An Efficient Biocatalytic Process for Simvastatin Manufacture

Nominated for the 2012 Presidential Green Chemistry Challenge Award

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Recent milestones

- Proof of concept demonstrated and process optimized 2007
- Optimized process using further evolved enzyme (Codexis) 2009
- Simvastatin-ammonium produced biocatalytically at 400 kg scale 2010
- Enzyme manufacturing scaled up to 150-200 kg batch scale 2010
- Process and Composition of Matter patent applications published 2011
- Process has been scaled up and >10 mT simvastatin produced 2011

Eligibility

The nominated technology was co-developed by Prof. Tang and Codexis. Prof. Tang is eligible for the academic award, and Codexis is eligible for the award in Focus Area 1, "An industry sponsor for a technology that uses greener synthetic pathways".

Focus Areas

This technology can be classified into all three focus areas: the use of greener synthetic pathways, the use of greener reaction conditions, and the design of greener chemicals.

Location

The new simvastatin manufacturing process was conceived and enabled in the United States and then scaled-up in Austria (enzyme manufacture) and India (simvastatin manufacture).

Abstract:

Simvastatin is an important drug for treating cardiovascular diseases. Simvastatin is a semi-synthetic derivative of lovastatin, a fungal natural product, and contains an additional methyl group at the C2' position of the side chain. Until the discovery of the novel, green synthetic pathway nominated here, introduction of this methyl group in lovastatin required multistep chemical synthesis due to the need to protect/deprotect other functionalities in the lovastatin molecule.

Generally, two different routes have been described for the conversion of lovastatin into simvastatin. In the first route lovastatin is hydrolyzed to the triol monacolin J, followed by protection via selective silylation, esterification with dimethylbutyryl chloride and deprotection. The other route involves protection of the carboxylic acid and alcohol functionalities followed by methylation of C2' with methyl iodide followed by deprotection. Both processes are inefficient despite considerable optimization (<70% overall yield), mass intensive as a result of the need for protection/deprotection, and require the use of copious amounts of toxic and hazardous reagents. Therefore, a shortened reaction sequence in which the need for protection/deprotection was circumvented was expected to i. be more efficient resulting in a higher atom economy, ii. reduce waste generation in the manufacture of this product, and iii. provide overall less hazardous process conditions.

Dr. Yi Tang's group at UCLA conceived a new simvastatin manufacturing process and enabled it by first identifying both a biocatalyst for regioselective acylation and a practical, low-cost acyl donor. Codexis licensed the intellectual property for this process from UCLA and subsequently optimized the enzyme and the chemical process for the commercial manufacture of simvastatin.

An Efficient Biocatalytic Process for Simvastatin Manufacture

Introduction:

Statins are important drugs for treating cardiovascular disease. Six statins have been approved by the FDA, three of which (lovastatin, simvastatin and pravastatin) are natural product derived. Lovastatin was the first FDA approved statin and is a secondary metabolite produced by fermentation of *Aspergillus terreus.* Simvastatin is a semi-synthetic derivative of lovastatin, containing a dimethyl group at C2' of the side chain (Fig 1). This subtle structural modification renders simvastatin more potent in the reduction of total and low-density lipoprotein cholesterol (LDL-C) with decreased hepatoxicity and reduced side effects. In 2005 simvastatin (brand name Zocor®) was Merck's best selling drug and second largest selling cholesterol lowering drug in the world with ~$5 billion in sales. Since Zocor went off patent in 2006, simvastatin rapidly became the second highest selling generic drug with sales ~ $2 billion in 2008. Annually, it is estimated that over 300 mTs of simvastatin are produced worldwide.

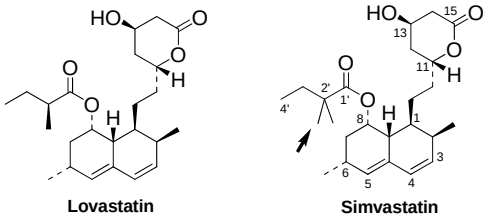

Fig. 1*: Chemical structure of lovastatin (left) and its semi-synthetic derivative simvastatin (right) with additional methyl substituent (arrow).*

The Challenge:

Manufacturing of simvastatin involves multiple steps to preserve other key chemical functionalities of the molecule. Simvastatin manufacturing processes are generally classified as "hydrolysis/esterificiation" routes (Fig. 2) or "direct methylation" routes (Fig. 3), both of which require protection/deprotection steps.

