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Review

Biocatalytic synthesis of atorvastatin intermediates

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ABSTRACT

Atorvastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, and this drug leads to decreased levels of low density lipoprotein (LDL) cholesterol. Lower LDL cholesterol has direct relationship in reducing mortality from coronary heart diseases. Lipitor® (atorvastatin calcium) was the first drug to reach the annual sales of 10 billion dollars in USA and currently is the top selling pharmaceutical product globally. Atorvastatin has a side chain containing two chiral centers as its pharmacophore and it can be synthesized either from chiral pool precursors, by using metal catalysts; or more preferably by the application of free or immobilized enzymes and whole cell biocatalysts for carrying out either asymmetric synthesis or racemic resolution. Biocatalytic synthesis methods for chiral atorvastatin intermediates employ a wide variety of biocatalysts such as alcohol dehydrogenase, 2-deoxy-D-ribose 5-phosphate aldolase, nitrilase, lipase, etc. and each of these biocatalytic processes is discussed in detail in this paper.

Contents

1. Introduction

Atorvastatin belongs to a class of drugs known as statins and these drugs act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This results in the inhibition of the reductive conversion of HMG-CoA into mevalonate, which is an early rate limiting step of cholesterol biosynthesis. This leads to decreased levels of low density lipoprotein (LDL) cholesterol. Lower LDL cholesterol has direct relationship in reducing mortality from coronary heart diseases. Lipitor® (atorvastatin calcium) was the first drug to reach the annual sales of 10 billion dollars and currently is the top selling pharmaceutical product in the world.

Atorvastatin has a (3*R*,5*S*)-dihydroxyhexanoate side chain and synthesizing the intermediate for this side chain is challenging because it has two chiral centers, and greater than 99.5% e.e. and 99% d.e. are required [1]. Synthesis of such type of intermediates are carried out by adopting a general methodology (Scheme 1) of first synthesizing an intermediate **4** having a single hydroxyl group of required absolute configuration, and subsequently, by carrying out syn selective reduction [2], followed by protection of the hydroxyl groups, an isopropylidene-protected building block **2** having two stereogenic centers is obtained. This intermediate **2** is chief building block for the atorvastatin as well as for other statins such as rosuvastatin and pitavastatin. It is apparent from the above discussion that the key step in the synthesis of atorvastatin side chain is synthesis of a chiral intermediate **4**. This has earlier been synthesized from the chiral pool such as by using maltodextrin [3], L-malic acid [4] or by asymmetric synthesis catalyzed by ruthenium and rhodium complexes [5]. All these conventional approaches have

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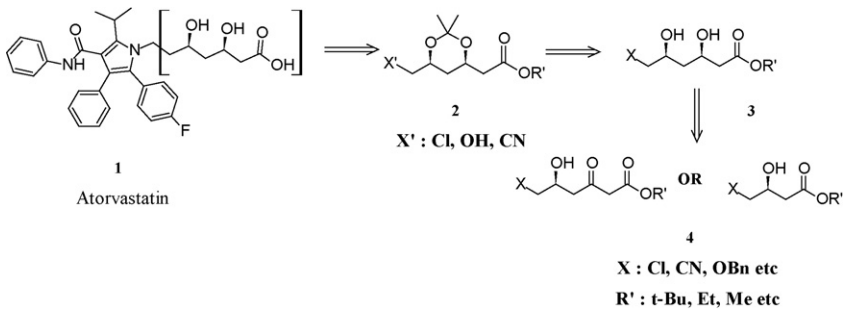


Scheme 1.

