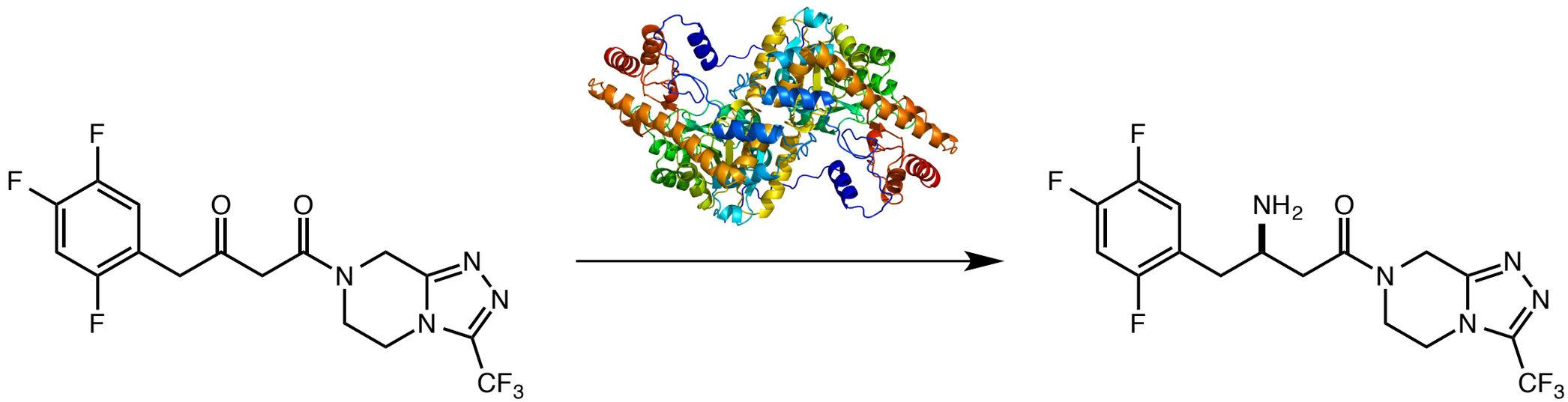
Protein-Engineered Biocatalysts in Industry



Stefan McCarver

Group Meeting

June 18th, 2015

Protein-Engineered Biocatalysts in Industry

Diverse applications of enzymes

World enzyme market in 2003

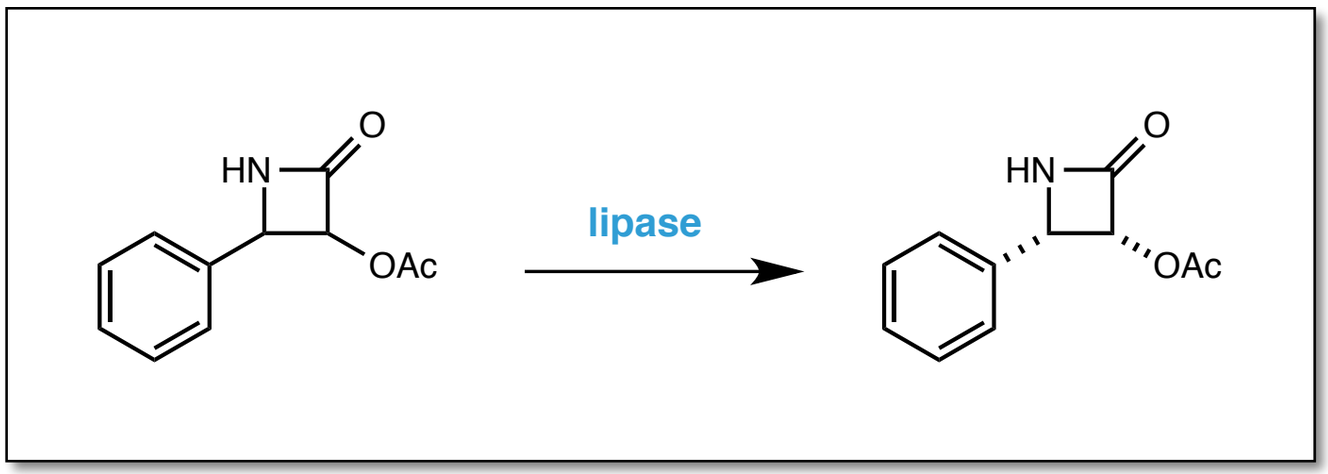
Product	USD (Millions)
Detergents	789
Foods	634
Agriculture and feed	376
Textile processing	237
Pulp, paper, leather, and chemicals	222