According to information obtained from US4,444,784, for the "hydrolysis/deprotection" route, and US4,820,850, for the "direct methylation" route, both routes utilize excess hazardous and

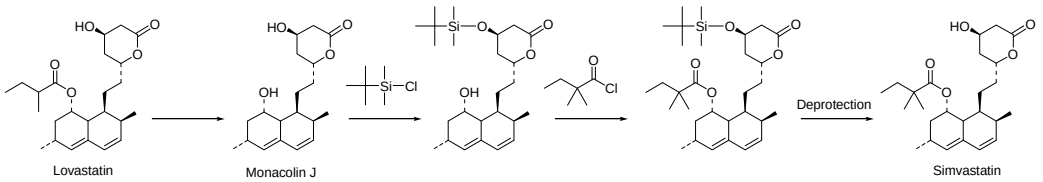

Fig. 2*: Simvastatin synthesis via the "hydrolysis/esterification" route involves hydrolysis of the ester bond to generate monacolin J with a free hydroxyl at the 8 position, followed by selective TBDMS protection, esterification with the desired dimethylbutyryl side chain and deprotection.*

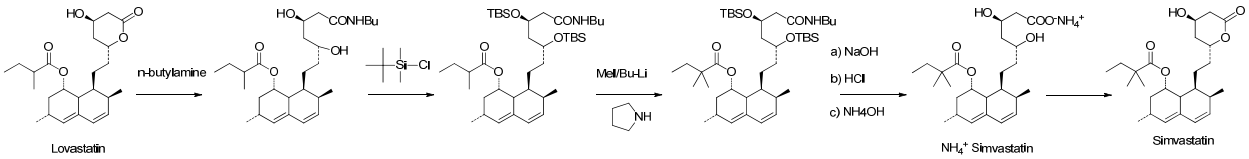

Fig. 3*: Simvastatin synthesis via the "direct methylation" route involves protection of the carboxylic and alcohol functions, alkylation using methyl iodide and deprotection to form the simvastatin-ammonium salt. This is subsequently converted to the simvastatin lactone.*

toxic reagents (see below) and copious amounts of solvents. Based on this and the low overall yields of the two processes (estimated at <70%) a higher yielding, less waste-generating manufacturing process for simvastatin was deemed to be still highly desirable.

The Approach:

To completely redesign the simvastatin manufacturing process using more efficient and greener chemistry, we decided to follow nature's example and use enzyme technology. Towards this end, we embarked on a protein engineering approach to first identify a biocatalyst for the conversion of lovastatin to simvastatin, and then to improve it towards commercial use. The technology we envisioned is shown in Fig. 4: following the quantitative hydrolysis of lovastatin to monacolin J acid, an enzyme is used to regioselectively acylate the C8 position of monacolin J with a dimethylbutyryl group to yield simvastatin. A novel, non-natural acyl donor was identified to enable this approach. This process circumvents the (de)protecttion steps required for chemical synthesis and is significantly more efficient, cost effective and environmentally friendly.

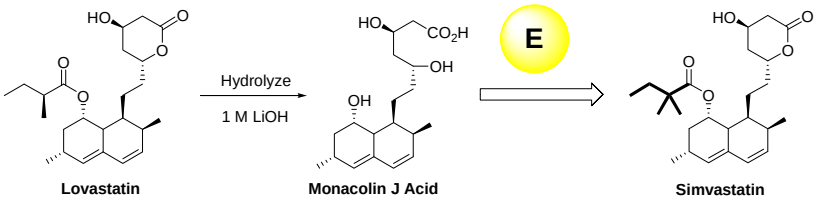

Fig. 4*: Enzymatic synthesis of simvastatin from monacolin J.*

The Solution:

We first identified LovD as an acyltransferase that selectively transfers the 2-methylbutyryl side chain from a fungal polyketide synthase (LovF) to the C8 alcohol of monacolin J sodium or ammonium salt (Fig. 5) and cloned the corresponding gene (the Tang group at UCLA - *Chem. Biol.* **2006**, 13: 1161; *J. Am. Chem. Soc.* **2009**, 131:8388). This reaction proved to be completely regioselective as only the C8 position was acylated, proving that protective chemistry was not required. The natural side chain donor in *Aspergillus terreus* is 2-methylbutyryl-LovF, an acyl substrate bound to a large enzyme (270 kDa) via a thioester. Since this acyl donor is neither readily available nor scalable for commercial manufacturing, we evaluated the substrate specificity of LovD towards different acyl groups and thioester acyl carriers. We demonstrated that LovF is not required for substrate recognition by LovD and that simple thioesters, e.g. with *N*-acetylcysteamine (SNAC) can be used for the conversion of monacolin J to lovastatin and simvastatin, albeit with low catalytic turnover (k_{cat} for simvastatin conversion was 0.02 min^{-1}). This represented the first demonstration of enzymatic synthesis of simvastatin. LovD can therefore be considered as a simvastatin synthase and a starting point for developing the desired green manufacturing process.

With LovD in hand we developed an *E. coli*-based biocatalytic approach to synthesize simvastatin. We synthesized a series of commercially accessible dimethylbutyryl thioesters and tested their ability to support the transacylation reaction. Dimethylbutyryl-*S*-methylmercapto-propionate (DMB-SMMP) was identified as a catalytically superior acyl donor to DMB-SNAC, thereby establishing a novel route to simvastatin (Fig. 5). DMB-SMMP is very efficient as an acyl donor in the LovD catalyzed reaction with a k_{cat} = 0.75 min^{-1}, more than 30-fold faster than that of DMB-SNAC (*Appl. Environ. Microbiol.* **2007**, 73:2054). DMB-SMMP binds readily to LovD and its binding is not competitively inhibited by high concentrations (>30 mM) of mona-

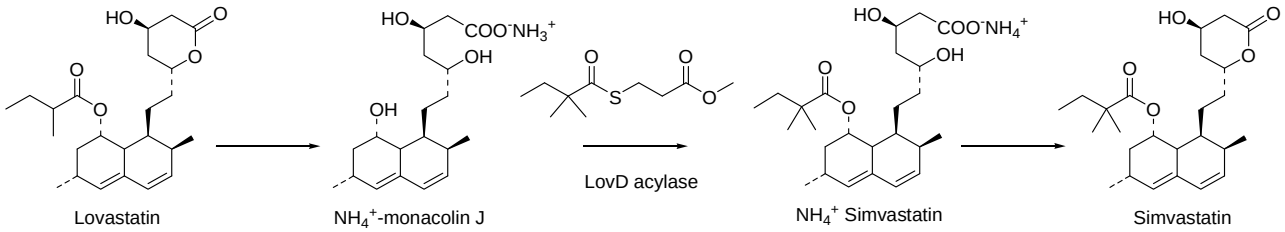

Fig. 5*: LovD enabled synthesis of simvastatin. Lovastatin is hydrolyzed and converted to the ammonium salt of monacolin J which is subsequently acylated using DMB-SMMP and LovD as biocatalyst.*

colin J. Furthermore, DMB-SMMP can be prepared in a single step from readily available, inexpensive precursors to provide a safer alternative to the typically used acyl donors and the reactions conditions they are used in. In addition, the methyl 3-mercaptopropionate by-product is hydrolyzed under reaction conditions and the acid has been shown to be recyclable.

Having a practical acyl substrate available, we next explored whether an *E. coli* strain overexpressing LovD can be used as a whole-cell biocatalyst for the synthesis of simvastatin. The *E. coli* host was first optimized by eliminating a competing esterase (encoded by *bioH*), which rapidly hydrolyzed the methyl ester bond of DMB-SMMP (*Metab. Eng.* **2007,** 9:379). In a typical laboratory run (1-2 L), the optimized *E. coli* strain harboring the LovD expression plasmid was grown to high cell density in minimal medium. DMB-SMMP and monacolin J were then added to the fermentor and progress of the reaction followed by HPLC. Complete (>99%) conversion of monacolin J to simvastatin (10 g/L) was observed in less than 24 hours. The synthesized simvastatin was readily isolated from the fermentation broth by ethyl acetate extraction at acidic pH. A two step extraction process followed by precipitation with ammonium hydroxide gave nearly pure (>98.5%) simvastatin ammonium salt. An additional recrystallization step afforded >99.5% pure simvastatin in 91% yield from monacolin J acid. Using directed evolution technology we developed LovD variants that were better expressed (*Biotechnol. Bioeng*. **2009**, 102:20), and more active for the *in vivo* process (*Chem. Biol*. **2009**, 16:1064). The structure of LovD was also determined and the basis of catalysis was elucidated (*Chem. Biol*. **2009**, 16:1064).

