Review of Literature

2.1 Statins- the HMG CoA reductase Inhibitors

Statins are a class of molecules with polyketide structure, which inhibit the rate limiting enzyme of cholesterol biosynthesis pathway, HMG-CoA reductase (HMGR). This activity makes these molecules suitable for therapeutic use (Alberts et al. 1980). The statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the most effective class of drugs to treat hypercholesterolemia. They act by interfering in the cholesterol biosynthesis by inhibiting the conversion of HMG-CoA into mevalonate. This inhibition leads to a decrease in the total cholesterol. They are compounds of natural origin that are biosynthesized as secondary metabolites of filamentous fungi and act as competitive inhibitors of HMG-CoA reductase. They are bulky and literally get "stuck" in the active site of the enzyme, thus preventing the enzyme from binding with its substrate, HMG-CoA and block the cholesterol synthesis pathway.

2.1.1 Classification of Statins

The statins are classified into two groups:

Type 1 statins: This class of statins are naturally produced and have substituted decalin-ring structure, which was also present in the first ever discovered statin: Mevastatin. The common statins that belong to this group are: Lovastatin, Pravastatin and Simvastatin.

Type 2 statins: This class of statins is synthetic in nature and the main differences between the type 1 and type 2 statins is the replacement of the butyryl group in type 1

statins by the fluorophenyl group in type 2. This group is responsible for additional polar interactions that cause tighter binding to the HMGR enzyme. The most common statins that belong to this group are: Fluvastatin, Atorvastatin and Rosuvastatin.

2.1.2 Chemical Structure of Statins

All natural statins are of very similar chemical structure. They possess a common main polyketide portion, a hydroxy- hexahydro naphthalene ring system, to which different side chains are linked at C8 (R1) and C6 (R2) (Fig 2.1). Lovastatin (or mevinolin, monacolin K, and Mevacor, Merck) contain a methylbutyric side chain (R1) and a 6-α methyl group (R2), which is lacking in mevastatin (or compactin, ML-236B, and CS-500). Pravastatin (or eptastatin and Pravachol, Bristol-Myers Squibb/Sankyo) has the β-hydroxylactone in the 6-hydroxy sodium salt form and is the C6-hydroxy analogue of mevastatin. Simvastatin (or Synvinolin and Zocor, Merck) contains an additional methyl group in the 2' position of the side chain (A Endo, Hasumi, and Negishi 1985; Kimura et al. 1990; Komagata et al. 1989)

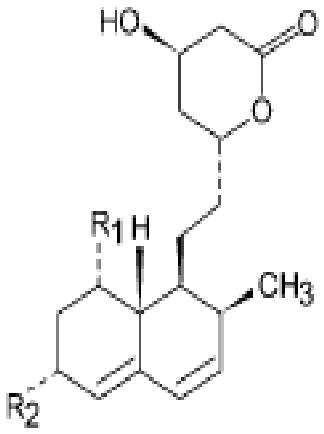
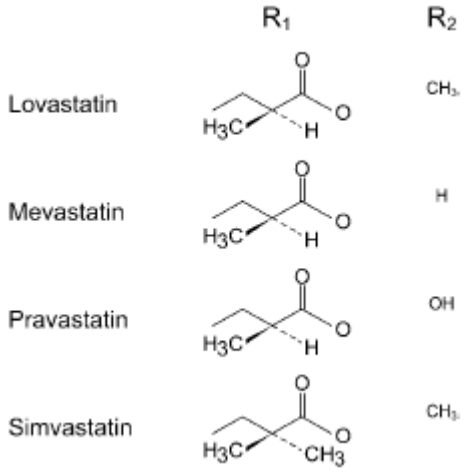


Figure 2.1(a) Base structure of statins (naphthalene ring and β-hydroxylactone)



(b) Statin side chains linked at C8 (R1) and C6 (R2) of the base structure

Adapted from (M Manzoni and Rollini 2002)

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The structures of the synthetic statins atorvastatin (Lipitor, Parke-Davis), fluvastatin (Lescol, Novartis), and cerivastatin (Baycol and Lipobay, Bayer) are dissimilar, and quite different from the natural statins (Fig. 2). Only the HMG CoA-like moiety, responsible for HMG CoA reductase inhibition, is common to both natural and synthetic statins. Unlike lovastatin and simvastatin, synthetic statins are obtained in hydroxy acid form. Fluvastatin (Levy, Troendle, and Fattu 1993), derived from mevalolactone, was the first entirely synthetic statin available, while atorvastatin, pyridine derivatives, is a new generation of highly purified statins (Bakker-Arkema et al. 1996).