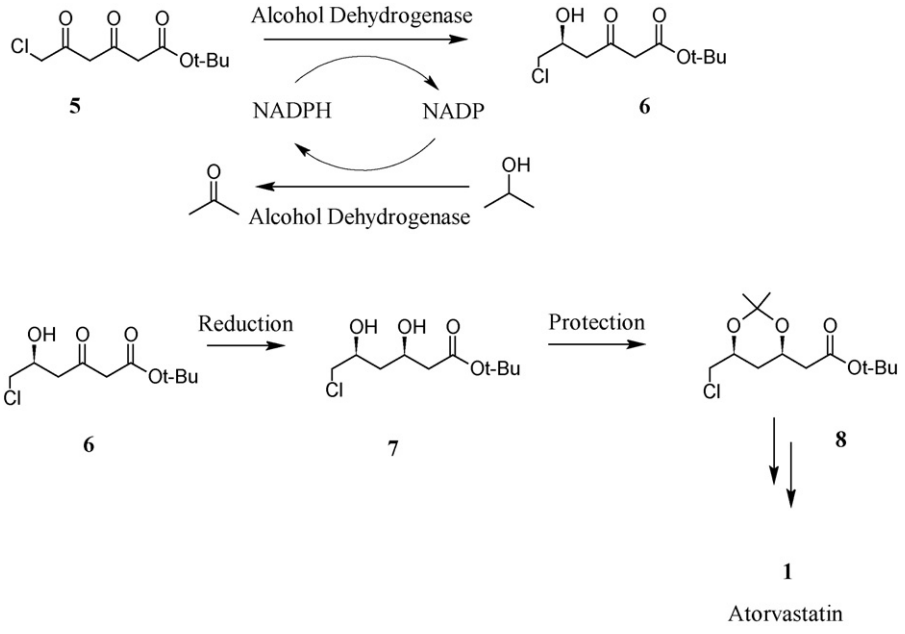
drawbacks such as high costs of catalysts and drastic reaction conditions as is the case for metal catalysts or having a large number of process steps, as is the case of synthesis using existing chiral pool. A more attractive and advantageous strategy is to employ an enzyme catalyzed asymmetric reaction or a kinetic resolution for synthesizing the desired intermediate **4**. Due to the high regio- and stereo-selective nature of biocatalysts, the generation of byproducts is reduced, in addition to this, most enzymatic processes usually operate at ambient reaction conditions, thus reducing the energy consumption vis a vis traditional synthesis methods. Hence biocatalytic synthesis methods tend to be more 'greener' than the conventional synthesis methods [6], and this green credential of biocatalysis further enhances the acceptance of this strategy to the industry. Moreover, certain biocatalytic processes such as those employing *Lactobacillus kefir* [7] or *Acinetobacter sp.* [8] as whole cell biocatalyst directly synthesize an intermediate **3** having two stereogenic centers with hydroxyl groups of required configuration. This further eliminates the need to carry out the syn selective reduction step, and thereby reducing the number of process steps required.

Biocatalytic synthesis methods for atorvastatin intermediates employ a wide variety of biocatalysts such as alcohol dehydrogenase, aldolase, nitrilase, lipase, halohydrin dehalogenase, etc. and each of the enzyme catalyzed processes is described in subsequent sections.

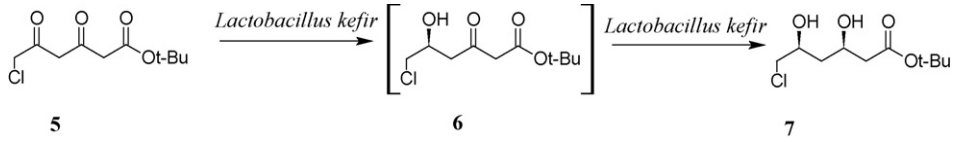
2. Alcohol dehydrogenase

Wolberg et al. [9,10] reported a novel method for the synthesis of hydroxy ketoester **6** (Scheme 2) a precursor to atorvastatin by using 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. The synthesis of the **6** was carried out on a gram scale using highly regio- and enantioselective reduction of the diketoester **5** catalyzed by NADP(H)-dependent alcohol dehydrogenase. Diketoester **5** is reduced exclusively in the δ-position (C5) by this enzyme to give hydroxyl ketoester **6** in >99.5% e.e. and isolated yield of 72%, with the reaction time being 24 h. Alcohol dehydrogenase itself recycles its cofactor by oxidation of 2-propanol to acetone and thus application of cofactor regeneration systems that require additional enzymes is avoided. This oxidation of 2-propanol is supposed to be driving force of the desired reaction. Hydroxy ketoester **6** is readily elaborated into dihydroxy ester **7** by carrying out syn selective reduction, and via further protection step the intermediate **8** can be synthesized. The latter is a central chiral building block for atorvastatin synthesis. The process was scaled up to 100 g scale [36] by using a fed-batch reactor, with the conversion of more than 90% attained in a total reaction time of 24 h.