Based on these proof-of-concept studies this technology was made available for license and Codexis continued to optimize the enzyme for commercial scale use. Using enzyme optimization technology, we developed LovD variants that enabled efficient simvastatin manufacture (Table 1). Over the course of nine iterative rounds of *in vitro* evolution 216 libraries were constructed and 61,779 variants were screened to result in a new LovD variant with improved activity, in process stability, and tolerance to product inhibition. The amino acid sequence of the new variant has 30 mutations and as such is 7% different from the natural enzyme. The Codexis team further developed a one-pot approach in which monacolin J is first converted to the water-soluble ammonium salt that is converted biocatalytically to the water-insoluble simvastatin ammonium salt (SAS), which is readily collected by filtration and used directly to make simvastatin lactone API. The ~1000-fold improved enzyme and the new process not only pushed the reaction to completion at high substrate loading, but also minimized the use of acyl donor as well as the use of solvents for extraction and separation of the product. As a result, SAS is synthesized in >97% yield at a loading of 75 g/L monacolin J. The technology was scaled-up in early 2010 to 400 kg batch size by our first commercial manu-

Parameter	Performance at initiation of evolution program	Final performance
[Monacolin J]	3 g/L	75 g/L
[Thioester]	>3 eq.	1.1 eq.
LovD loading	10 g/L (natural LovD)	0.75 g/L (evolved LovD)
Reaction time	18 hrs	36 hrs
Conversion	50%	97%

Table 1*: Comparison of biocatalytic simvastatin manufacturing processes using natural and evolved LovD. The overall volumetric productivity on an enzyme basis was improved over 300-fold from 0.2 to 67 g/L.day.*g_{lovD}*. At the same time the amount of thioester required for complete conversion was greatly reduced.*

facturing partner (who prefers to remain undisclosed). A second API manufacturer (Arch Pharmalabs) was brought on-line in late 2010. Enzyme manufacture has been established at 30,000L scale and over 10 mT simvastatin has now been produced using this biocatalytic process. Customers have evaluated biocatalytically-produced simvastatin and confirmed that it met their stringent specifications.

The Significance:

The economic attractiveness of our biocatalytic acylation process stems from its greenness compared to the previous processes (Table 2). As a result of the simple nature of the biocatalytic process, the number of steps is reduced by avoiding the protection/deprotection strategy. The

Route	"hydrolysis/deprotection"	"direct methylation"	Biocatalytic process
Number of steps	4	5	3
Reagents:			
➢ Protection:			None
- TBDMS-Cl	2.5 equivalents	2.25 equivalents	
- Butylamine		16.6 equivalents	
➢ Deprotection:			None
- $Bu_4N^+F^-$	2.3 equivalents		
- HF (50% aq)		15 equivalents	
➢ Reagents			None
- imidazole	2 equivalents	2.5 equivalents	
- n-BuLi/hexane		2.3 equivalents	
- pyrrolidine		2.3 equivalents	
Side chain	4 eq. DMB-chloride	4 eq. methyl iodide	1.1 eq. DMB-SMMP
Solvents	Multiple and significant volumes	Multiple and significant volumes	Small volumes of a single solvent
Non-solvent waste: kg/kg Product	>25	>25	<1

Table 2: *Reagent use for the manufacture of simvastatin from lovastatin using the different chemical routes and the biocatalytic route. The information for the chemical processes is derived from gram-scale syntheses (18-50 g starting material; US4,444,784 and US4,820,850) which to our knowledge is the largest scale information publicly available. Comparison of E-factors for the different processes is problematic as we do not have good visibility on large scale process characteristics for the two chemical processes. The E-factor for the biocatalytic step including water and solvents (MTBE) is 25 (0.6 if all water and solvent is recycled).*

use of hazardous and toxic materials is greatly reduced and the formation of 1000s of mTs of non-solvent waste will be prevented with the pending expanded implementation of this process.

