activity via inhibition of geranylgeranylation, this leads to eNOS upregulation [31,32]. Remarkably, some beneficial effects of statins were displayed before cholesterol levels were reduced [30], and it can be assumed that those effects, dependent on the enhancement of eNOS expression and/or activity, result in a decline of platelet activation, attenuation of adhesion molecules expression, decrease of inflammatory cytokine production, and increase of reactive oxygen species (ROS) [33]. Therefore, pleiotropic effects of statins include the reduction of haemostasis by reducing platelet activation and the pro-coagulation cascade; the increase of fibrinolysis and the anticoagulation cascade; the improvement

of endothelial function; the increase of NO bioavailability; as well as antioxidant, immune modulatory, and anti-inflammatory activities and stabilization of atherosclerotic plaques [27,29,34–37]. Thus, the therapeutic effects of statins are nowadays present in areas such as cardiovascular health, regulation of the immune system, anti-inflammatory and immunosuppressive properties, prevention and treatment of sepsis, treatment of autoimmune diseases, osteoporosis, kidney and neurological disorders, and even in cancer therapy; some of these therapeutic areas will be commented on.
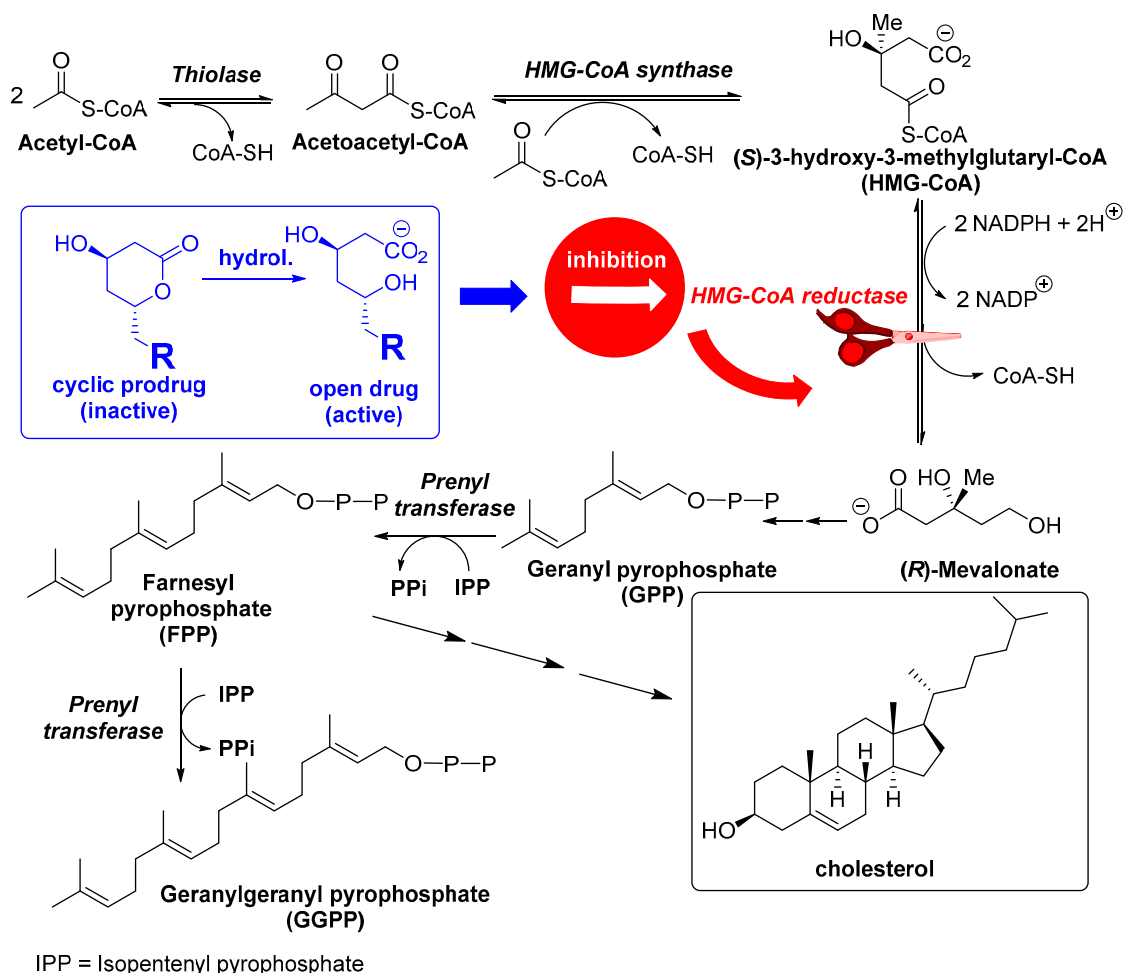

Figure 2. Mevalonate pathway.

2.1. Cardiovascular Effects

Aside from the main mechanism of action of lowering cholesterol levels, statins are also useful in the treatment of some other cardiovascular disorders, including acute coronary syndrome, heart failure, cardiac arrhythmias, aortic stenosis, peripheral arterial disease, cerebrovascular disease, and essential hypertension, as recently reviewed (see papers by Mihos et al. [38], Oesterle et al. [39], and references therein). In fact, chronic administration of statins is believed to produce what is known as PIC ("pre-ischemic conditioning"), protecting the myocardium during ischemic insult and injury [40], as a consequence of an increase in nitric oxide availability and immunomodulation; thus, statins increase the production of nitric oxide and blunt the formation of superoxide radicals via the upregulation of eNOS and stabilization of its mRNA, leading to an improved vascular function and a reduction in vascular inflammation [34]. In this sense, recent studies show the effectiveness of statins' cardiovascular primary prevention [41], also for elderly people [42], and point towards the special benefits of fluvastatin [43].

2.2. Immunomodulatory Effects

The main objectives of autoimmune therapies are to re-establish immunological homeostasis and reduce autoimmune damages. Different studies are increasingly suggesting that an imbalance between Th17 and Treg cells, as well as the incorrect release of potent pro-inflammatory mediators by Th17 cells, are crucial for the pathogenesis of a number of autoimmune disorders [44]. Thus, a new immunotherapeutic strategy could be based on increasing Treg or inhibiting Th17 differentiation/effector functions. In this respect, statins show an outstanding potential, especially considering the increasing evidence that they might inhibit Th17 differentiation/effector functions and conversely promote Treg differentiation/suppressive function selectively in the setting of autoimmune diseases [44]. Small GTPases have been centrally implicated in regulating the development and functions of T and B lymphocytes as well as of dendritic cells (DC) [45,46]. Thus, as a consequence of the inhibition of GTPases prenylation, statin-based therapy can be a potential alternative for the treatment of autoimmune diseases [44,47,48]. In fact, positive effects of statin treatment have been reported in numerous autoimmune diseases such as multiple sclerosis [49,50], systemic lupus erythematosus [51–53], autoimmune myocarditis [54–56], or rheumatoid arthritis [44,57–59].

2.3. Neurological Disorders

This is probably one of the most attractive therapeutic areas in which the use of statins introduces interesting advances. Pleiotropic effects of statins via GTPases inhibition might have potential therapeutic implications in many neurological disorders, as the current connection between neurodegenerative diseases and vascular risk factors is becoming more and more evident [30,60]; therefore, statin treatment could display beneficial effects in neurological disorders such as stroke, Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), primary brain tumours, or depression.

2.3.1. Stroke

The risk factors for cerebrovascular disease are well known and largely variable and, in this sense, reduction of serum cholesterol levels could be highly beneficial for reducing the hazard of suffering a cerebrovascular accident (CVA), also named stroke [30]. Anyhow, an indubitable link between high cholesterol level and stroke risk is difficult to establish, because controversial data from several clinical studies have been published in the literature, some of them finding no relationship between cholesterol and stroke [61,62], while in some other cases, the beneficial effects are indeed observed [63,64]. A possible explanation for these discrepancies could be based on the fact that stroke can be either ischemic or haemorrhagic, and there are evidences supporting an association between elevated cholesterol and increased risk of ischemic stroke, but also showing a relationship between low cholesterol levels and increased risk of haemorrhagic stroke [30]. So, while disagreements are still present on the usefulness of statins in the primary prevention of acute stroke, there is a wide consensus on the positive aspects of statins treatment in secondary prevention after stroke or transient ischemic attack for diminishing the menace of suffering a new stroke [65–68]. Even in haemorrhagic stroke, some data from recent studies suggest that statin therapy could improve the outcome after spontaneous intra-cerebral haemorrhage and statin therapy should be not discontinued [69–71]. In any case, the most feasible explanation for reduction in clinical events reported for patients treated with statins is the stabilization of atherosclerotic plaques, which are generated by lipids deposition and migration and proliferation of vascular smooth muscle cells (see report from Malfitano et al. [30] and references cited therein for a more detailed explanation).