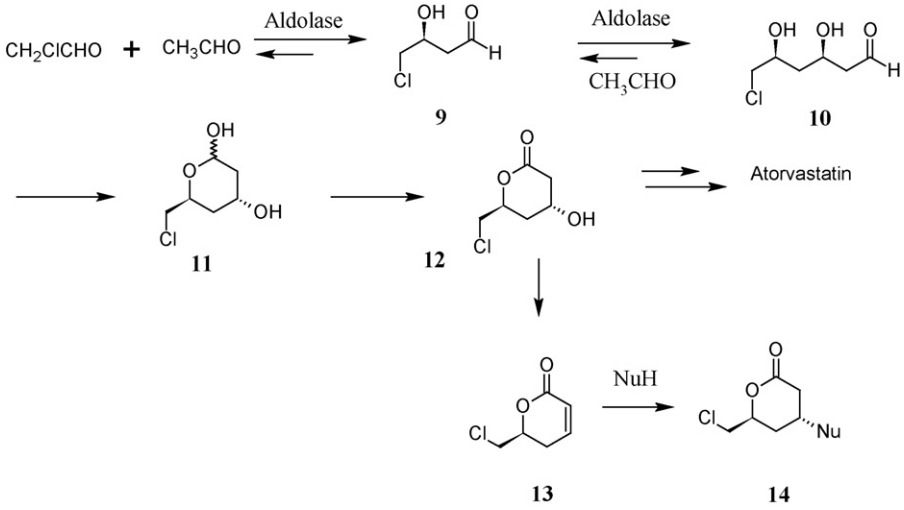
Another approach (Scheme 3) for the synthesis of **7** was by using *L. kefir* as whole cell biocatalyst [7]. It is known that this organ-



Scheme 2.



Scheme 3.



Scheme 4.

ism contains two different types of alcohol dehydrogenase, which in a highly stereo- and regio-selective manner, converts **5** into the dihydroxy ketoester **7** via the intermediate **6**. The cofactor NADP(H) was regenerated by the usual glucose metabolism of the cell. **7** Was obtained with 99% e.e. in a total yield of 47.5% with reaction time being 22 h.

3. Aldolase

The application of 2-deoxy-D-ribose 5-phosphate aldolase in carrying out one pot reactions for synthesizing chiral compounds from easily available achiral bulk chemicals was shown on a milligram scale by Gijsen and Wong [12,13]. The synthesis of intermediate **11** was carried out in a reaction mixture consisting of 133 mg of chloroacetaldehyde and 264 mg of acetaldehyde in total reaction volume of 20 ml (Scheme 4). The atorvastatin intermediate **12** can be easily formed by oxidation of **11**. However aldolase shows low affinity to chloroacetaldehyde and is rapidly inactivated at required aldehyde concentrations and hence very high quantity of aldolase is required. Another factor was that, due to reversible nature of aldol reactions, very long reaction time of 6 days was used in their process. Hence the process was unpractical for scale up.