The merits of the nominated technology vis-à-vis the selection criteria specified in the Nomination Package for 2011 Awards

Focus areas: The nominated technology addresses all three focus areas of the Presidential Green Chemistry Challenge program:

1. It provides a **greener synthetic pathway** for the manufacture of simvastatin by using a novel enzyme enabling a biocatalytic, green-by-design, regioselective acylation that obviates the need to use hazardous and toxic reagents that generate substantial waste, while at the same time improving the overall yield.
2. The process is run under **greener reaction conditions** compared to the traditional processes in that it is run at ambient temperature and atmospheric pressure. No solvents other than water are used during the biocatalytic process.
3. A new and **green chemical was designed** for this new process. The acylating thioester DMB-SMMP is a safer acylating reagent and the thiopropionate by-product can be recycled at scale.

Science and Innovation:

- The nominated technology is original, never employed before:
  - A new biocatalytic process was conceived and enabled using molecular biology methodology and chemical process development including screening of various side-chain donors.
- The nominated technology is scientifically valid.
  - The technology has undergone repeated peer review and has been published in high impact journals (referenced in the text).
  - It is also proven as a result of its successful adoption for commercial scale manufacturing.

Human health and environmental benefits:

The nominated technology offers several human health and/or environmental benefits:

- Reduced use of toxic and hazardous substances:
  - Avoidance of *tert*-butyl dimethyl silane chloride, a moisture sensitive, flammable solid.
  - Avoidance of methyliodide, a possible carcinogen and compound that can cause lung, liver, kidney and central nervous system damage.
  - Avoidance of *n*-butyl lithium, a pyrophoric reagent that requires handling under cryogenic conditions (-30^{o}C),
  - Reduction in solvent use due to the aqueous nature of the reaction conditions.
  - Several co-products are avoided and the only new co-product is methyl 3-mercapto-propionic acid, which will be recycled at commercial scale.
- Improved energy efficiency as the reaction is run at ambient temperature.

The merits of the nominated technology vis-à-vis the Twelve Principles of Green Chemistry

1. Waste prevention:
   - The highly selective biocatalytic acylation provides producyt in substantially higher yield and circumvents the need for protection/ deprotection reactions that result in significant waste generation.
   - The main waste streams are aqueous and directly biodegradable.

	• Use of extraction solvents is greatly reduced.
2. Atom Economy:	• The high yield of the reaction, the high purity of the product, the avoidance of protection/deprotection reactions and the low solvent usage result in more of the total atom and mass that is put into the process is recovered in the product.
3. Less Hazardous Chemical Syntheses:	• In this process, the use of hazardous chemicals such as TBDMS, MeI, and *n*-butyl lithium is avoided.
4. Design Safer Chemicals:	• The acyl donor used was designed for use in this process and provides safety advantages over the traditional chemistries.
5. Safer Solvents and Auxiliaries:	• Water is used as a safe and recyclable solvent.
6. Design for Energy Efficiency:	• The reaction runs at or near ambient temperature and at atmospheric pressure. • The high activity of the evolved biocatalysts minimizes the fermentation capacity requirement, thereby reducing the energy and water needs, as well as the CO_2 output of the catalyst manufacturing process.
7. Use of Renewable Feedstocks	• The catalyst is produced efficiently from renewable resources.
8. Reduce Derivatives	• No protection/deprotection needed.
9. Catalysis	• The new process is enabled by a regioselective catalyst preventing the need for protection/deprotection reactions.
10. Design for Degradation	• The major waste streams generated by this process are directly biodegraded in biotreatment facilities.
11. RTA for pollution prevention	• The process is monitored in real-time via the pH-stat controlled addition of NH_4OH.
12. Inherently Safer Chemistry for Accident Prevention	• The chemicals used in this process are readily used safely. Hazardous reagents are avoided. The reaction is run at ambient temperature.

Broad applicability:

- The nominated technology is a practical, cost-effective approach to green chemistry:
  - It is a practical operation to manufacture simvastatin.
- The nominated technology embodies features that can be transferred readily to other processes and facilities:
  - Regioselective catalytic acylation reactions are highly desirable as they circumvent the need for extensive protection/deprotection methodology. The nominated technology demonstrates a new biocatalytic acylation process for the manufacture of simvastatin and enabled the development of an economic, green-by-design biocatalytic process.

References to Patent Applications:

WO2007/139871 entitled “Methods and Materials for Making Simvastatin and Related Compounds”, published Dec. 6, 2007; both WO2011/41231 entitled “Variant LovD Polypeptides and their uses” and WO2011/41233 and entitled “Improved LovD acyltransferase mediated acylation” published April 7, 2011. Corresponding national phase applications have been filed.