2.3.2. Alzheimer's Disease (AD)

AD is a chronic neurodegenerative syndrome caused by the appearance of brain senile plaques composed of aggregated forms of β-amyloid peptide (Aβ), and it is the most common cause of

dementia in elderly people, with a new case globally occurring every seven seconds [72]. Emerging evidence suggests a link between cholesterol and AD [37,72–76], and extensive studies have been published stressing the therapeutic utility of pleiotropic effects of statins, showing a dose-dependent beneficial effect on cognition, memory, and neuroprotection [72] by different mechanisms, such as altering the properties of plasma membrane by a reduction in cholesterol levels and a modulation of secretase activities, thus decreasing amyloid precursor protein (APP) processing [77], or by altering neuronal activity via modification of GTPases prenylation [28,74,78]. On the other hand, a possible effect of statins in cholinergic neurotransmission has been also described; in fact, simvastatin inhibits acetyl cholinesterase (AChE) activity in rats [79] and prevents the blockade caused by AChE inhibitors at α 7-nicotinic AChE receptors [80], thus increasing cholinergic neurotransmission. In this sense, Ghodke et al. [81] reported that statins treatment for 4 months, but not for 15 days, showed noteworthy enhancement in mice memory function, whereas a high cholesterol diet showed significant diminishing of memory. However, long-term statin treatment showed a significant decrease in serum cholesterol level as well as brain AChE level. Moreover, a high cholesterol diet showed a significant decrease in memory function with an increase in serum cholesterol level as well as brain AChE level. Thus, they concluded that there was no direct correlation between brain cholesterol level, as well as HMG-CoA activity with memory function regulation, although there is tangible link between plasma cholesterol level and AChE level, and long-standing plasma cholesterol alteration may be essential to regulate memory function through the AChE modulated pathway. Finally, a simvastatin-related rise of butyryl cholinesterase (BuChE) activity in mice brain, which may be a potential adverse effect in patients with AD, has been recently reported [82].

Another feasible mechanism for explaining statins' neuroprotective effect considers an activation of the heme oxygenase/biliverdin reductase (HO/BVR-A) system [37]. Statins can also be active in AD treatment because of their protecting effect against glutamate toxicity over primary cortical neurons [83,84]. Low-dose administration of statins avoids aberrant neuronal entry into mitosis [85], promotes anti-apoptotic pathways [86], and impairs inflammation [87], although higher doses of statins have been shown to induce toxic effects [88]. Recently, some studies point towards the utility of simvastatin administration in the improving of hippocampus-dependent spatial memory in mice, due to an activation of Akt (protein kinase B), via a depletion of FPP and inhibition of farnesylation [89,90]. This same group has recently shown how simvastatin administration potentiates the contribution of *N*-methyl *D*-aspartate receptor (NMDAR) to synaptic transmission, by increasing the surface distribution of the GluN2B subunit of the NMDAR without affecting cellular cholesterol content [91]. The influence of statins in these ionotropic glutamate-receptors, and the succeeding utility of these drugs on treatment of AD and other mental disorders, is undoubtedly a very attractive and innovative research field [91,92].

Lamentably, although most evidence consistently confirms how statins do afford neuroprotection and improve disease pathology in animal models [93,94], results are rather controversial or even disappointing in human trials [72,95–98], thus a very careful study design and analysis will be essential in the future [95].

2.3.3. Parkinson's Desease (PD)

PD, the second most common chronic neurodegenerative disorder in adults over the age of 65 years [99], is a progressive neurodegenerative disorder characterised by the presence of intracellular protein aggregates (Lewy bodies) and the loss of dopaminergic neurons from the *pars compacta* component of the *substantia nigra* in the midbrain; PD-related clinical manifestations of dopamine deficiency (gait, tremor, rigidity, and bradykinesia) are the most archetypical symptoms of this disease. There are several studies showing that some statins (simvastatin, but neither atorvastatin nor lovastatin) may reduce the incidence of PD in patients aged over 65 years [100]. Compared with discontinuation of statins, continuation of lipophilic statin use has been associated with a reduced risk of PD, particularly in the elderly [101]; nevertheless, in patients with existing PD, 10-day treatment of simvastatin

(40 mg/day) showed no significant effects on dyskinesia, functional impairment, or involuntary movement [102].

As inflammation is accepted to be a main contributor to the PD aetiology, the anti-inflammatory action of statins could be a rational explanation for their activity [30]; in fact, simvastatin has been reported useful for reversing the loss of striatal dopamine activity and the production of nitrosylated free radicals, thus inducing neuro-protection [103,104], by decreasing the release of inflammatory mediators from microglia. Also, some studies in rats have shown that simvastatin can protect against loss of NMDA receptors produced by 6-hydroxydopamine (6-OHDA) [105]; also using the 6-OHDA model in rats, Wang et al. [106] recently described the beneficial effect of simvastatin in reducing abnormal involuntary movements known as L-DOPA-induced dyskinesia, commonly observed in patients chronically treated with L-DOPA. Finally, simvastatin and pravastatin can decrease the dopaminergic neuronal loss induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) via inhibition of p21(Ras)-induced NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [107].

Anyhow, as mentioned in the previous paragraph, more definitive evidence from prospective and clinical studies is required before drawing any conclusions about statins efficacy for treatment of PD.

2.3.4. Depression

As well as for the previous neurological disorders discussed so far, there are reported discrepancies about statins' effect in depression, with some studies reporting positive effects of statins in reducing depression and depression-like symptoms in animals [108–115] or humans [116,117], while some others stated no relationships [118–120]. These divergences require more detailed studies, also for elucidating the possible mechanism of the positive effects, which, in some cases, have been associated with a modulation of NMDA receptors [121] or peroxisome proliferator-activated receptor gamma (PPAR-γ) receptors, by NO inhibition [122].

2.3.5. Epilepsy

In several studies, a reduced risk of developing epilepsy after age 50 has been reported [123–128], and the mechanism for this neuroprotective effect has been associated with a decrease in the association of subunit 1 of NMDA receptors to lipid rafts [129], as well as inhibition of calcium-dependent calpain activation, ROCK inhibition, the activation of the PI3K pathway, and increased APP cleavage [124], or the increased expression level of eNOS [130]; a recent publication by Scicchitano et al. [131] summarises the currently available data concerning statin effects in modulating epileptic seizure activity (sometimes adversely) and epileptogenesis in different experimental models, as well as in clinical studies [123,132].

2.4. Cancer

There are many studies dealing with the potential antitumor efficacy of statins, reporting effects in different cancer cell lines, as well as the possible risks of cancer development caused by statins treatment and the results of different clinical trials [133–138]. Once again, the molecular mechanisms explaining statins' effects are quite different, and clinical trials are not reporting conclusive results; in fact, although some large scale meta-analyses seem to indicate that statins do not have significant effects on cancer incidence [133,139,140], in some other cases, some beneficial effects associated with statins' administration in the treatment of different cancers have been described [141–144].

What is really clear is that there is not just one mechanism explaining the anticancer activity of statins, because depending on the type and dosages of statin used, the type of cancer cells, and the time of exposure of cells to statins, different effects leading to cell-cycle arrest, induction of apoptosis, or changes in molecular pathways are reported [138]. Concerning cell modifications, a common scheme is followed, starting with an arrest of cells in the G1 [145,146] or S-phase [147], and this inhibition of cell-cycle progression is mediated by cyclins (such as cyclin D1 [148]), cyclin-dependent kinases (CDKs, such as $p21^{WAF1/CIP1}$ [148], p27 [149], CD4 [148], or p53 [150,151]), and inhibitors of CDKs [145].

Simultaneously, inhibition of G-protein prenylation is produced, leading to the arrest of proliferation and/or induction of apoptosis in cancer cells [152,153], by an increase in caspases activity [147,154,155]; henceforward, inhibition of prenylation is a promising way to impede progression of cancer (see the recent review of Matusewicz et al. [138] and cites therein). On the other hand, it has been also reported that a substantial reduction in the amount of cholesterol leads to a reduction in the content of membrane lipid rafts in the cell membrane, altering cell signalling [156,157], and loosing membrane integrity; in this sense, it is known that the membrane of breast cancer and prostate cancer cells has higher level of cholesterol and lipid rafts, so these cells are more susceptible to apoptosis promoted by statins compared with normal cells [158,159]. In another feasible mechanism, statins are associated with inhibition of phosphorylation of caveolin-1 (Cav-1), the integral membrane protein that binds and transport cholesterol, which promotes tumour cell survival and resistance to chemotherapy by different mechanisms [160].

Anyhow, an exhaustive recompilation of all other mechanisms proposed to explain the action of statins in cancer treatment would be out of the scope of this manuscript and can be found in recent reviews [135,136,138,161]. However, once again, while the correlation between data obtained in vitro with those other ones reported in animal models is very high, clinical trials are not that irrefutable in their conclusions, and more detailed studies are demanded.

3. Biocatalyzed Synthesis of Statins

As previously indicated in the Introduction, the use of biocatalyzed steps for preparing homochiral synthons useful for the synthesis of statins is a smart strategy for gaining both efficiency and sustainability. In this section, we will present some examples.