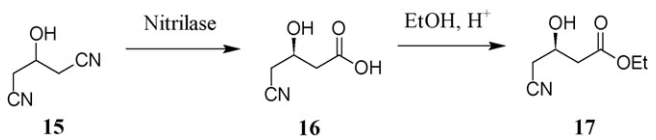
Subsequent studies by Liu et al. [14] used a mutant aldolase, which resulted in increased yield of **12** to 43%, in comparison to 25% for the wild type aldolase, however the other reaction conditions remained same. The process was substantially improved upon and scaled up by Greenberg et al. [15] of Diversa Corporation by improving the aldolase using high throughput screenings of environmental DNA libraries for chloroacetaldehyde resistance and for higher productivity, and several improved variants were identified. The process was further improved by using a fed-batch approach, to overcome the challenge of significant substrate inhibition. All this improvements resulted in the synthesis of **12** on a 100 g scale in total reaction time of 3 h with e.e. of >99.9% and a 10-fold reduction in catalyst load over previous method.

DSM pharma also operates an aldolase catalyzed synthesis of **12** on an industrial scale [16,17]. Jennewein et al. [18] of DSM pharma chemicals have also reported improved aldolase variants by using directed evolution and high throughput screenings. Recently researchers at DSM [11] have reported the conversion of **12** into an unsaturated lactone **13**, and by the subsequent addition of various nucleophiles such as AcSH, HCN, $MeNO_2$, etc., a range of different pyranoside derivatives **14** is obtained, thus increasing the versatility of the process in synthesis of other pharmaceutical intermediates.

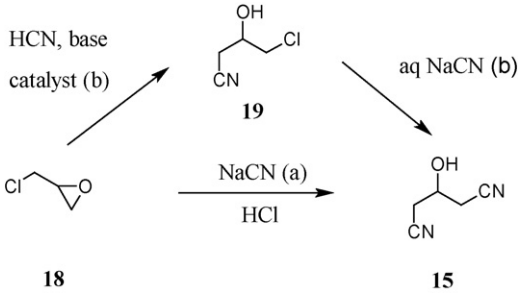
4. Nitrilase

The researchers at Diversa Corporation [19] created a large genomic library (>200) of nitrilases by extracting DNA from samples in various natural habitats and expressed them in host organism *E. coli*. These nitrilases were then employed to catalyze out various different enantioselective reactions. It was found that nitrilase from their library could carry out desymmetrization of the 3-hydroxyglutaronitrile **15** (Scheme 5) to atorvastatin intermediate (*R*)-4-cyano-3-hydroxybutyric acid **16**. The process was operated at 1 g scale with the substrate **15** concentration taken at 240 mM. The isolated yield of **16** was 98% yield with 95% e.e. in the total reaction time of 22 h. The subsequent esterification product of **16** is an atorvastatin intermediate.

When attempts were made to carry out the above process at more practical conditions [20], such as higher substrate concentrations of 2 M and 3 M, the e.e. values were 89.2% and 87.6%, respectively. These low enantiopurity does not meet the require-



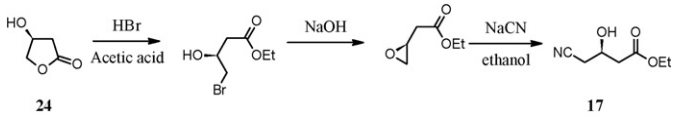
Scheme 5.



Scheme 6.

ments for the atorvastatin intermediate. The possible solution was to improve the enantioselectivity of the nitrilase. The method adopted by Desantis et al. [20] to achieve this, was a proprietary directed evolution technique [21], called gene site saturation mutagenesis. This essentially is a technique of replacing each amino acid of a protein with each of the other 19 naturally occurring amino acids, and expression of these genes in the host organism *E. coli*. The wild type nitrilase is 330 amino acids in length, and hence the total mutant nitrilases in this technique contained over 30,000 clones, and each were screened to identify mutant enzymes with improved enantioselectivity. The most effective mutant nitrilase catalyzed the synthesis of **16** at 3 M concentration with a yield of 96% and e.e. of 98.5% in total reaction time of 15 h. This was a dramatic improvement, both in terms of enantioselectivity, and reaction time, in comparison with the wild type nitrilase.