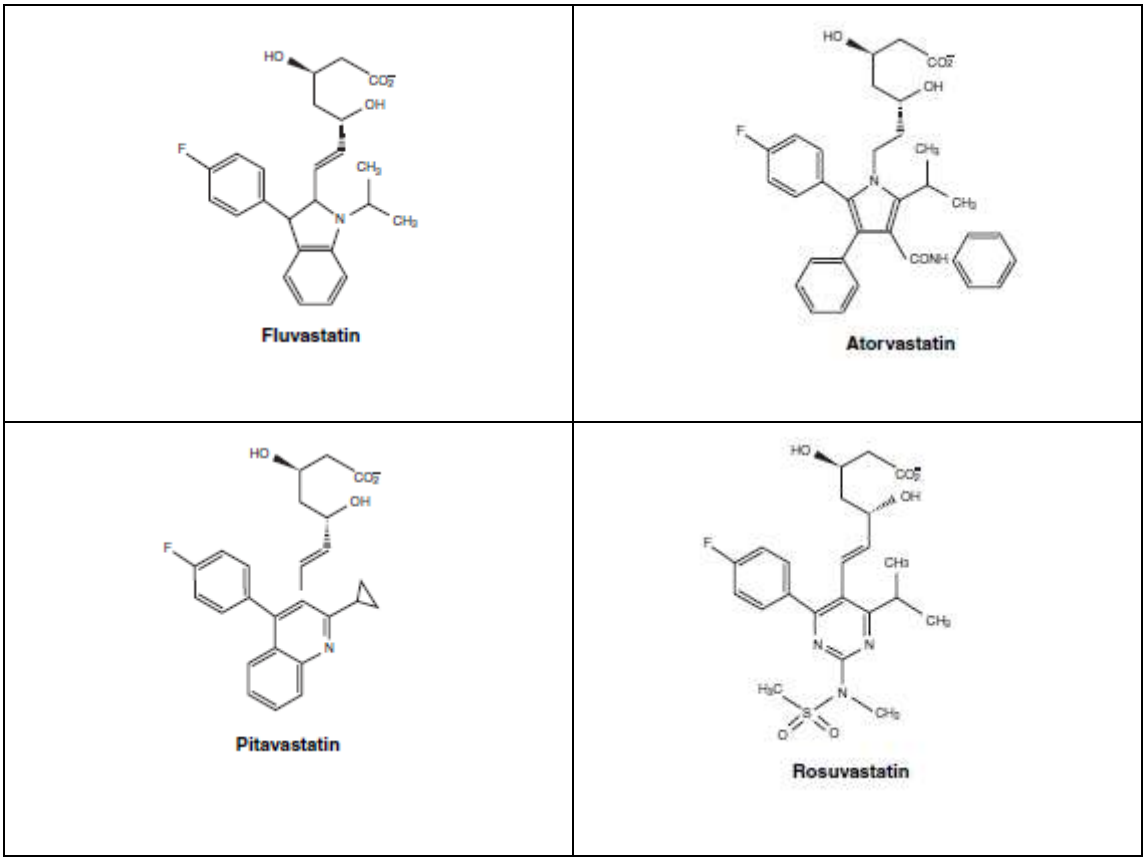

Figure 2.2: Chemical structure of synthetic statins, adapted from (Schachter 2005)

2.1.3 Mechanism of Action

The cholesterol biosynthetic pathway, starting from acetyl-CoA units, involves more than 25 enzymes, but the rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate by HMG-CoA reductase (EC. 1.1.1.34). The mechanism involved in controlling plasma cholesterol levels by statins is the reversible inhibition of HMG-CoA reductase, related to the structural homology of the acid form of the statins to HMG-CoA, the natural substrate of the enzymatic reaction (Barrios-González and Miranda 2010; M Manzoni and Rollini 2002) (Fig. 3). The statins occupy the HMG-CoA binding site of the enzyme, thus blocking substrate access to the active site of the enzyme. The tight binding of statins is due to the large number of van der Waals interactions between inhibitors and HMG-CoA reductase (Istvan and Deisenhofer 2001).

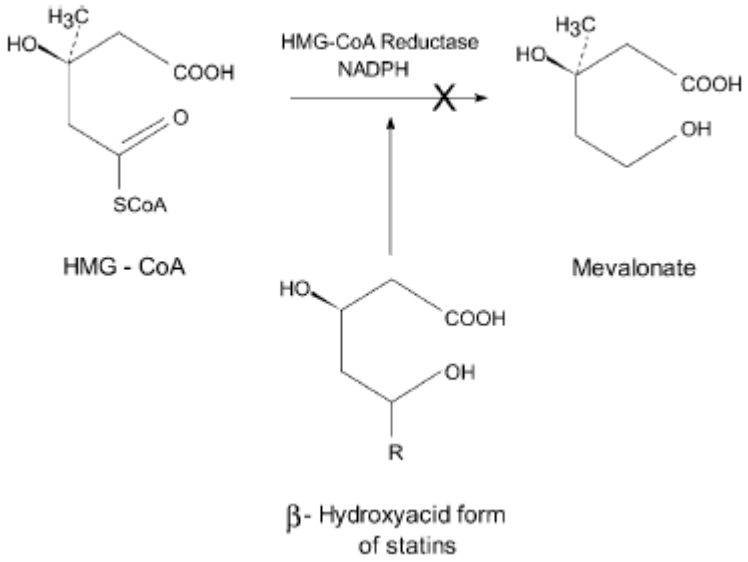

Figure 2.3: Mechanism of action for hypocholesterolemic activity of statins, adapted from (Manzoni and Rollini 2002)

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2.2 Historical Perspective: Discovery of Statins

In the 1950s and 1960s, it became apparent that elevated concentration of plasma cholesterol was a major risk factor for the development of coronary heart disease, which subsequently led to the development of drugs that could reduce plasma cholesterol. Natural products with a powerful inhibitory effect on HMG CoA reductase, including ML236B (compactin), were first discovered by the Japanese microbiologist Akira Endo in a fermentation broth of *Penicillium citrinum* in the 1970s, during a search for antimicrobial agents (A Endo, Kuroda, and Tsujita 1976). The prototype compound compactin was developed by Sankyo, and was shown to be highly effective in reducing concentration of total and LDL cholesterol in the plasma of patients with heterozygous familial hypercholesterolaemia (Mabuchi et al. 1981; and 1983). In 1978, Alberts, Chen and others at Merck Research Laboratories found a potent inhibitor of HMG-CoA reductase in a fermentation broth of *Aspergillus terreus*. They named their discovery mevinolin; later, the official name was established as lovastatin (Alberts et al. 1980). Lovastatin produced a profound reduction of apolipoprotein-B-containing lipoproteins, especially LDL cholesterol and, to a lesser extent, plasma triglycerides, and a small increase in HDL cholesterol.Observed tolerability continued to be excellent, with very few patients withdrawing from treatment due to adverse effects. In November 1986, Merck applied for regulatory approval of lovastatin. In February 1987, a US FDA advisory panel fully considered the various safety issues arising out of the animal toxicology studies. The panel voted unanimously for the approval of the drug, and FDA approval was obtained on 31 August 1987. Lovastatin had patent protection only in certain other countries, all of which later granted approval. Since lovastatin had been

commercialized, 6 statins, including 2 semi-synthetic statins (simvastatin and pravastatin) and 4 synthetic statins (fluvastatin, atorvastatin, rosuvastatin and pitavastatin) have been introduced to the market. The second entrant, simvastatin, which differs from lovastatin only in that it has an additional side chain methyl group, was initially approved for marketing in Sweden in 1988 and subsequently worldwide. Pravastatin (discovered by Sankyo after the failure of compactin) followed in 1991, fluvastatin in 1994, atorvastatin in 1997, cerivastatin in 1998 (withdrawn later), and rosuvastatin in 2003 (Akira Endo 2008; 2010). As noted above, lovastatin is a fermentation product. Simvastatin is a semisynthetic derivative of lovastatin, and pravastatin is derived from the natural product compactin by biotransformation, whereas all other HMG-CoA reductase inhibitors are totally synthetic products. The generic names for all HMG-CoA reductase inhibitors end with 'statin', and the members of this class are today often referred to as 'statins', as opposed to the formal, class name 'HMG-CoA reductase inhibitors'. Table 2.1 below summarizes the history of discovery and development of statins.