3.1. Simvastatin

Lovastatin (Mevacor **2**, ATC Code C10AA02, DrugBank Code DB00227, Figure 1) is a naturally-occurring fungal polyketide produced by *Aspergillus terreus* [162], while simvastatin (**3**, ATC Code C10AA01, DrugBank Code DB00641, Figure 1) is a semisynthetic analogue of **2** and is more effective in treating hypercholesterolemia, because of the fact that the substitution of the α-methylbutyrate side chain with α-dimethylbutyrate significantly increases the inhibitory properties of **2**, while lowering undesirable side effects [10].

Because of the economic importance of simvastatin, as mentioned in the Introduction, various multistep syntheses of **3** starting from **2** have been described; thus, a widely used process (route #1) starts with the hydrolysis of the C8 ester in **2** to yield the triol Monacolin J **10**, followed by selective silylation of the C13 alcohol to yield **11**, esterification of C8 alcohol with dimethylbutyryl chloride to furnish **12**, and deprotection of C13 alcohol to finally yield **3** [163] (Figure 3).

In another option, namely route #2 [164], lovastatin **2** was treated with n-butylamine and TBSCl to obtain **13**, which was alkylated with another methyl group to furnish **14**, and finally transformed into **3** by hydrolysis and lactonization. Both multistep processes shown in Figure 3 were laborious, thus contributing to simvastatin being nearly five times more expensive than lovastatin [165].

Some enzymatic transformations using lipases and esterases were investigated as alternatives to chemical hydrolysis leading to Monacolin J **10** [166,167]. However, the requirement of regioselective esterification of the C8 alcohol invariably involves protection of other reactive alcohol groups in **10**, and generally leads to lowered overall yield. Therefore, a specific reagent that is able to selectively acylate C8 of **10** is important for the efficient synthesis of simvastatin **3** and additional statin analogues.

In this sense, Tang and co-workers [22] described an acyltransferase (LovD) able to catalyse the last step of lovastatin biosynthesis, as shown in Figure 4, by transferring a 2,2-dimethylbutyryl acyl group from dimethylbutyryl-S-methylmercaptopropionate (DMB-SMMP, **16**) regioselectively to the C8 hydroxyl of Monacolin J **10**, the immediate biosynthetic precursor of simvastatin. The reaction proceeds via a ping-pong mechanism, and LovD is inhibited by Monacolin J at moderate substrate concentrations. LovD displayed broad substrate specificity toward the acyl carrier, the acyl substrate, and the decalin

core of the acyl acceptor. This same group developed a one-step, whole-cell biocatalyzed process for the synthesis of Simvastatin from Monacolin J using an *Escherichia coli* strain overexpressing LovD, leading to >99% conversion of monacolin J to simvastatin without the use of any chemical protection steps [165]. The process was scaled up for gram-scale synthesis of simvastatin, also showing that simvastatin synthesized via this method could be readily purified from the fermentation broth with >90% recovery and >98% purity, as determined by high-performance liquid chromatography.
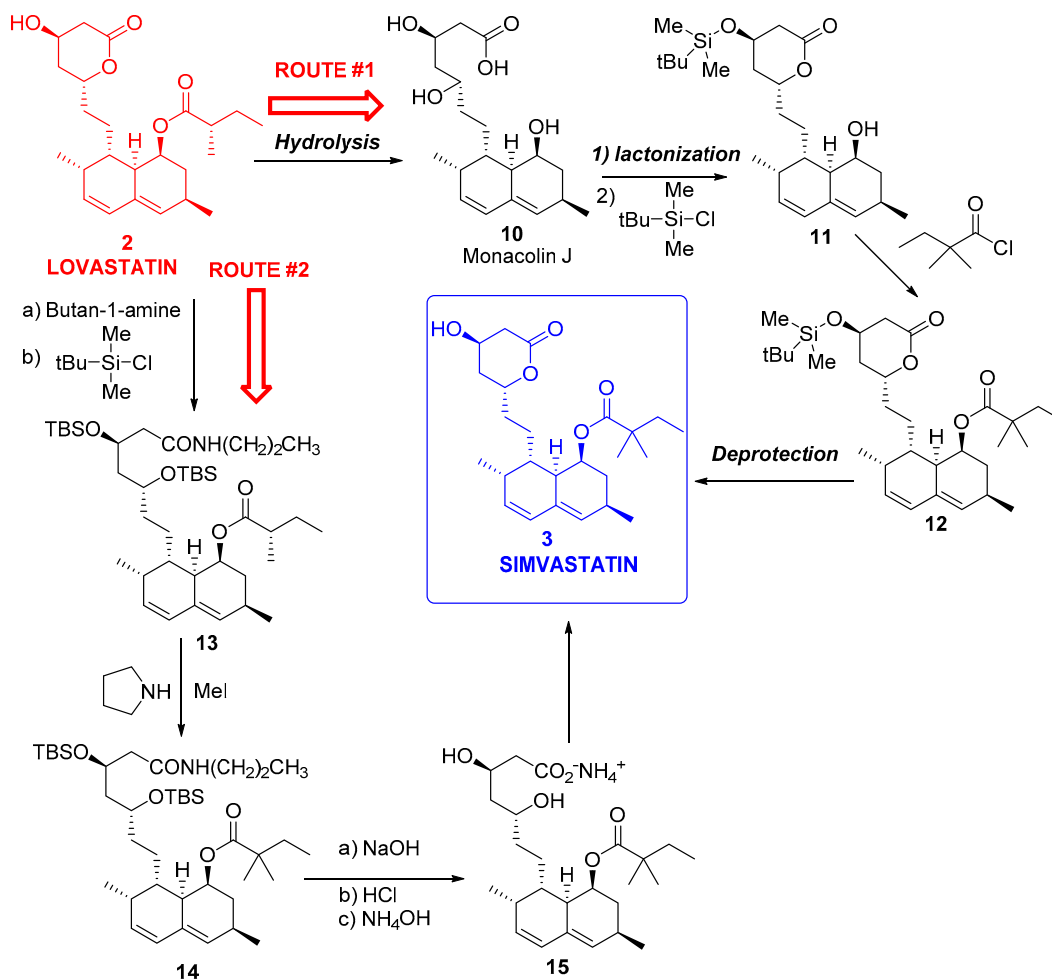

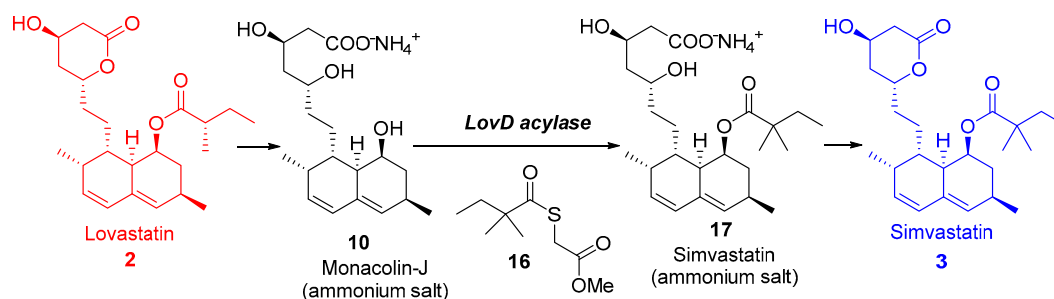**Figure 3.** Chemical transformations of lovastatin **2** into simvastatin **3**.

Figure 4. Biocatalyzed transformations of lovastatin **2** into simvastatin **3**.

Codexis improved not only the enzyme (previously modified used directed evolution at lab scale in an *E. coli*-based biocatalytic platform [168]) but also process chemistry to enable a large-scale simvastatin manufacturing process, by carrying out nine iterations of in vitro evolution, creating 216

libraries and screening 61,779 variants to develop a LovD variant with improved activity, in-process stability, and tolerance to product inhibition. The approximately 1000-fold improved enzyme and the new process pushed the reaction to completion at high substrate loading and minimized the amounts of acyl donor and of solvents for extraction and product separation. This process possesses many advantageous characteristics from a Green Chemistry point of view:

- Catalyst is produced efficiently from renewable feedstock.
- Reduced use of toxic and hazardous substances like tert-butyl dimethyl silane chloride, methyl iodide, and n-butyl lithium.
- Improved energy efficiency as the reaction is run at ambient temperature and at near atmospheric pressure.
- Reduction in solvent use because of the aqueous nature of the reaction conditions.
- The only by-product (methyl 3-mercaptopropionic acid) is recycled.
- The major waste streams generated are biodegraded in bio treatment facilities.
- Codexis' process can produce simvastatin with yields of 97%, significant when compared with <70% with other manufacturing routes.

For these reasons, Codexis and Prof. Tang obtained the U.S. Environmental Protection Agency's Green Chemistry Presidential Award in 2012 [169], inside the category of Greener Synthetic Pathway. Recently, identification of the complete biosynthetic pathway leading to monacolin J has been reported [170].

3.2. Biocatalyzed Synthesis of the Lateral Chain of Superstatins

Different biocatalytic routes have been proposed and implemented at industrial scale for the stereoselective preparation of the lateral chain (bearing the stereocentres) of superstatins. Thus, we would use the preparation of atorvastatin as a reference to illustrate how different biotransformations can be included in the overall protocol.