Bergeron et al. [22] of Dow pharma employed the Nitrilase from the Diversa Corporation, to develop a scalable process of synthesizing **17** from a low cost starting compound epichlorohydrin **18**. The one pot, one step reaction (Scheme 6a) for the synthesis of **15** was found to be unsuitable for the application on a plant scale, due to the precise pH control required for the reaction to proceed efficiently. Hence they devised a two step process to synthesize **15** (Scheme 6b). The yield for the synthesis of **19** was 93% and for the synthesis of **15** was 68%. The isolation of **15** was carried out by extraction with 2-methyl-propan-1-ol. The nitrilase catalyzed step to get **16** was carried out with 252.5 g of substrate **15** at 3 M concentration. The product **16** was obtained with e.e. of 98.8% and 81% overall yield in reaction time of 16 h. The extraction of **16** from the reaction mixture was difficult due to its water solubility and the presence of the cell mass in the mixture. The isolation method used was acidification and filtration with Celite filter aid followed by extraction



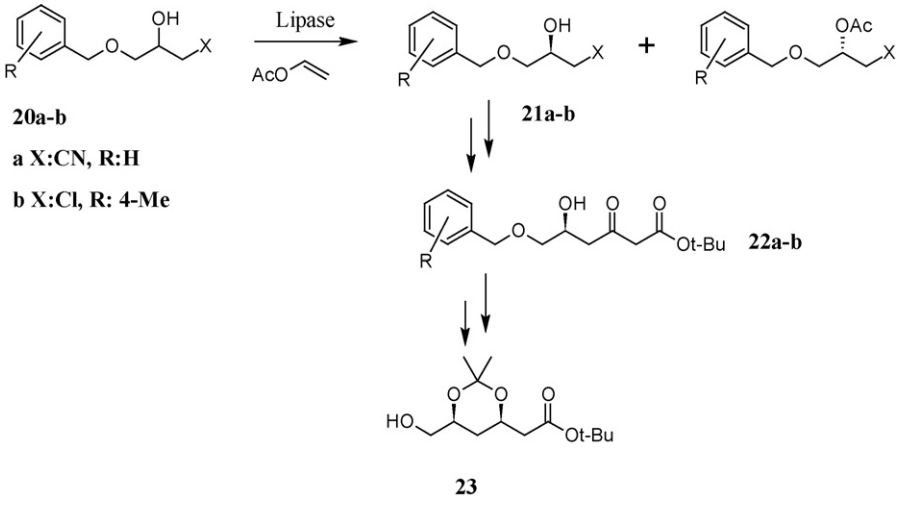
Scheme 8.

with methyl ethyl ketone. This process represents a scale up of over 200 times over previous reported method of Desantis et al. [20]. To lower the cost of the catalyst, i.e., nitrilase, it was expressed in the strain of *Pseudomonas fluorescens* by the Pfenex Expression Technology, developed by The Dow Chemical Company. The nitrilase was obtained in quantity of more than 50% of the total cell protein. This high level of expression directly contributes in easier downstream processing; thereby further lowering of the costs.

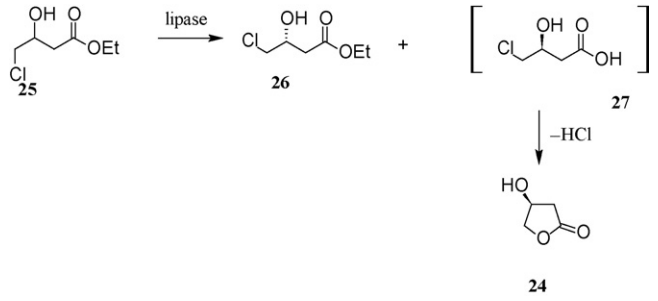
5. Lipase

Sun et al. [23] carried out the kinetic resolution of **20a** by transesterification (Scheme 7) with lipase from *Argribacter sp.* on a gram scale with 98% e.e. of **21a**, in an isolated yield of 40% with the reaction time of 45 h. The atorvastatin intermediate **22a** was then synthesized with 77% yield by chemical methods. They later reported [24] the synthesis atorvastatin intermediate kaneka alcohol **23** by employing **20b** as the enzymatic substrate, and lipase from *Alcaligenes sp.*, also it was observed that the reaction time for this step reduced to 30 h, with e.e. remaining the same. Their scheme for the synthesis of is rather unsuitable for scale up, not only because as this being kinetic resolution, hence maximum yield theoretically is just 50%, but also due to the large number of intermediate process steps with high reaction times after the enzymatic synthesis of **21a–b**.