Table 2.1: Chronological history of statins discovery and development:

Year	Major Discovery
1970s	The cholesterol controversy, Phase 1, which lasted until 1984.Discovery of compactin, the first potent inhibitor of cholesterol synthesis.
1978	Discovery of lovastatin from fermentation broth.
1980	Lovastatin shown to be effective in healthy volunteers in early clinical trials; compactin withdrawn from clinical trials, causing suspension of further trials.
1984	Clinical trials with lovastatin resume.
1987	Lovastatin becomes available for prescription after FDA approval, first of the class.

1994	Unequivocal reduction of mortality with simvastatin in 4S trial resolves the cholesterol controversy.
1995-1998	Four five-year clinical outcome trials with pravastatin and lovastatin all show reduction of coronary events with very few adverse effects.
2001	Withdrawal of cerivastatin due to excessive risk of rhabdomyolysis.
2002	Heart Protection Study confirms safety of simvastatin in five-year trial in 20,000 patients and demonstrates clinical benefit in a broad array of patient types, including those with low cholesterol levels.
2003	The statin Crestor (rosuvastatin) was approved by the FDA in 2003 for use in treating high cholesterol.
2006	The U.S. Food and Drug Administration approved generic Pravastatin.
2007	Statin use was first time found to prevent gallstones forming, particularly in women who have diabetes.
2009	FDA approval for Pitavastatin.
2010	Rosuvastatin was approved by the FDA for the primary prevention of cardiovascular events.
2011	Sitagliptin and Simvastatin combination -approved by FDA for diabetes with high cholesterol.
2012	Statins tied to reduced cancer deaths.
2013	Liptruzet (ezetimibe and atorvastatin) approved by FDA to cut cholesterol.

2.3 Therapeutic Applications of Statins

. It is proved that the lives of millions of people have been extended through statin therapy with more than 30 million people worldwide are taking statins. Statins were developed for, and currently represent the mainstay of, hypercholesterolemia dyslipidemia treatment. There are ample evidences that support the use of statins to lower cholesterol for primary and secondary prevention of coronary artery disease. The Scandinavian Simvastatin Survival Study (4S) demonstrated as early as 1994 that statin therapy could reduce the all-cause mortality rate in a secondary prevention

population (Pedersen et al. 2004). These results were subsequently confirmed by other landmark clinical trial, CARE (Sacks et al. 1996). In 1995 the West of Scotland Coronary Prevention Study (WOSCOP) extended the benefit of statin treatment to primary prevention by pravastatin application in hypercholesteromic men (Shepherd et al. 1995). Lovastatin was used in the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) to further extend the benefit of primary prevention to a healthy, mixed gender cohort (Downs et al. 1998).

In past few years, increasing evidences have accumulated suggesting that inhibitors of HMG-CoA reductase, or statins, have therapeutic 'pleiotropic' effects independent of cholesterol lowering. These include anti-inflammatory and antioxidative properties, improvement of endothelial function and increased endothelial nitric oxide synthetase expression and nitric oxide bioavailability, which might contribute to the therapeutic benefits, observed with statin therapy. Notably, important immunomodulatory effects of statins have been demonstrated to be independent of lipid lowering (Kwak et al. 2000). Recently it has been shown that statins protect against DNA shortening by telomerase activation and promote healthy aging free of age-related diseases like heart disease, diabetes and cancer. With all these multiple benefits, statins are proving to be much more powerful drugs than anyone ever thought. Table 2.2 summarizes therapeutic applications of statins with underlying mechanism of particular benefit.

Table 2.2: Therapeutic applications of statins with possible underlying mechanism

S. No	Disease	Possible underlying mechanism	Reference
1	Cholesterol Lowering	Inhibits the mevalonate pathway by blocking the rate limiting step of pathway via inhibiting HMGR	Goldstein and Brown 1990
2	Cardiovascular Disease (CVD)	Plaque stabilization, improvements in endothelial-mediated responses with better local regulation of the coronary arterial tone and an immunosuppressive effect.	Brown et al. 1993; Anderson et al. 1995
3	Anti Inflammatory & Anti oxidant	Reduce the plasma levels of inflammatory markers like CRP due to an inhibition of IL-6 in the vascular tissues. Inhibit the ability of macrophages to oxidise LDL.	Giroux, Davignon, and Naruszewicz 1993
4	Bone Regeneration	Promote osteoblastic & inhibit osteoclastic activity.	Park 2009
5	Cancer	Simvastatin on LNCaP and PC3 cells showed its ability to inhibit serum-stimulated Akt activity and reduced expression of PSA. Akin to this, inhibited serum-induced cell migration, invasion, colony formation, and proliferation.	Kochuparambil et al. 2011
6	Alzheimer's Disease (AD)	Modulation of amyloid-precursor protein (APP) cleavage by altering membrane cholesterol levels in vitro. It completely rescued cerebrovascular reactivity, basal endothelial nitric oxide synthesis, and activity-induced neuro metabolic and neurovascular coupling.	Eckert, Wood, and Müller 2005; Tong et al. 2012
7	Parkinson's Disease	Simvastatin exposure inhibited the activation of p21ras (necessary for the neurotoxic chemical to produce Parkinson's) in the microglial cells. The statin also blocked the neurotoxin from activating nuclear factor-kappa B, "a transcription factor required for the transcription of most of the pro inflammatory molecules.	Ghosh et al. 2009
8	Infectious Diseases	Improved susceptibility to endothelial nitric oxide synthase stimulation and reduced endothelial adhesion of leukocytes, results in improved survival after sepsis.	Merx et al. 2005
9	Renal Diseases	Slow progression of Chronic Kidney Diseases by improving the lipid profile as well as by affecting inflammatory cell-signaling pathways that control vascular cell migration, proliferation, and differentiation.	Campese and Park 2007
10	Acute Lung	Vascular-protective changes in EC	Singla and Jacobson