The chemical synthesis of atorvastatin originally described by researchers at Warner-Lambert Company [171], shown in Figure 5, started from a chiral building block, ethyl (*R*)-4-cyano-3-hydroxybutyrate **18**, also known as "hydroxynitrile" (**HN**), and the second stereogenic centre of **20** was obtained by diastereomeric induction, using cryogenic borohydride reduction of a boronate derivative of the 5-hydroxy-3-keto intermediate **19** derived from **HN**.
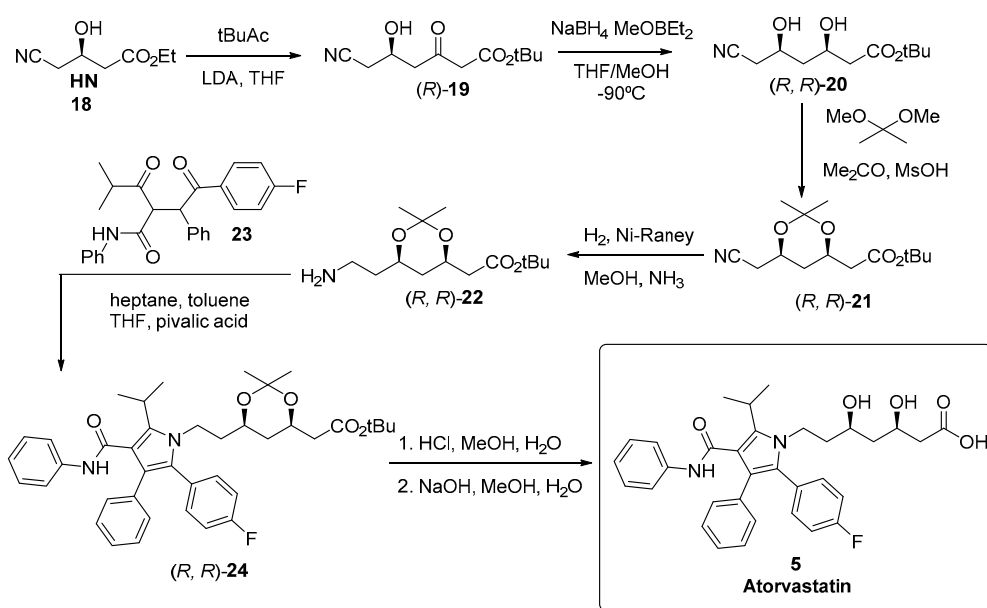

Figure 5. Chemical synthesis of atorvastatin **5**.

Taking this procedure as model, different strategies for generating the desired chirality can be envisaged from a biocatalytic retrosynthetic scheme [172], as depicted in Figure 6, in which purely chemical steps are denoted by a red **C**, while those syntheses feasible to be biocatalyzed are represented by a blue **BT** and a number, corresponding to the type of biocatalyst used. Thus, **route #1** creates the desired chirality by a stereoselective desymmetrization of dinitrile **25** using a *nitrilase* (**BT-1**), while **route #2** requires the preparation of **HN 18** via a bioreduction of ketoester **27**, so a *ketoreductase* (**BT-2**) is the biocatalyst required for that aim. Anyhow, in this synthetic path, another bioreduction should be used for avoiding the previously mentioned borohydride reduction of intermediate **19**, using another *ketoreductase* (**BT-3**), so this can be considered **route #3**. Finally, if an *aldolase* (**BT-4**) is the enzyme selected, it is possible to envisage **route #4** as an alternative through cyclic intermediate **28**. These different routes will be discussed in the following sections.

3.2.1. Hydrolases as Catalysts for the Preparation of the Lateral Chain of Atorvastatin and Other Superstatins

As shown in **route #1**, a nitrilase-catalyzed enzymatic desymmetrization of prochiral 3-hydroxyglutaronitrile **25** and subsequent esterification of the resulting (*R*)-3-hydroxy-4-cyanobutyric acid (*R*)-**26** can lead to **HN**. The use of enzymatic protocols for hydrolysing nitriles is a green alternative compared with chemical methodologies [173], because of the harsh reaction conditions required, demanding either strong mineral acids (e.g., hydrochloric or phosphoric acid) or bases (e.g., potassium or sodium hydroxide) and relatively high reaction temperatures. Moreover, chemical procedures sometimes give low yields because of both unwanted by-product formation and the generation of concentrated contaminating waste salt streams (e.g., 6 mol L^{-1}) when the acid or base is neutralized prior to disposal [174].
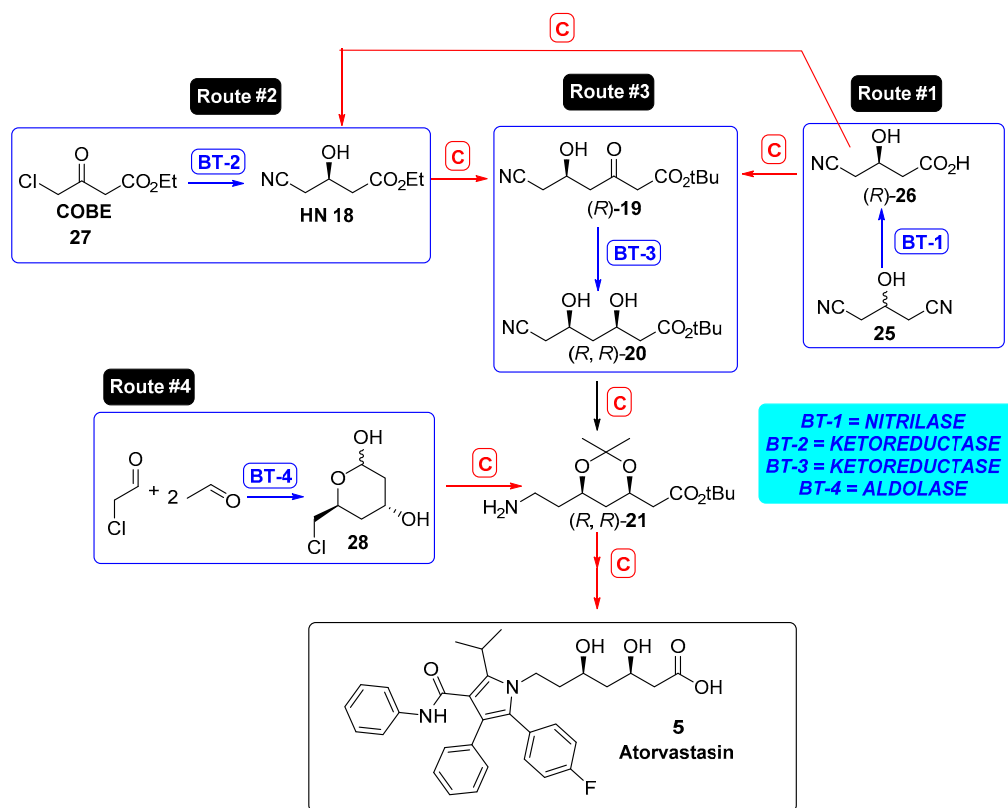

Figure 6. Biocatalytic retrosynthetic routes to atorvastatin. **BT** represents biotransformation step, while **C** stands for chemical processes.

Thus, researchers at Diversa described a wild type nitrilase enzyme that catalysed the desymmetrization of **25** at high substrate concentration (3M) at lab-scale reaction, with an enantiomeric excess (ee) of 88%. [175]. A mutant nitrilase, obtained by directed evolution using gene site saturation mutagenesis (GSSM), and showing Ala190His single mutation, resulted in an excellent biocatalyst; hence, after a 15 h reaction at 20 °C, (*R*)-**26** was isolated in 96% yield, with an excellent ee of 98.5% and a volumetric productivity of 619 g L^{-1} d^{-1} [176]. Subsequently, Dow Chirotech, a subsidiary of Dow Chemical Company, developed the Diversa nitrilase further into a biocatalysis process [177] and used the Pfenex expression system (a *Pseudomonas fluorescens*-based host expression system) to overproduce the enzyme. In this way, optimal reaction conditions for desymmetrization of **25** were as follows: 3 M (330 g L^{-1}), pH 7.5, 27 °C, under 16 h reaction time. A conversion of 100% and 99% product ee was obtained, and the so-formed (*R*)-**26** was consequently esterified to give **HN**. Overall, a highly efficient three-stage synthesis of **HN** starting from low-cost epichlorohydrin (required to produce **25**) was achieved with an overall yield of 23%, 98% ee, and 97% purity [177]. Recently, an enzymatic method has been described for the synthesis of ethyl (*R*)-3-hydroxyglutarate from **HN** using free and immobilized recombinant *Escherichia coli* BL21(DE3)pLysS harbouring a nitrilase gene from *Arabidopsis thaliana* (AtNIT2) [178]. The hydrolysis of **HN** proceeded with the freely suspended cells of the biocatalyst under the optimized conditions of 1.5 mol L^{-1} (235.5 g L^{-1}) substrate concentration and 6.0 wt % loading of wet cells at pH 8.0 and 25 °C, with 100% conversion obtained in 4.5 h. Furthermore, immobilization of the whole cells enhanced their substrate tolerance, stability, and reusability. Under the optimized conditions (100 mmol L^{-1} tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 8.0, 25 °C), the immobilized biocatalyst could be reused for up to 16 batches, with a biocatalyst productivity of 55.6 g gwet cells^{-1} and a space–time productivity of 625.5 g L^{-1} d^{-1}.