(*S*)-3-hydroxy-gamma butyrolactone **24** is a useful C4 chiral synthon, and is used as precursor for the synthesis of nutraceuticals such as L-carnitine. Wang and Hollingsworth [25] have described a facile method for the synthesis of atorvastatin intermediate **17** from **24** in high overall yield of about 80% (Scheme 8). **24** has been synthesized earlier with high enantiopurity from the microbial resolution of racemic **25** using cell extracts of recombinant *E. coli* cells coding genes for *Enterobacter sp.* [26] and *Rhizobium sp.* [27]. A novel (*S*)-4-Chloro-3-hydroxybutyrate hydrolase was found responsible of this reaction; however, there was no significant similarity between this enzyme and lipase or esterase. **24** can also be synthesized by lipase catalyzed hydrolysis (Scheme 9) of racemic **25** in aqueous phase [28]. The lipase steriospecifically hydrolyzed only



Scheme 7.

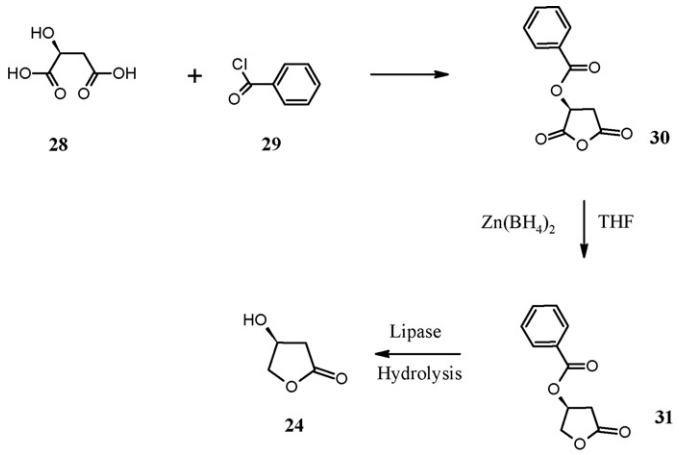


Scheme 9.

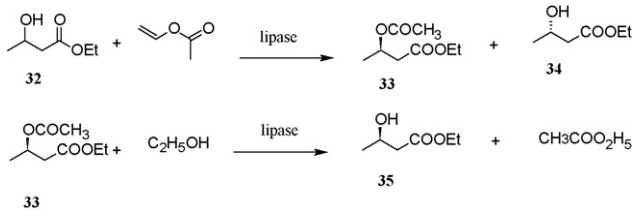
the (*S*)-enantiomer, however, the resulting acid **27**, is unstable, and it readily loses one HCl molecule to give the corresponding lactone **24** of high enantiopurity (>99% e.e.). However, the enantiopurity of **24** rapidly decreased when the process is operated at yields of more than 40%.

Lee et al. [4] of LG chemicals has recently reported the synthesis of **24** using a three step synthesis method (Scheme 10). The initial two steps in this synthesis scheme are carried out in a straightforward manner. In the second step, zinc borohydride was chosen as the preferred hydrogen carrier due to its cheaper cost then other hydrides such as sodium borohydride. In the third step, it was desired that the lactone ring does not get hydrolyzed under reaction conditions. Undesired byproducts were obtained when hydrolysis was carried out both in acidic and basic conditions. Hence more than 15 different lipases, esterases, proteases, etc. were screened for their efficiency in carrying out enzymatic hydrolysis. The best results were obtained for *Candida rugosa* lipase and it was immobilized on amberlite XAD-7 as polymeric support and then employed for carrying out hydrolysis of **31** to obtain **24** with e.e. of 99.8%. This enzymatic hydrolysis was observed to be non-stereoselective in nature, and when racemic malic acid was used, the product **24** was a racemate too, and hence the application of a chiral pool precursor L-malic acid for this process was necessary. This process has been scaled up to a ton scale, with an overall yield of over 80%, and reaction time of 14 h.