	Injury (ALI)	phenotype can be attributed to statin-induced inhibition of mevalonate production and the resultant changes in Rho GTPase activity and localization	2012
11	Rheumatoid Arthritis (RA)	Adjuvant therapy associated with other conventional therapeutic methods used in RA. Statins improves endothelial function in patients with RA. Its beneficial effect may be attributed to lowering C-reactive protein and TNF-alpha concentrations.	Tikiz et al. 2005
12	Aging	By telomerase activation, statins may represent a new molecular switch able to slow down senescent cells in tissues and be able to lead healthy lifespan extension.	Boccardi et al. 2013
13	Cognitive disorders	Increase the soluble RAGE level by inducing RAGE (receptor for advanced glycation end products) shedding, and by this way might prevent the development of RAGE-mediated pathogenesis.	Quade-Lyssy et al. 2013
14	Pancreatic Diseases	Associated with a lower risk of pancreatitis in patients with normal/ mildly elevated triglyceride levels	Preiss et al. 2012
15	Pregnancy Complication	Prevent preeclampsia by decreased release of the anti-angiogenic molecule sFlt-1 from macrophages & increased release of VEGF and PlGF to restore angiogenic balance.	Girardi 2013
16	Healing Disorders	Decreasing farnesyl pyrophosphate, promote neovascularisation and reducing bacterial load	Stojadinovic et al. 2010
17.	Multiple sclerosis	Attributed to the immunomodulatory properties of statins and to their induction of a bias toward Th2 cell anti-inflammatory cytokine production	Davignon and Leiter 2005

2.4 Market Potential of Statins

Since the first human trial of HMG CoA reductase inhibitor in 1978, the growth in importance of this drug class, both financially and medically, has been staggering.

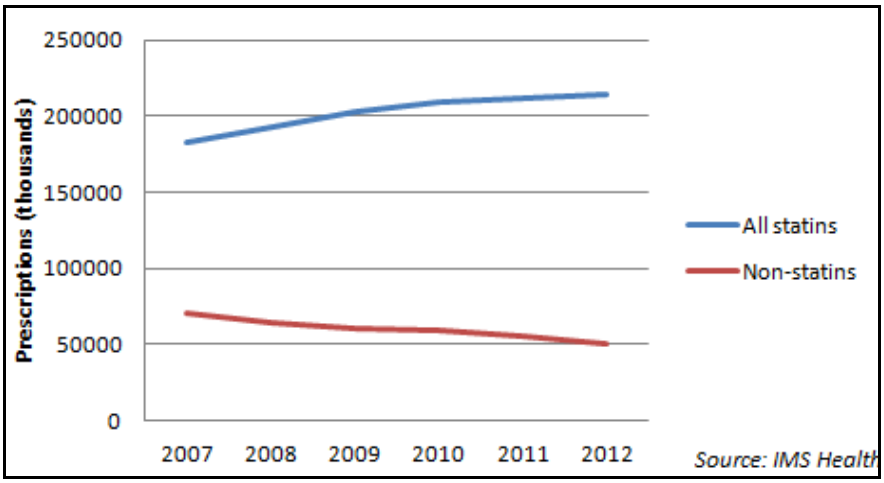

Figure 2.4: Yearly prescription of statins

Sales of statins have dominated the pharmaceutical market in recent years, consistently ranking as the number one class of drugs, peaking in 2006 with annual sales of $23 billion, so are called “the golden child” for pharmaceutical companies. The statins command an immense market potential with an average sale of US$20 billion annually. Statins have been at the top of the list of global best-selling drugs in the therapeutic treatment of hypercholesterolemia. So it’s not surprising to learn that 36m Americans take a statin pill every day, generating annual sales of $15.5bn for the manufacturers, and making two statins supplement drugs - Lipitor and Zocor - the top two best-selling medicines in the USA. Statin use has been increasing by an average of 12 per cent a year, and it’s a trend that is likely to continue as almost all of us as being at risk from raised cholesterol levels due to changed eating habits and sedentary life style. Statins the largest selling drug worldwide and its market in India is presently estimated at about Rs. 300 crore. Biocon, India's largest and USFDA qualified producer and exporter of statins, is the market leader. In fact, 57% of the prescriptions for cholesterol medicines are for two statins: simvastatin once sold by Merck under

the brand name Zocor, and atorvastatin, sold by Pfizer under the brand Lipitor. Between 2011 and 2012, when atorvastatin went generic and cheaper versions became available, use of the drug increased by 20%.

2.5 Biosynthesis and Bioconversion of Statins

Statins are natural compounds of fungal origin that are produced as secondary metabolites. During evolution, some fungi such as mushrooms developed a defence mechanism in which they produce statins to block the biosynthesis of cholesterol. Since bacteria require cholesterol-like compounds to grow, statins could fend off invading bacteria by shifting the bacteria's ability to generate these compounds.

The microorganisms used for statin production broadly belongs to three main groups, *Aspergillus*, *Penicillium* and *Monascus* spp. Lovastatin (mevinolin or monacolin K) was obtained from a strain isolated from the soil and classified as *A. terreus* at CIBE Laboratories in Madrid (Alberts et al. 1980). A few years later, lovastatin was also obtained from 17 strains of different species of the genus *Monascus*. However, commercial production of lovastatin is based on *A. terreus* batch fermentation and most of the past research work in on this species (Matilde Manzoni et al. 1998; Kumar et al. 2000; Rodríguez Porcel et al. 2007; Bizukojc and Ledakowicz 2008).