Hydrolases are also useful for preparing (*S*)-3-hydroxy butyrolactone (*S*)-**32**, another enantiopure intermediate to furnish **HN** (Figure 7). In fact, opening of (*S*)-**32** with HBr/EtOH will yield the corresponding ethyl (*S*)-4-bromo-3-hydroxybutanoate ((*S*)-BHBE, (*S*)-**33**) [179], later transformed into **HN** via S_N2 when treated with sodium (or potassium) bromide. Although (*S*)-**32** can be produced from chiral pool raw materials (lactose or malic acid), it can be conveniently obtained by enzymatic hydrolysis of the racemic ethyl 4-chloro-3-hydroxybutanoate (CHBE, *rac*-**29**) in the aqueous phase [180]. The lipase stereoselectively hydrolysed only the (*S*)-enantiomer; however, the resulting acid (*S*)-**30** is unstable, and it readily loses one HCl molecule to give the corresponding lactone of high enantiopurity (>99% ee). However, the enantiopurity of the lactone rapidly decreased when the process was operated at yields of more than 40%. The hydrolysis of the enantiopure benzoic ester of (*S*)-hydroxybutyrolactone (*S*)-**31** has also been described using lipase from *Candida rugosa* (CRL) immobilized on amberlite XAD-7 as polymeric support, with ee of 99% [181]. This enzymatic hydrolysis was observed to be non-stereoselective in nature, because the enzymatic hydrolysis of the racemic benzoic ester yielded the racemic lactone, so that a chiral pool precursor (L-malic acid) for this process was necessary. Anyhow, this method has been scaled up to a ton scale, with an overall yield of over 80%, and a reaction time of 14 h [182]. Recently, a platform pathway for the production of 3-hydroxyacids has been described as an alternative biosynthetic route to generate the enantiopure lactone [183].
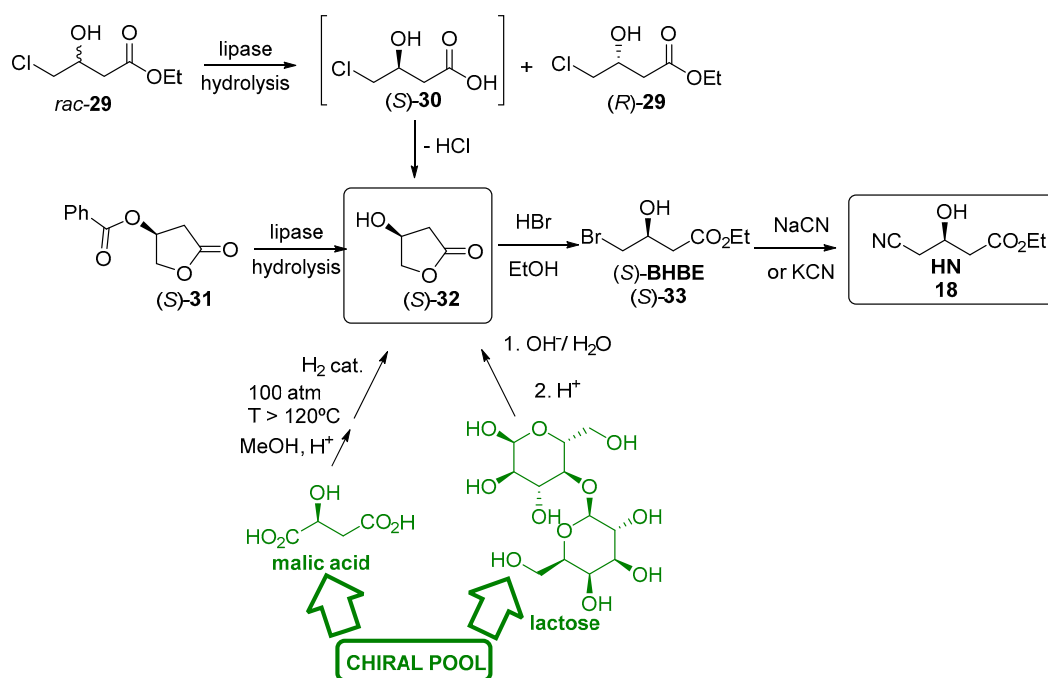
Figure 7. Preparation of **HN 18** starting from (*S*)-3-hydroxy butyrolactone (*S*)-**32**.

More recently, new biocatalytic approaches employing hydrolases have been described for furnishing the lateral chain of superstatins. Actually, Figure 8 shows a synthetic scheme for preparing rosuvastatin **9**. As can be seen, conjugated ketoester **36** is subsequently transformed into final calcium rosuvastatin **9** by different steps (silyl ether cleavage, diastereoselective Narasaka-Prasad [184,185] *syn*-reduction using diethylmethoxy borane leading to ester **37**, and finally ester hydrolysis and salt formation).
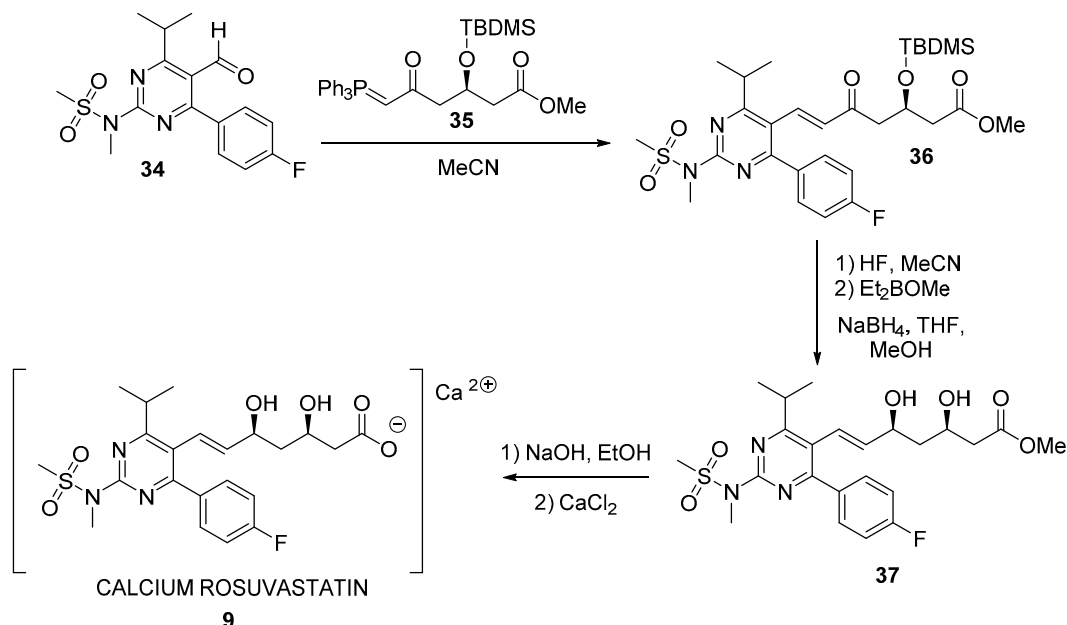

Figure 8. Final steps in the chemical synthesis of rosuvastatin **9**.

Aldehyde **34** can be easily obtained [186], while the preparation of enantiopure ylide **35** is much more complicated. Thus, several examples can be found in the literature starting from racemic diethyl 3-hydroxyglutarate, which had to be previously transformed in an activated derivative to react with

the corresponding methyltriphenylphosphonium ylide to finally yield **35**; although this route has been described using an enzymatic desymmetrization step [187,188], different side reactions were observed to decrease either the final yield or the enantiomeric excess. Recently, a bi-enzymatic process has been described for obtaining enantiopure monoester (*R*)-**40** (Figure 9), combining a stereoselective hydrolysis of prochiral **38** to obtain (*R*)-**39** with high yield and enantiopurity, and a subsequent removal of the acetyl group with cephalosporin acetyl esterase [189].
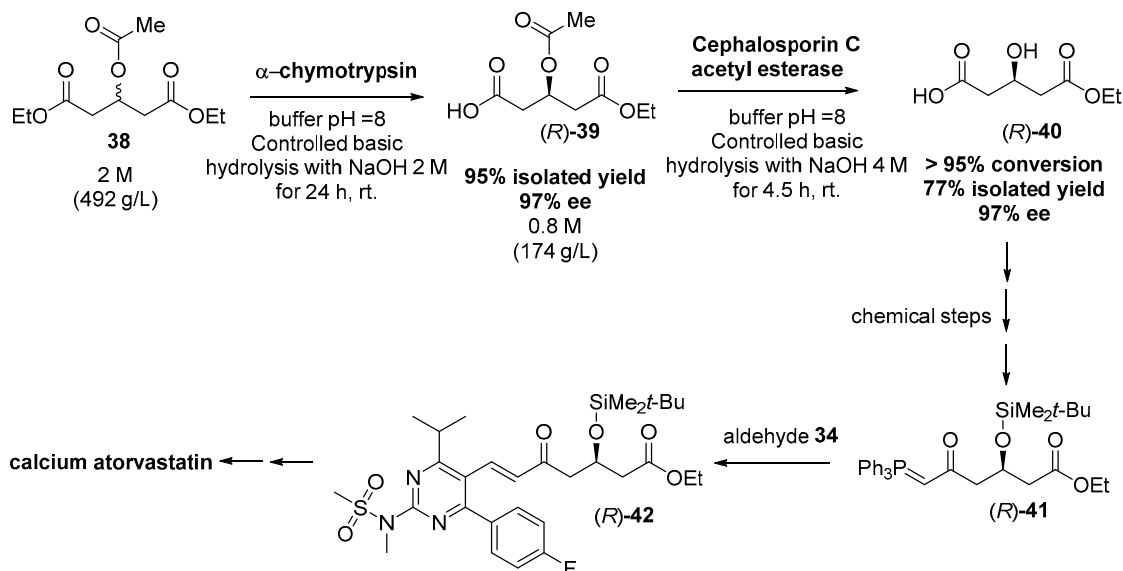