Fishman et al. [29] developed a two step enzymatic process for the production of (*R*)-ethyl-3-hydroxybutyrate **35** (Scheme 11) and scaled up to a multikilogram scale. Both enantiomers **34** and **35** were obtained at 99% chemical purity and over 96% e.e. with an overall process yield of 73%. The first reaction involved an acetylation of racemic **32** with vinyl acetate for the production of **34** via the selective acetylation of (*R*)-22. In the second reaction, **33** was subjected to alcoholysis with ethanol to derive enantiopure



Scheme 10.



Scheme 11.

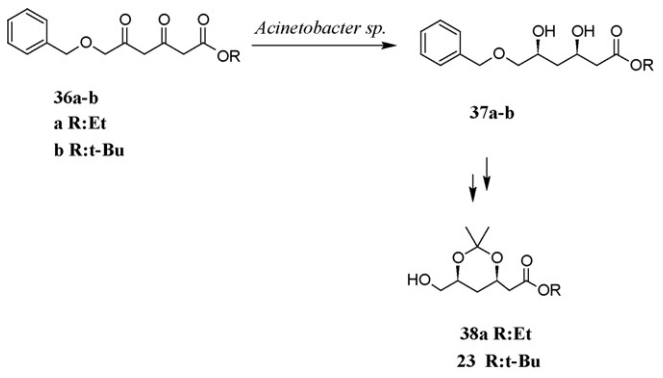
35. Immobilized *Candida antarctica* lipase B was employed in both stages, with high productivity and selectivity. The immobilized lipase was packed in a column and the reactants were circulated through the enzyme bed sequentially until each of the targeted conversions was reached. The desired products were separated from the reaction mixture after each of the two stages by fractional distillation.

6. Ketoreductase and halohydrin dehalogenase

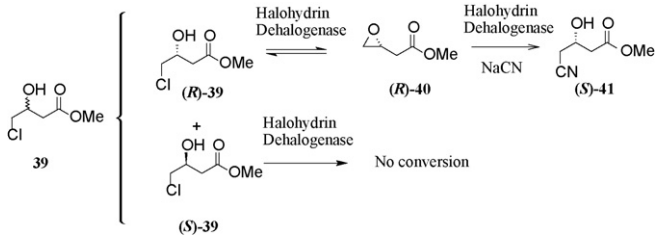
The researchers at Bristol-Myers Squibb [8] synthesized the dihydroxy ethyl ester **37a** on a gram scale with high e.e. (>97%) from the corresponding diketoester, by using *Acinetobacter sp.* as whole cell biocatalyst (Scheme 12). Ketoreductases secreted by these strains were responsible for this highly enantioselective reduction. Subsequently [30] the tert butyl ester **37b** and the corresponding kaneka alcohols **38a**, **23**, were also synthesized. Complete conversion of the substrates **36a–b** occurred to give the corresponding dihydroxy esters **37a–b** in 24 h, with the e.e. 99.3% and d.e. 63.3%. This crude product was further purified by flash chromatography and crystallization to obtain the purified product with 99% e.e. and 99% d.e. They later identified the gene encoding for desired ketoreductase and cloned them into *E. coli* [31]. They then used the cell extracts of these recombinant *E. coli* cells to carry out stereoselective reduction of **36a**, and observed a very high yield of 99.3% for **37a** with >99.9% e.e. and 99.8% d.e. with reaction time being just 3 h.