Early biogenetic investigations of statins carried out on 14C-labelled monacolin J and L, employing a strain of *Monascus ruber*, suggested that these compounds are the precursors of lovastatin and, consequently, can be classified as isolated intermediate metabolites in the lovastatin biosynthetic pathway (A Endo, Hasumi, and Negishi 1985). Monacolin L is the first to be synthesized

from nine molecules of acetate and is, in turn, converted to monacolin J by hydroxylation; monacolin K is then derived from monacolin J. The monacolin X, i.e., the α- methyl-β-ketobutyryl ester of monacolin J, is converted to Lovastatin. The lovastatin biosynthetic pathway starts from acetate units (4- and 8-carbons long) linked to each other in head-to-tail fashion to form two polyketide chains. as shown in the figure below (Figure 2.5). The methyl group present in some statins in the side chain or at C6 is derived from methionine, as frequently occurs in fungal metabolism, and is inserted in the structure before the closure of the rings (Shiao and Don 1987). Hence, lovastatin is derived from acetate via a polyketide pathway (Moore et al. 1985). Pioneering genetic research by Reeves, McAda, and workers at MDS Panlabs Inc., identified a type I polyketide synthase (PKS) gene essential for lovastatin biosynthesis by *A. terreus* which is now known as Lovastatin nonaketide synthase (LNKS) (Hendrickson et al. 1999). LNKS is a multi-domain enzyme containing seven activities and functions in a manner similar to animal fatty acid synthases (FAS) and bacterial type I PKS, viz., the ketosynthase (KS) which performs decarboxylative claisen condensation for chain elongation (Kennedy et al. 1999); the malonyl-CoA:ACP acyltransferase (MAT) selects and transfers the extender unit in the form of malonic esters, while acyl carrier protein (ACP) serves as the tether (or bind) for the extender unit and the growing chain.

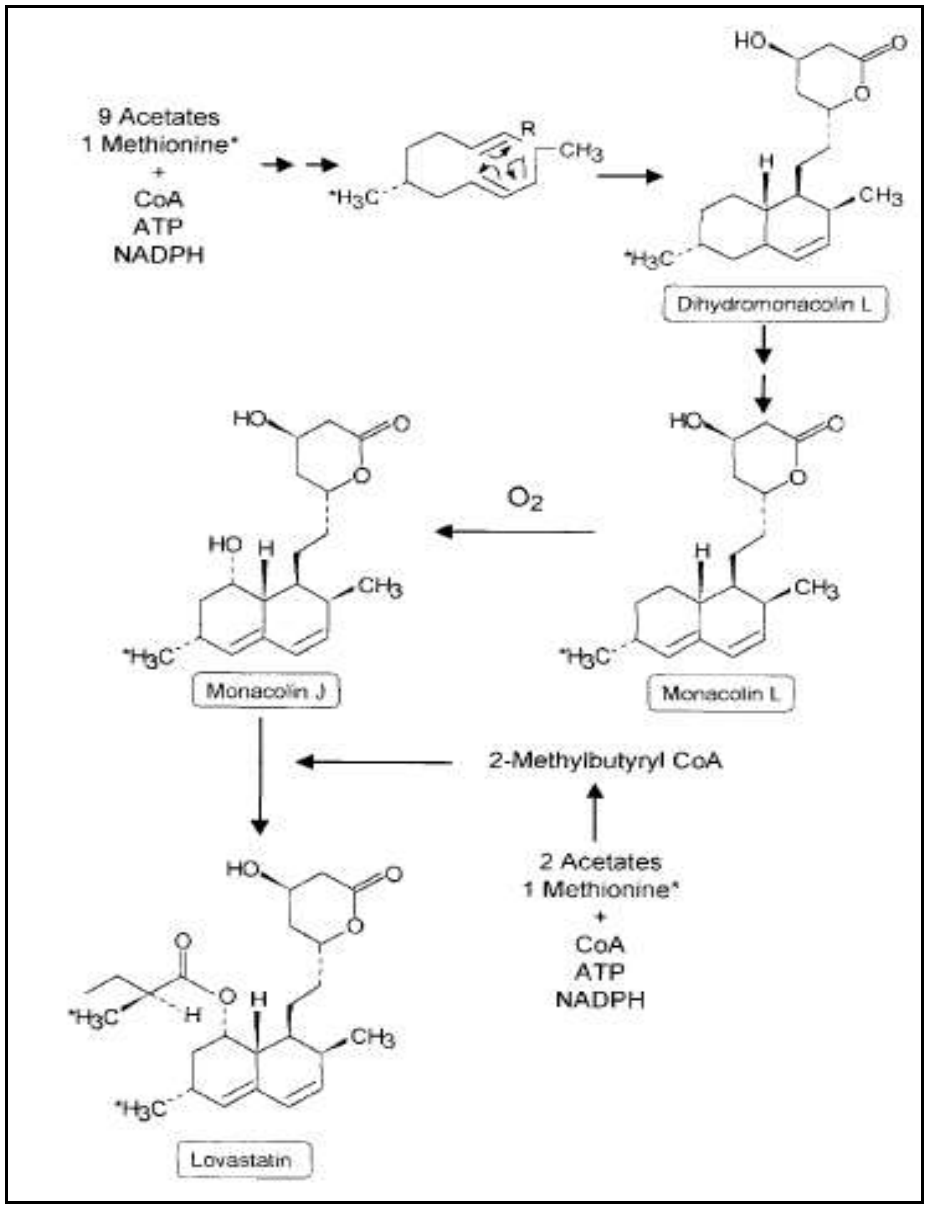

Figure 2.5: Lovastatin biosynthetic pathway

In addition, tailoring enzymes such as ketoreductase (KR), dehydratase (DH), methyltransferase (MT) and enoylreductase modify the carbon backbone and introduce structural diversity. Kennedy et al showed that complete lovastatin gene cluster of 18 genes is over 64 kb in size as shown in figure 2.6.