Figure 9. Two-enzymatic system for synthesizing chiral intermediates for Rosuvastatin, as described by Metzner et al. [189].

Furthermore, these same authors have optimized the overall procedure, using a smart engineering approach with an enzyme recycling of chymotrypsin and immobilized cephalosporin C acetyl esterase, with excellent volumetric productivity, transferring this technology to Sandoz for its industrial implementation [190].

3.2.2. Ketoreductases as Catalysts for the Preparation of the Lateral Chain of Atorvastatin and Other Superstatins

As commented before, **HN 18** was the starting point for the first synthesis of atorvastatin (Figure 5). For preparing **HN**, apart from the hydrolytic procedures described in Figure 7, some different purely chemical methodologies have been also described [191], and are depicted in Figure 10.
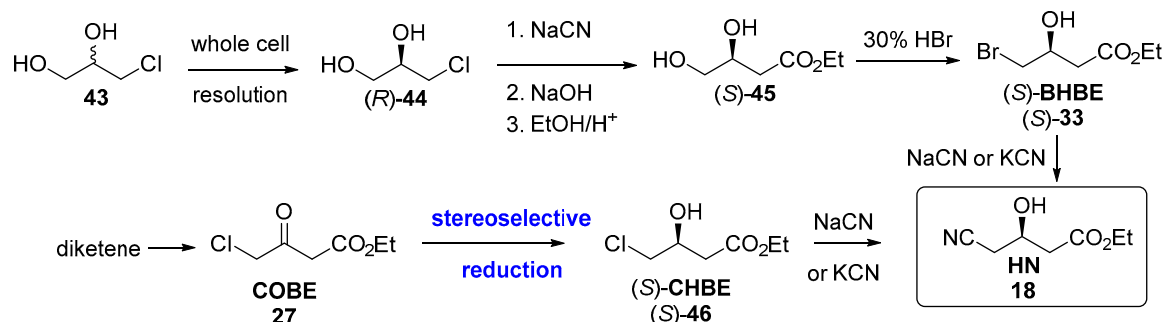

Figure 10. Different chemical methodologies for the preparation of **HN 18**.

The first synthetic protocols involved kinetic resolutions of prochiral 1,3-dichloropropan-2-ol **43** using microbes, and transformation to dihidroxyester (*S*)-**45** and subsequently to bromohydrine

(*S*)-**33** [179]. Later routes have involved asymmetric reduction of ethyl 4-chloroacetoacetate (COBE, **27**), produced from diketene, to furnish ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE, (*S*)-**46**), using either chemical or biocatalytic reductions, as previously shown in **route #2**, Figure 6. Finally, the corresponding halohydrin ((*S*)-**33** or (*S*)-**46**) could be converted to **HN** by treatment with cyanide.

In this sense, the enzymatic asymmetric reduction of 4-bromo-3-oxobutyrate esters has hardly been investigated compared with the corresponding chlorine analogue, because of the lower reactivity and enantioselectivity of enzymes towards brominated compounds, although (*S*)-4-bromo-3-hydroxybutanoate esters would be better substrates for the ulterior cyanide treatment; anyhow, some examples can be found in the literature, starting from methyl 4-bromo-3-oxobutyrate (BAM), using *Escherichia coli* engineered cells containing a mutant β-keto ester reductase (KER-L54Q) from *Penicillium citrinum* and a cofactor-regeneration enzyme such as glucose dehydrogenase (GDH) or *Leifsonia sp.* alcohol dehydrogenase (LSADH) [192,193].

Regarding chlorine containing oxoesters, the seminal paper of Patel et al. using glucose-, acetate-, or glycerol-grown cell (10% w/v) suspensions of *Geotrichum candidum* SC 5469 [194] to produce (*S*)-**46** in reaction yield of 95% and optical purity of 96%, starting from 10 mg mL^{-1} of **27**, showed how the bio-reduction could be an interesting alternative to asymmetrical chemical reduction. Furthermore, the optical purity of (*S*)-**46** was increased to >99% by heat treatment of cell suspensions (55 °C for 30 min) prior to conducting bio-reduction at 28 °C.

Ye et al. [195] have reviewed a list of different yeast able to reduce **27** to furnish (*S*)-**46**, such as *Candida etchellsii* [196], *Candida parapsilosis* [197], *Candida magnoliae* [198], *Saccharomycopsis lipolytica* [196], or *Candida macedoniensis* [199], but in many cases, the stereoselectivity values obtained were not very high. Also, fungi as *Aureobasidium pullulans* CGMCC 1244 [200], *Cylindrocarpon sclerotigenum* IFO31855 [201], *Penicillium oxalicum* IFO 5748 [197], *Botrytis allii* IFO9430 [197], or *Pichia stipitis* CBS 6054 [202] can produce (*S*)-**46** with a higher enantiomeric excess compared with yeasts. This same group, through genome database mining of this yeast *Pichia stipitis*, found two carbonyl reductases (PsCRI and PsCRII) leading to (*S*)-**46** with >99% enantiomeric excess, which were subsequently characterized, cloned, and expressed in *E. coli* [195]. On the other hand, Cai et al. [203] described a substrate-coupled biocatalytic process based on the reactions catalyzed by an NADPH-dependent sorbose reductase (SOU1) from *Candida albicans* in which **27** was reduced to (*S*)-**46**, while NADPH was regenerated by the same enzyme via oxidation of sugar alcohols (sorbitol, mannitol, or xylitol). Optimization of COBE and sorbitol proportions yielded 2340 mM of (*S*)-**46** starting from 2500 mM **27** with an enantiomeric excess was 99%. This substrate-coupled system maintained a stable pH and a robust intracellular NADPH circulation, so that pH adjustment and the addition of extra coenzymes were unnecessary, thus making this system very attractive. The bio-reduction of **27** and the scaling up of the process using *Escherichia coli* cells expressing a reductase (ScCR) from *Streptomyces coelicolor* to afford enantiopure (*S*)-**46** has recently being described [204], at substrate loading of 100 g/L, while the concentration of coenzyme NAD$^+$ was limited to 0.1 mM based on cost considerations, other reaction parameters were optimized as 25 °C and pH 6.5, with a biocatalyst dose of 10 kU/L in the presence of isopropanol (1.5 equiv of **27**) as co-substrate for regenerating NADH. The reaction was performed in a toluene−aqueous biphasic system (1:1, v/v), with agitation at the maximal linear rate of 0.88 m/s. Finally, the bio-reaction was performed on a pilot scale using a 50 L thermostatised stirred-tank-reactor, affording (*S*)-**46** in 85.4% yield and 99.9% ee, and a total turnover number (TTN) of 6060 for the cofactor NAD$^+$. The specific production was calculated to be 36.8 g product/g dcw, which is the highest value reported to date among the whole-cell-mediated processes for producing (*S*)-**46**. Furthermore, from the point of view of sustainability, for this bio-reduction, the reaction and extraction solvent (toluene) was recycled with a loss of only 4.1%, so that the E factor (kg waste per kg product) for the process was determined as 1.8 if the process water was excluded, which was much lower than that value (2.3) obtained from the process using isolated ketoreductase, glucose dehydrogenase as the biocatalyst for cofactor regeneration, and glucose as the co-substrate [179]. The main contributors to the low E factor were the loss of the solvent toluene (46.1%), the use of excessive isopropanol, and

the formation of coproduct acetone (combined ca. 35%). If water was also included, then the E factor would be 13.4.

Very recently, a recombinant *Escherichia coli* harbouring both the carbonyl reductase and glucose dehydrogenase has been described [205]. The recombinant *E. coli* was cultured in a 500-L fermenter, and the biocatalytic process for the synthesis of (*S*)-**46** in an aqueous-organic solvent system was constructed and optimized with a substrate fed-batch strategy. Concentration of **27** reached to 1.7 M, and (*S*)-**46** was obtained after a 4 h reaction in a 50-L reactor with yield of 97.2% and enantiomeric excess (ee) of 99%. Finally, (*S*)-**46** was extracted from the reaction mixture with 82% yield and 95% purity.