Elenkov et al. [37] had attempted to synthesize the atorvastatin intermediate **(*R*)-41** by a sequential kinetic resolution of the racemate **39** using halohydrin dehalogenase (Scheme 13). It was observed that the conversion of **(*S*)-39** to the atorvastatin intermediate **(*R*)-41** did not take place. However conversion for **(*R*)-39** was fast, and the product **(*S*)-41** was rapidly formed. Hence, **(*S*)-39** and **(*S*)-41** were isolated in >95% e.e. from the reaction mixture by column chromatography.

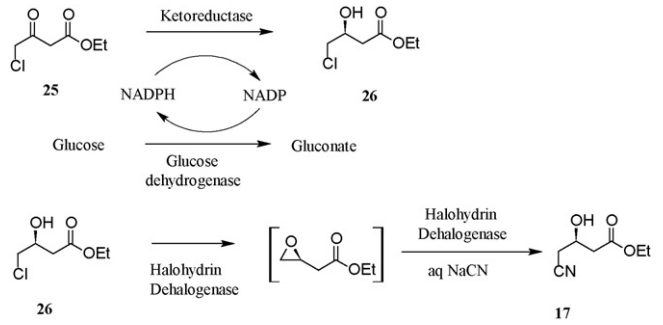
The researchers at Codexis have developed a two step process [32,33] for the synthesis of atorvastatin intermediate **17** (Scheme 14). Complete conversion of the substrate **25** takes place in the ketoreductase catalyzed first step of the process. The reaction



Scheme 12.



Scheme 13.



Scheme 14.

mixture of this step is then extracted with ethyl acetate, dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to obtain the product **17** in high chemical purity. The cofactor NADP(H) regeneration strategy consists of the glucose dehydrogenase catalyzed conversion of glucose to gluconate. In the second step of the process, halohydrin dehalogenase catalyzes the complete conversion of **26** into its cyano derivative **17** with high enantiopurity >99.9% e.e. via the intermediate epoxide formation. The product separation of **17** too consisted of solvent extraction followed by filtration and evaporation. This easy separation of the products without the need to carry out fractional distillation is a major advantage for the process. Note that the mutant form of halohydrin dehalogenase used in this process and those used by the Elenkov et al. [37] has complementary stereoselectivities. All the enzymes used in this process were produced by fermentation of recombinant *E. coli* cells. The genes encoding ketoreductase and halohydrin dehydrogenase were taken from *Candida magnoliae* and *Agrobacterium radiobacter* respectively, and for glucose dehydrogenase, genes from both *Bacillus megaterium* and *Bacillus subtilis* were cloned separately in *E. coli* cells.

Halohydrin dehalogenase for this process was developed by employing directed evolution approach in generating enzymes that meet predefined process-design criteria. This was done in conjunction with recombination-based directed evolution by incorporating a strategy for statistical analysis of protein sequence activity relationships [34]. This combination facilitated mutation-oriented enzyme optimization. On a biocatalyst basis, the halohydrin dehalogenase evolved through this combination process has improved the volumetric productivity of the cyanation reaction by approximately 4000-fold. Presently Lonza Inc., a fine chemical contract manufacturer for Pfizer, is operating this process on an industrial scale. Codexis Inc. also won the 2006 Presidential Green Chemistry Challenge Award from the United States Environmental Protection Agency (USEPA) under the focus category "Greener Reaction Conditions" for the development of this process.

7. Conclusion

The ability of biocatalysts to convert cheap achiral compounds into high value chiral pharmaceutical intermediates has kept research interest in this field high, and apart from atorvastatin intermediates, many other pharmaceutical intermediates are slated to the manufactured on a large scale using biocatalysts [35]. In this regard, biocatalysis is becoming an important method for the production of pharmaceutical intermediates. The major challenges for biocatalysis in general, are the relatively low volumetric productivity of enzyme catalyzed reactions, leading to higher costs of downstream processing, as well as difficulty for scale up. However the progress made in the directed evolution techniques such as site saturation mutagenesis, high throughput screening techniques, etc. has tremendously increased the volumetric productivity, leading to effective scale up with lower reaction times; and with ever increasing number of commercially marketed enzymes, the acceptability of the biocatalytic reactions is bound to increase in future.

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