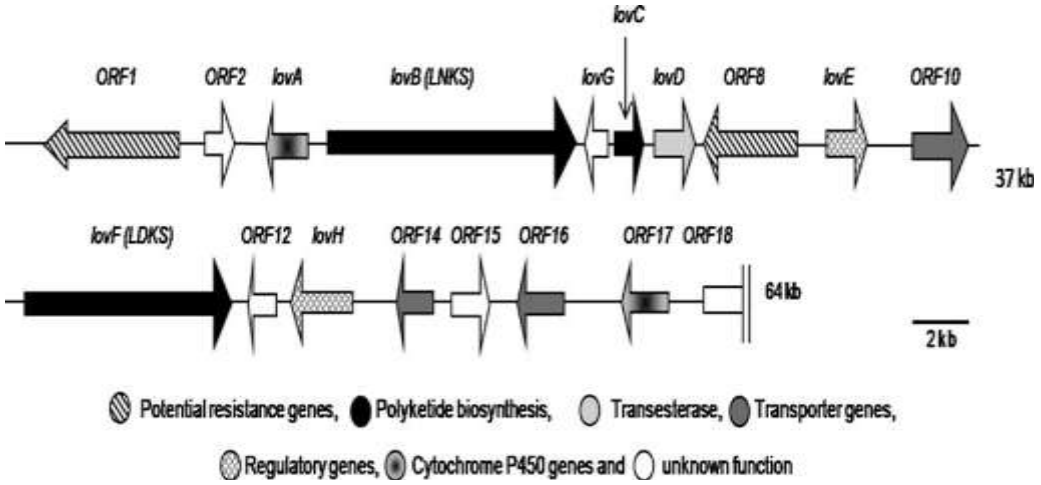

Fig. 2.6 Lovastatin biosynthetic gene cluster (Kennedy et al. 1999; Hutchinson et al. 2000)

The lovB gene encodes NKS, while the lovF encodes a diketide synthase (DKS). The presence of methyltransferase domains in the LNKS and in the DKS indicated that in both these cases the methyl groups are likely to be added (S-adenosyl-methionine) while the polyketide is being synthesized. Furthermore, the function of the genes could be largely predicted by sequence comparison. Additional understanding of their function was obtained by a loss-of-function mutation strategy, through disruptions of the individual genes of the cluster. In addition, lovE encodes a transcription factor regulatory protein with the typical binuclear Zn^{++} finger motif. Its disruption mutants did not produce lovastatin or its intermediates, while the over-expression resulted in increased metabolite production. It is assumed that lovE regulates Lovastatin production at the transcriptional level. However, there is also a second gene (lovH) with a similar structure (Hutchinson et al. 2000). On the other hand, lovA and ORF 17 encode putative cytochrome 450 monooxygenases. The transformation to monacolin J requires CYP450 oxygenases, probably encoded by genes lovA and ORF 16. The

five carbon side unit side chain is synthesized by the other polyketide synthase, product of lovF (also known as LDKS), through a single condensation between an acetyl-CoA and a malonyl-CoA. The LDKS consists of seven linearly arranged domains, in order: KS, MAT, DH, MT, ER, KR and ACP. The condensed diketide undergoes methylation and reductive tailoring by the individual LovF catalytic domains to yield α-S-methylbutyryl tyioester covalently attached to the phosphopantetheine arm of the acyl carrier protein domain of LovF (Kennedy et al. 1999). Gene lovD encodes the 2-mehtylbutyryl/monacolin J transesterase that catalyzes the last step that joins together the two polyketide components of lovastatin, i.e., transacylates, the acyl group from LovF to the C8 hydroxyl group of Monacolin J to yield Lovastatin.

One particularly unique feature of this type of highly reducing PKSs is the lack of a built-in offloading domain that facilitates the release of completed products. This is in sharp contrast to bacterial type I or fungal non-reducing PKSs, in which a dedicated thioesterase domain is appended at the end of the mega-synthase and catalyzes the release of polyketides. As mentioned before, transfer of the diketide side chain from LovF to monacolin J was proposed to be catalyzed by a dissociated acyltransferase LovD (shown in Figure 2.7).

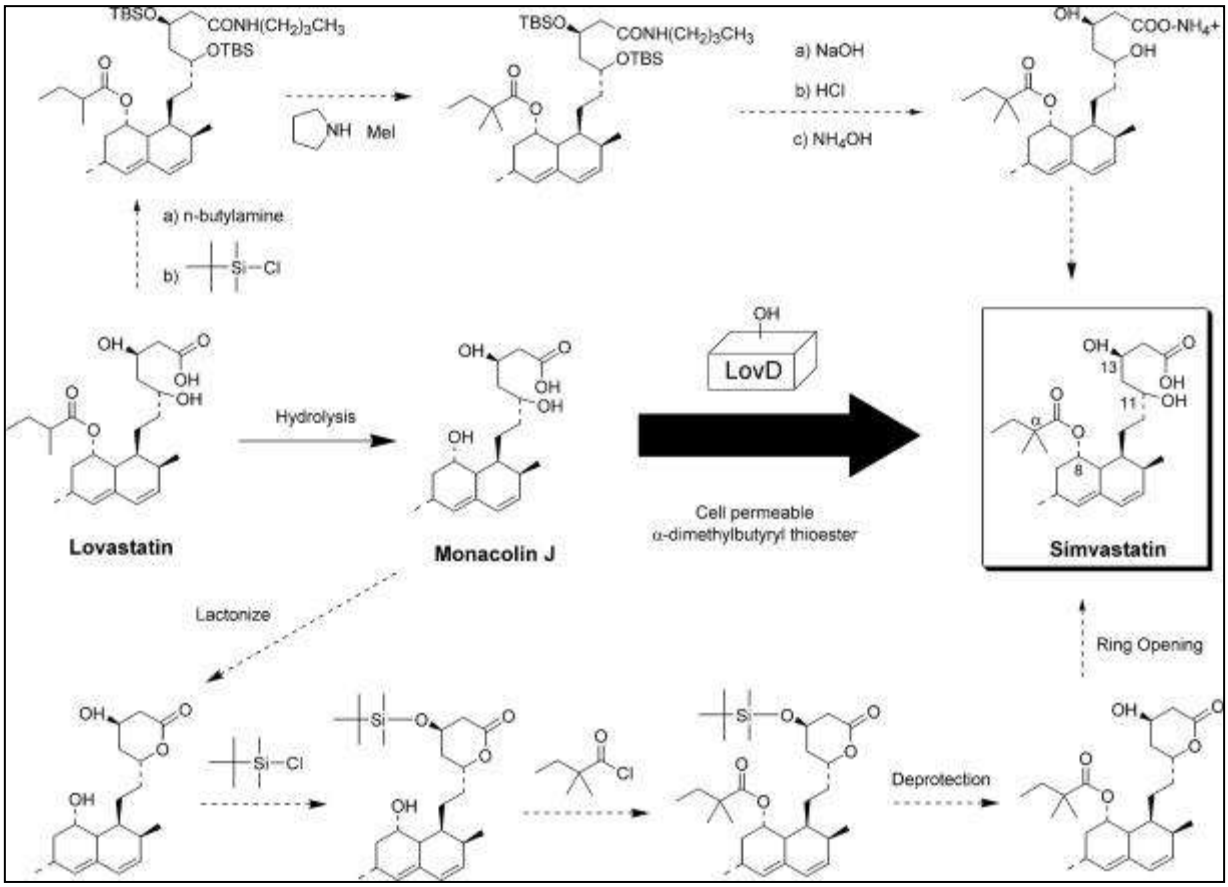

Figure 2.7: Enzymatic conversion of Monacolin J to Simvastatin (thick arrow)