Nevertheless, because of the great overall demand of **HN** required for atorvastin synthesis (estimated to be in excess of 100 mT [179]), it is highly desirable to reduce the wastes and hazards involved in its manufacture, while reducing its cost and maintaining or, preferably, improving its quality. This has been successfully carried out on a multiton scale by Codexis by means of a three-enzyme two-step process, the detailed description of which is depicted in Figure 11.
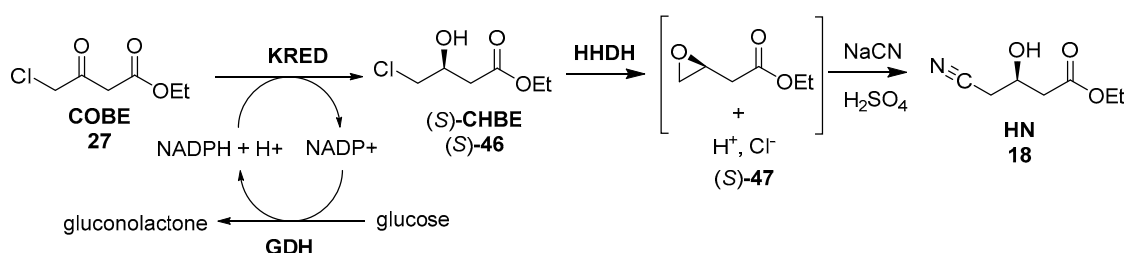

Figure 11. Codexis synthesis of **HN**.

Hence, the first step involves the biocatalytic reduction of **27**, using a ketoreductase (KRED) in combination with glucose and an NADP-dependent glucose dehydrogenase (GDH) for cofactor regeneration, leading to (*S*)-**46** in 96% isolated yield and >99.5% ee. In the second step, a halohydrin dehalogenase (HHDH), an enzyme capable of catalysing the elimination of halides from vicinal haloalcohols, resulting in epoxide ring formation [206], was employed to catalyse a nucleophilic substitution of chloride by cyanide, using HCN at neutral pH and ambient temperature. The efficiency and greenness of this protocol (Codexis was awarded the U.S. Environmental Protection Agency's Presidential Green Chemistry Challenge Award in 2006 for this work [207]) is based on the fact that all previous manufacturing routes to **HN** shown in Figure 10 involved, as the final step, a standard but troublesome S_N2 substitution of halide with cyanide ion in alkaline solution (pH = 10) at high temperatures (80 °C), being this reaction substituted in the Codexis protocol. In fact, in the S_N2 chlorine substitution, both (*S*)-**46** and **HN** are base-sensitive molecules, and extensive by-product formation is observed, leading to high E values [179]. Moreover, the product is a high-boiling oil, and a troublesome high-vacuum fractional distillation is required to recover **HN**, resulting in further yield losses and waste, and clearly contravening the first and sixth principles of Green Chemistry [208]. Thus, conducting the cyanation reaction under milder conditions at neutral pH, by employing the enzyme, HHDH, is the key step for increasing the greenness of the overall process.

Coming back to the Codexis protocol, awkwardly, both the wild-type KRED and GDH as well as HHDH displayed very low activities, so that in the first experiments, huge enzyme loadings were required to obtain an economically feasible reaction rate, thus leading to troublesome emulsions, which hampered the subsequent downstream processing. Additionally, severe product inhibition and poor stability under operating conditions were observed. To enable a practical large-scale process, the three enzymes were optimized by in vitro enzyme evolution using gene shuffling technologies according to predefined criteria and process parameters, resulting in an overall process in which the volumetric productivity per mass catalyst load of the cyanation process was improved ~2500-fold, comprising a 14-fold reduction in reaction time, a 7-fold increase in substrate loading, a 25-fold reduction in enzyme use, and a 50% improvement in isolated yield [179].

Also using bio-reductions, some other strategies have been developed for the preparation of chiral building blocks for statins synthesis. Thus, Figure 12 illustrates **route #3**, previously shown in Figure 6, depicting bioreduction of the corresponding 6-substitued-3,5-dioxohexanoates **48** to furnish (*R* or *S*)-**49** (similar to (*R*)-**19**, Figure 6). As depicted in Figure 5, the homochiral intermediate (3*R*,5*R*)-**20** was originally prepared by diastereoselective chemical reduction of (*R*)-**19**, using NaBH$_4$ and MeOBEt$_2$ and, so as to obtain a high diastereoselectivity (>99.5% de), an extremely low temperature (−90 °C) and pyrophoric triethyl borane were demanded [209], with a concomitant extensive energy consumption and substantial amount of waste formation. Another alternative chemical route using chlororuthenium(II) arene/β-amino alcohol as the catalyst for the reduction was described [210], although the diastereoselectivity was insufficient (80% de).
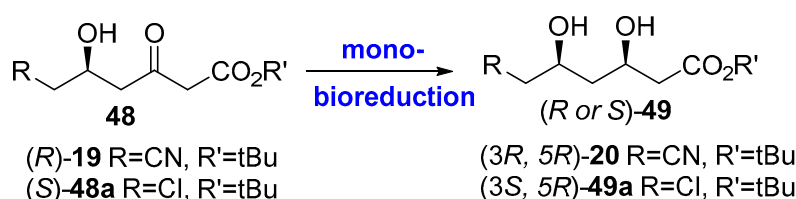

Figure 12. Bio-reductions to produce chiral building blocks for statins.

Therefore, the use of a ketoreductase is highly desirable to develop green and sustainable bioreduction. This process has been described [211,212] using NADP(H)-dependent alcohol dehydrogenase of *Lactobacillus brevis*. This enzyme was overexpressed in a recombinant *E. coli* and the cell extracts were then employed for carrying out the biocatalytic reactions on a gram scale, to reduce (*S*)-**48a** to give the corresponding (3*S*, 5*R*)-**49a** in >99.5% de and isolated yield of 72%, at 24 h. Alcohol dehydrogenase itself recycles its cofactor by a substrate coupled methodology, by oxidation of 2-propanol to acetone. This process was scaled up to 100 g [213] using a fed-batch reactor, with the conversion of more than 90% attained in a total reaction time of 24 h. For the same substrate, Liu and co-workers have reported the use of a ketoreductase from *Rhodosporidium toruloides*, wild-type and genetically evolved, under different reaction conditions [214–217], while Xu et al. used a ketoreductase from *Klebsiella oxytoca* [218]. On the other hand, for reducing (*R*)-**19** (up to 300 g L^{-1}), the ketoreductase from *L. brevis* overexpressed in *E. coli* cells has also been employed [219], coupled to glucose-GDH for cofactor recycling, yielding (*R*,*R*)-**20** in >99.5% de and 351 g L^{-1} d^{-1} space–time yield under the optimized conditions. Very recently, the same group has evolved the ketoreductase in order to improve the activity and thermostability of the enzyme [220]; thus, by coexpressing both the mutant ketoreductase and GDH, they describe the bioreduction of (*R*)-**19** to (*R*,*R*)-**20** at 40 °C in only 6 h, leading to values of >99.5% de and 1050 g L^{-1} d^{-1} space–time yield. Other similar bioreductions have also been reported using ketoreductases from other sources, such as *Rhodotorula glutinis* (whole cells [221]); engineered cells containing overexpressed NADPH-dependant ketoreductase from *Saccharomyces cerevisiae* and GDH [222,223]; a wild-type ketoreductase from *Kluyveromuces lactis* XP1461 (NADH-dependant) expressed in *E. coli* [224], subsequently improved by site-saturation mutagenesis [225]; or the ketoreductase from *Candida albicans* XP1463, also expressed in *E. coli* cells [226].

In a similar way, the double reduction of dioxoesters **50** (Figure 13) would directly lead to the target dihydroxyester **51**. For this purpose, whole cells of *Lactobacillus kefir*, which contain two different types of alcohol dehydrogenase, are able to convert **50b** into the dihydroxy ester (3*R*, 5*S*)-**51a** (99% ee in a total yield of 47.5% after 22 h, [227]) and the cofactor NADP(H) was regenerated by the usual glucose metabolism of the cell.
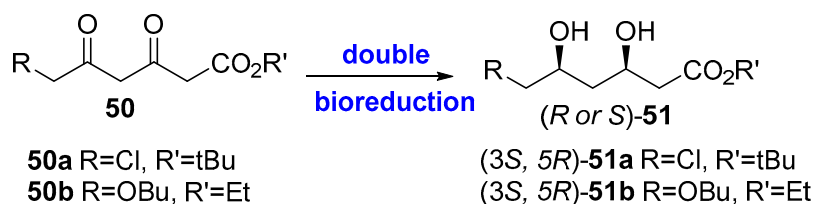
Figure 13. Bio-reductions of dioxoesters to produce chiral building blocks for statins.

The double bio-reduction has been also described using isolated enzymes, from *Acinetobacter* species; in fact, Patel et al. originally described the bio-reduction of **50b** using both whole cells and cell extracts from *Acinetobacter calcoaceticus* [228], and some years later, they also cloned and overexpressed [229] the diketoreductase responsible for the double reduction, which was efficiently carried out with the engineered enzyme [230]. Similarly, a diketoreductase from *Acinetobacter baylyi* ATCC 33305 was cloned and heterogeneously expressed in *Escherichia coli* by Wu et al. [231], showing an excellent biocatalytic performance at substrate concentration around 100 g L^{-1} [232] for the double reduction of **50a**. Interestingly, the 3D structure of this enzyme was reported, and the details of the catalytic mechanism were explained [233–235].

3.2.3. Aldolases for the Preparation of the Lateral Chain of Atorvastatin and Other Superstatins

Aldolases can also be used in the preparation of chiral building blocks for statin synthesis. This would correspond to **route #4** in Figure 6. In fact, Gijsen and Wong [236,237] first described the use of 2-deoxy-D-ribose 5-phosphate aldolase (DERA) from *E. coli* in the preparation of intermediate **28**, in a reaction mixture consisting of 133 mg of chloroacetaldehyde and 264 mg of acetaldehyde in a total reaction volume of 20 mL (Figure 14). The atorvastatin intermediate lactone (4*R*, 6*S*)-**54** can be easily formed by oxidation of lactol **28**. However, aldolase showed low affinity to chloroacetaldehyde and was promptly inactivated at required aldehyde concentrations, so that a huge amount of aldolase was required. Furthermore, a very long reaction time of 6 days was required because of the reversible nature of aldol reactions, making this process unpractical for scaling up.
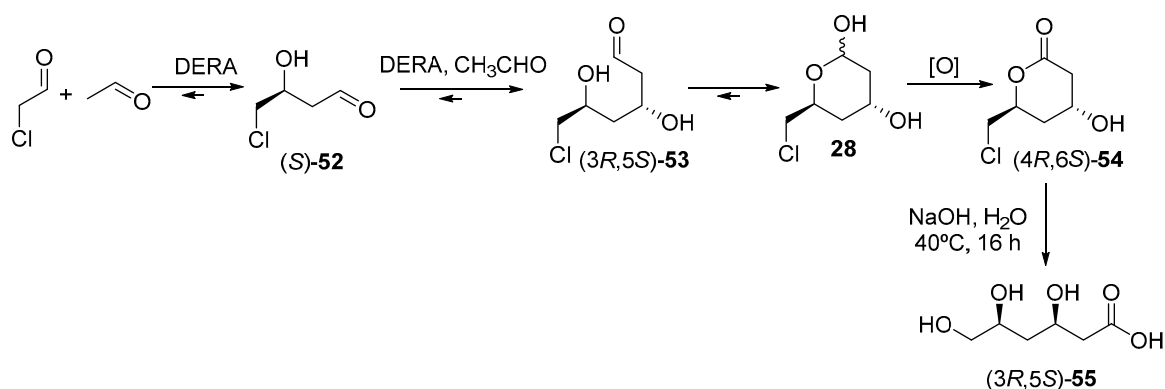

Figure 14. Aldolase-catalysed synthesis of chiral building blocks for statins.

Subsequent studies by Liu et al. [238] described a mutant aldolase, leading to an increased yield of (4*R*, 6*S*)-**54** to 43%, in comparison with 25% for the wild type aldolase, although the other reaction drawbacks were not overpassed. The process was markedly improved and scaled up by Greenberg et al. [239] of Diversa Corporation, by genetically modifying DERA by means of high throughput screenings of environmental DNA libraries, focussing on chloroacetaldehyde resistance and higher productivity; in a second step, the process was further improved by using a fed-batch bioreactor, in order to avoid significant substrate inhibition. Thus, the final synthesis of (4*R*, 6*S*)-**54** on a 100 g scale in a total reaction time of 3 h with an ee of >99.9% and a 10-fold reduction in catalyst load over the previous method [240]. More recently, the use of whole cells systems is being evaluated

for this process [241,242], as well as new strategies for improving DERA by genetic engineering [243]. Finally, a simple basic hydrolysis of lactone (4*R*, 6*S*)-**54** leads to the trihydroxyacid (3*R*, 5*S*)-**55**, which is the precursor [244] of the lateral chain of superstatins. On the other hand, scientists from Lek Pharmaceutical (a Sandoz company) have described the use of whole cells of *Escherichia coli* BL21 (DE3) overexpressing the native *E. coli* deoC DERA gene for production of chiral lactols such as **28** [241], with excellent volumetric productivity (up to 50 g L^{-1} h^{-1}), >80% yield, and >80% chromatographic purity with titers reaching 100 g L^{-1}. This process is highly cost effective and environmentally friendly, and its sustainability is even improved if the oxidation of **28** to (4*R*, 6*S*)-**54** is also catalysed with an enzyme, as this same group has reported using PQQ-dependent glucose dehydrogenases [245]. Ohshima and co-workers described the sequential aldol reactions depicted in Figure 14 using DERA isolated from thermophilic organisms, describing a relatively lower activity compared with the enzyme from *E. coli*, although this fact was compensated by a better synthetic yield caused by the increased acetaldehyde resistance shown by the thermophilic enzyme [246]. Shen and co-workers reported higher conversions when chloroacetaldehyde was used as the acceptor substrate, as compared with acetaldehyde [243], and thus the development of new DERAs from different microorganisms is an open research area, as reported in recent revisions [247,248].

In any case, compared with other chemical protocols, most pharmaceutical processes are performed on a smaller scale, with the production volume of 1000 to 10,000 tons per year and product concentration ranging between 50 and 100 g/L; hence, the main drawback is the transfer of the biocatalytic process from laboratory to a larger scale, especially with respect to retention times, which are greater on a larger scale (compared with those in the laboratory). A good example of industrial scale-up has been described by Ručigaj and Krajnc [242], who used acetoxyacetaldehyde and acetaldehyde as substrates, which are presented in an aldol reaction catalyzed by a crude DERA expressing culture lysate. By optimizing addition regimes of both reactants into a reaction mixture, the corresponding lactol was produced at near 77 g/L. The complete process was designed in a practical and economical manner and could be used further on an industrial scale. Another industrial scale, low temperature process was developed by DSM, leading to a final product concentration of 100 g L^{-1} [249].

4. Prognosis and Conclusions

It is easily foreseen that because our diet habits are becoming progressively unhealthier, with an increased uptake of fats and abandoning the traditional "Mediterranean diet", hypercholesterolemia and dyslipidaemia will be typical maladies in Western society. Thus, statins would be gradually more present in our lives, being a very important piece of the global pharmaceutical market, either as branded or generic drugs. This fact, combined with the plethora of other pharmacological activities, called pleiotropic effects, that are being ascribed to statins, as revised in Section 2, makes us predict an ever-growing market for this type of drug. Anyway, more detailed and careful studies are demanded in order to be sure about the real efficiency of pleiotropic therapeutic effects of statins, by clearly identifying those patients who could be the best ones for responding to the desired effect of statins, and by establishing the most effective dose, duration of use, and statin drug entity required. Besides, more accurate clinical trials have to be conducted in order to evaluate the real effect upon the desired target, by designing more effective and truthful biomarkers.

For statins' preparation, new and more sustainable protocols would be demanded; in this context, the substitution of chemical by biocatalyzed processes will certainly help to gain sustainability, because of the well-known green features of biocatalysis—synthetic routes conducted under mild reaction conditions; at ambient temperature; using water as reaction medium in many cases; and, last but not least, avoiding functional group activation and protection/deprotection steps usually required in traditional organic synthesis. Thus, we also foresee a growing increase in the use of biocatalysis and biotransformations for the preparation of statins, mainly promoted by the enhancement of biocatalysts' performance through chemical modification and genetic engineering.

In another context, very recently, a new type of drug has emerged for dealing with those patients already using statins, but not reaching low-density lipoprotein cholesterol levels, rather by genetic and environmental factors or by pathological states—known as statin-resistance [250,251]. These drugs are the inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9), a hepatic protease that becomes attached to low-density lipoproteins receptors (LDLRs), causing them to remain inside liposomes and get destroyed [252]. Nowadays, there are two PCSK9 inhibitors commercialized, both of them approved in 2015: alirocumab (Praluent®, from Sanofi) and evolucomab (Repatha®, from Amgen), both of them are used not as monotherapy, but are rather combined with a low cholesterol diet as well as with statins at maximally tolerated doses [253]. These two drugs are monoclonal antibodies, and their high price hampers their prior authorization practices and reduces their long-term adherence, so that the search for small molecules active as PCSK9 inhibitors is a "Holy Grail" in medicinal chemistry [254]. This situation leads us to think that (a) the statin market is not going to decrease, because they are going to be complemented (not substituted) with new drugs; and (b) as most of the new small molecules tested as PCSK9 inhibitors contain stereocenters in their structures [254], surely biocatalysis would become a very useful tool to facilitate more sustainable synthetic routes for their preparation.

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Conflicts of Interest: The authors declare no conflict of interest.

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