Xie et al. demonstrated that the protein interactions between LovF and LovD play a key role in facilitating rapid offloading of the diketide substrate from LovF to LovD and ensure efficient biosynthesis of lovastatin. Also that only the completely tailored, RSmethylbutyryl- ACP is accessible by LovD (that is, none of the acyl intermediates are transferred). A possible mechanisms that may account for this phenomenon is the methyl transfer, ketoreduction, dehydration, and enoyl reduction steps may take place very rapidly following exit of the acetoacetyl-ACP from the KS active site. Another possibility suggested is that the acyl-ACP may be inaccessible by LovD during the tailoring steps (Xie and Tang 2007).

Studies on the 13C incorporation in lovastatin and compactin, carried out in *P. citrinum* and *M. ruber*, indicated a similar pathway (A Endo, Hasumi, and Negishi 1985; Chakravarti and Sahai 2004). Later, Abe et al. 2002 cloned and characterized the compactin gene cluster from *P. citrinum* using similar strategies. Nine genes, mlcA to mlcH and mlcR (regulator) clustering in a 38-kb region, were transcribed when compactin was produced. The predicted amino acid sequences encoded by these nine genes were similar to those encoded by the genes for lovastatin biosynthesis. Later, the pathway-specific transcriptional activator MlcR-binding sequence was identified and located in the promoters of mlcA and mlcC, and other genes of the cluster. It is important to note that in the case of compactin too; the introduction of extra copies of the regulator gene mlcR enhanced the metabolite production (Baba et al. 2009).

With development in the research of lovastatin and compactin biosynthesis and genetics, greater attention is required towards the possibility of obtaining the biosynthesis of the semisynthetic derivatives (mainly simvastatin and pravastatin) by biotechnological processes. For example, simvastatin could be produced by direct fermentation with combinatorial biosynthesis methods, and could also be synthesized from monacolin J with the acyltransferase LovD. Simvastatin, a semisynthetic derivative of lovastatin, is marketed by Merck as Zocor and is the second-bestselling drug in the USA, with annual sales in 2005 tipping USD$ 12 billion (Kidd 2006). The molecular difference between lovastatin and simvastatin resides in the side chain on the C-8 carbon position. In this position, lovastatin carries a 2-methylbutyrate moiety, while simvastatin has a 2, 2-dimethylbutyrate (DMB) moiety. Simvastatin is traditionally prepared by direct alkylation of

lovastatin. However, here chemical reaction conditions are very rigid, and the final product is difficult to purify. Additionally the mounting pressure of labor protection and environment protection is very high. These multistep processes are laborious, thus contributing to simvastatin being nearly five times more expensive than Lovastatin. One biotechnological approach to its production would be the enzymatic synthesis of simvastatin from monacolin J, with the acyltransferase LovD. In 2007, Xie et al. cloned lovD gene from *Aspergillus terreus*, and overexpressed this enzyme in *E. coli*. In this way, they were able to characterize this acyltransferase that selectively transfers the α-methylbutyryl group (from the LDKS) to the C8 hydroxyl group of MCJ, to yield lovastatin (Fig. 2.7). The authors showed that LovD has broad substrate specificity towards the acyl group. Most notably, LovD was able to catalyze the direct acylation of monacolin J by acyl-CoA thioesters or membrane permeable thioesters acyl donors like N-acetylcysteamine (SNAC) thioesters. This work showed the feasibility of using LovD as a biocatalyst for a single-step synthesis of simvastatin using α-dimethylbutyryl-S-NAC (or α-dimethylbutyryl-S-methylthioglycolate) as a substrate. However, the acylation reaction proceeded with poor turnover, due to substrate inhibition at increasing concentrations of MCJ. This was a result of a weak binding of these acyl donors to lovD, so the second substrate (MCJ) became a competitive inhibitor of DMB-S-NAC. A different approach used was to engineer *A. terreus* so that it can directly produce simvastatin (instead of lovastatin) by fermentation. (Berg, Hans, and Streekstra 2007) used this latter approach by inactivation of lovF gene, to prevent internal accumulation of (lovastatin's side branch) 2 methylbutyrate (2 MB); together

with the engineering of the *A. terreus* strain so that it will synthesize DMB (the side chain of simvastatin). Initially, the authors showed that *A. terreus* (LovD) can also use DMB as a side chain and hence produce simvastatin instead of lovastatin, although DMB is not normally produced by this fungus. Instead, *A. terreus* synthesizes 2 MB by means of LovF (also known as LDKS); through a single condensation between an acetyl- CoA and a malonyl-CoA. However, the authors also observed that, in this reaction, *A. terreus* (LovF) can also incorporate methylmalonyl-CoA to produce DMB and then produce simvastatin, although in a small proportion, since LovF preferentially accepts malonyl-CoA.

Though compactin is not used as a medicine, it is an important source for producing pravastatin, a more efficient derivative (Sankyo Squibb). At first stage, a strain of *Penicillium citrinum* produces compactin (SmF). Pravastatin is normally produced in a second stage by biotransformation (hydroxylation) of compactin by *Streptomyces carbophilus*. (Ykema, Streekstra, and Luiten 1999) developed a one-step biosynthesis process to produce pravastatin, by transforming the *P. citrinum* strain with a *S. carbophilus* hydroxylase gene that converts compactin to pravastatin.

Recent studies under submerged fermentation conditions have been mostly performed with *A. terreus*, to some extent, with by *M. ruber*, *M. pilosus*, and *M. purpureus*, and with *P. citrinum*. It is noteworthy that inspite of several decades of research the industrial biotechnology field has utilized only a very small proportion of the natural microbial arsenal in the search for new producers of statins.

2.6 Microorganisms used for statin production

Various kinds of micro organisms like fungi, yeasts and actinomycetes were reported in the literature for the production of lovastatin and related HMG-CoA reductase inhibitors. But till today the best know organism for the biotechnological production of HMG-CoA reductase inhibitors are soil dwelling molds like *Monascus ruber* (Endo 1985) and *Aspergillus terreus* (Alberts *et al.,* 1980; Samiee *et al.,* 2003). Apart from *Aspergillus terreus* few other species of *Aspergillus* have also been reported such as *A. flavus* (Shindia 1997; Samiee *et al.,* 2003), *A. niger*, *A. repens*, and *A. versicolor* (Gunde-Cimerman *et al.*,1993), *A. oryzae* (Hajko *et al.,* 1994; Shindia 1997), *A. fischeri, A. parasiticus,*and *A.umbrosus* (Samiee *et al.*,2003).

Another major genus of the fungus which has been reported in the literature for production of lovastatin (monacolin K) and related compounds was *Monascus*. A wide variety of *Monascus sp.* were reported, as potential producers of this drug, particularly *M. ruber, M. purpureus, M. pilosus, M. vitreus, M. publigerus, M. anka* (Negishi *et al.*,1986), and *M. paxii* (Manzoni *et al.,* 1999).

Various other filamentous fungi have also been described in view of their lovastatin and related compounds producing ability such as *Penicillium citrinum* (Endo *et al.*,1976), *Penicillium brevicompactum* (Brown *et al.,* 1978), *P. funiculosom* (Samiee *et al.*,2003), *Phythium ultimum* (Endo *et al.*,1976), *Hypomyces chrysospermum* (Endo *et al.*,1986), *Paecilomyces* sp., *Eupenicillium* sp., *Trichoderma longibrachiatum* (Endo *et al.*,1986; Samiee *et al.*,2003), *T. viridae* (Samiee *et al.*,2003), *T. pseudokoningii*, *Phoma* sp., *Doratomyces nanus*, *Gymnoaseus umbrinus* (Endo *et al.*,1986), *Scopulariopsis* sp., *Paecilomyces varioti* (Gunde-Cimerman *et al.,* 1993a). Few investigators have also described higher basidiomycetous fungi, for lovastatin

production such as *Pleurotus sapidus*, *P. saca*, *Agaricus bisporus*, *Volvariella volvacea*, *Agrocybe aegerita*, *Trametes versicolor* (Gunde-Cimerman *et al.*, 1993a), *Pleurotus ostreatus* (Gunde-Cimerman 1993b; Alarcon *et al.*, 2005), *P. cornucopiae*, *P. Eryngii* (Gunde-Cimerman and Cimerman 1995). Few actinomycetes like *Phanerochate chrysogenum*, *Scopulariopsis brevicaulis*, *Doratomyces stemonitis* (Shindia 1997), *Acremonium chrysogenum* (Samiee *et al.*, 2003) have also been reported for production of lovastatin. Table 2.3 summarizes the recent fermentation studies for production of natural statins.

Table 2.3: Recent fermentation studies (2001-2013) for production of statins

S.No	Organism	Mode of production	Product	Reference
1	*A. terreus*	Submerged fermentation	Lovastatin	Casas López *et al.* 2004
2	*P. citrinum*		Compactin	Choi *et al*. 2004
3	*M. pilosus*		Lovastatin	Miyake *et al.*2006
4	*P. citrinum*		Compactin	Zaffer Ahmad *et al.*2006
5	*A. terreus*		Lovastatin	Rodriguez Porcel *et al.* 2007
6	*A. terreus*		Lovastatin	Gupta *et al.* 2009
7	*P. brevicompactum*		Mevastatin	M.Manzoni *et al* .2002
8	*M. purpureus*		Lovastatin	Sayyad *et al.*2007
9	*A. terreus*		Lovastatin	Jia *et al.*2009
10	*A. terreus*		Lovastatin	Kaur *et al.*2010
11	*A. terreus*		Lovastatin	Sorrentino *et al.*2010
12	*M. purpureus*		Lovastatin	Subhagar *et al.* 2010
13	*A. terreus*		Lovastatin	Li *et al.*2011
14	*A. terreus*		Lovastatin	Li *et al.*2011
15	*M. oryzae*		Lovastatin	Ahmed I.El-Batal *et al.* 2012
16	*Amycolatopsis sp*		Wuxistatin	Bin Zhuge, Hui *et al.*2008
17	*A. macra*		Pravastatin	Ajaz Ahmad *et al.*2013
18	*A. livida*		Pravastatin	
19	*A. madurae*		Pravastatin	
21	*A. flavipes*	Solid State fermentation	Lovastatin	H.R. Valera *et al.*2005
22	*M. ruber*		Lovastatin	Bao-Jun Xu *et al.*2005
23	*P. brevicompactum*		Compactin	Shaligram *et al.*2009
24	*A. terreus*		Lovastatin	J. Barrios-González *et al.* 2008
25	*M. purpureus*		Compactin	Panda *et al.*2009
26	*M. purpureus*		Lovastatin	Subhagar *et al.*2010
27	*M. pilosus*		Lovastatin	Masatoshi *et al.2009*
28	*A. terreus*		Compactin	Survase Shrikant A
29	*M. purpureus*		Lovastatin	Panda *et al.*2010
30	*A. terreus*		Lovastatin	Patil *et al.*2011
31	*A. terreus*		Lovastatin	Mohammad F J *et al.*2012
32	*A. terreus*		Lovastatin	Pei-lian *et al.* 2007
33	*A. tubingensis*		Lovastatin	Zhen-Jun Zhao *et al.*2013
34	*A. wentii*		Lovastatin	
35	*A. fumigates*		Lovastatin	
36	*P. chrysogenum*		Lovastatin	