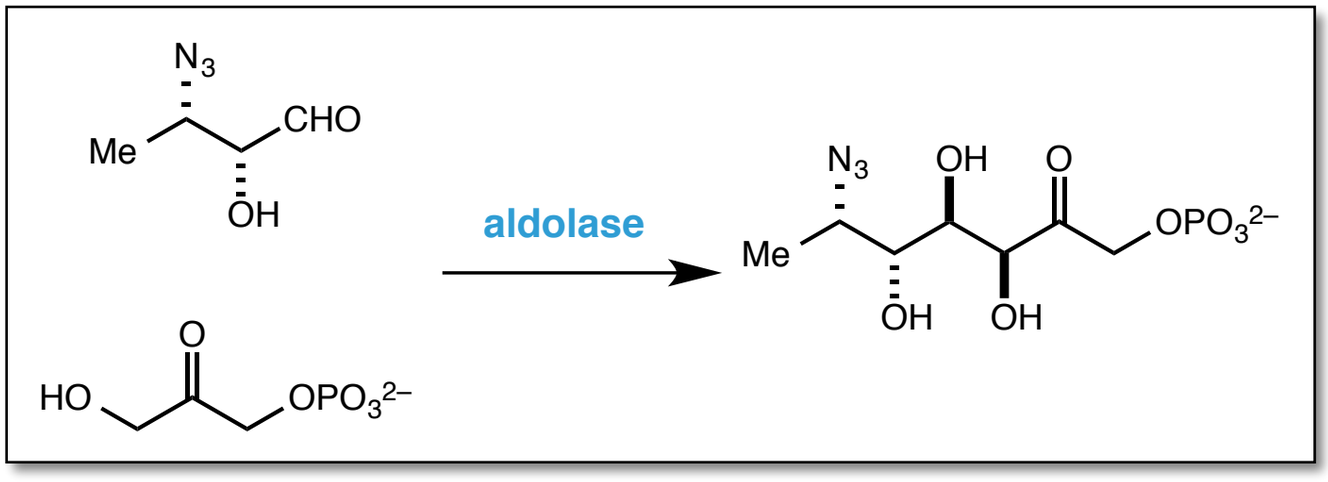
Sanchez, S.; Demain, A. L. *Org. Process Res. Dev.* **2011**, *15*, 224–230.

Protein-Engineered Biocatalysts in Industry

Diverse applications of enzymes

■ Enzymes can provide many advantages for chemical synthesis





Higher enantioselectivity and regioselectivity

Can be effective in both aqueous and organic media

Typically do not require protecting groups

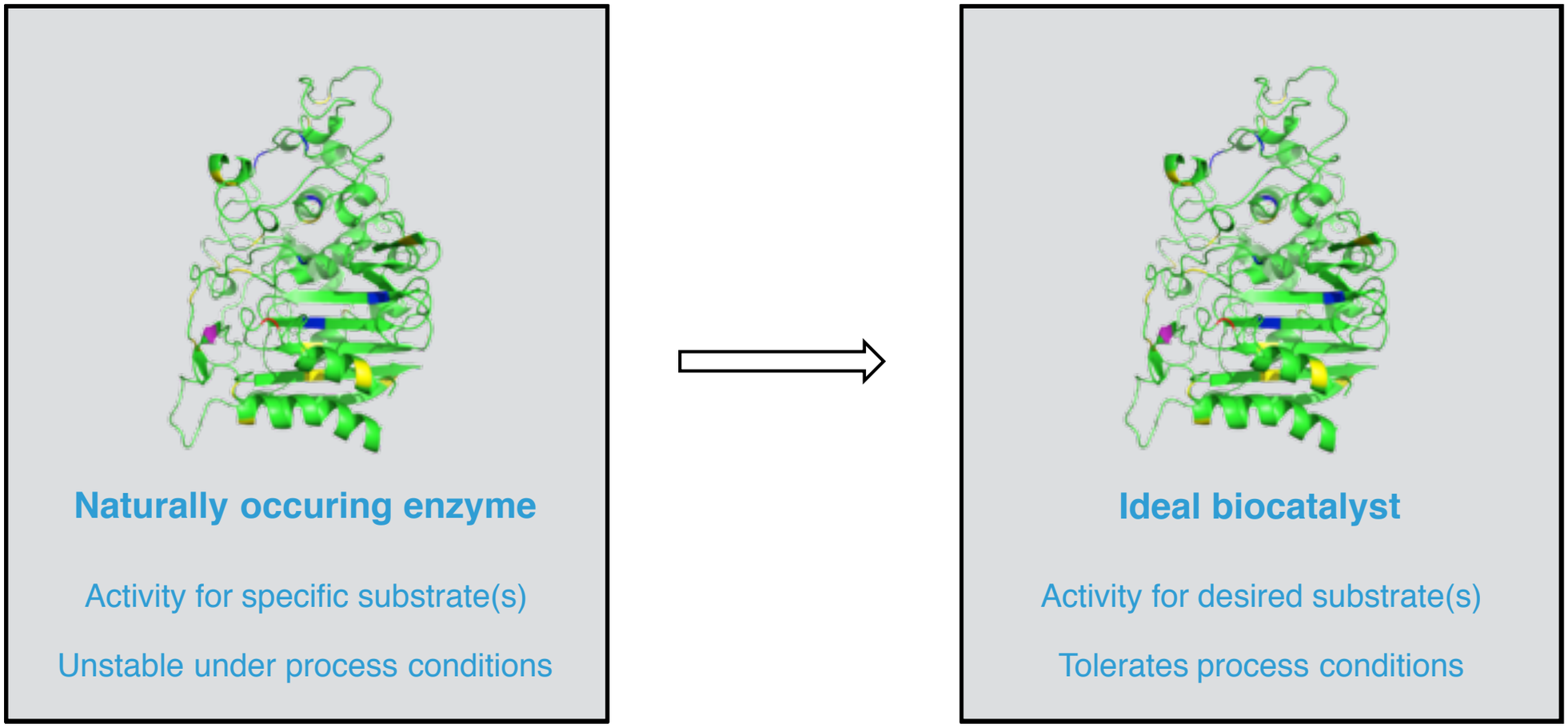
Operate under mild conditions with high efficiency

Koeller, K. M.; Wong, C.-H. *Nature* **2001**, *409*, 232–240.

Protein-Engineered Biocatalysts in Industry

Utilizing natural enzymes for chemical reactions

- Naturally occuring enzymes usually do not have desired catalytic reactivity



Naturally occuring enzyme

Activity for specific substrate(s)

Unstable under process conditions

Ideal biocatalyst

Activity for desired substrate(s)

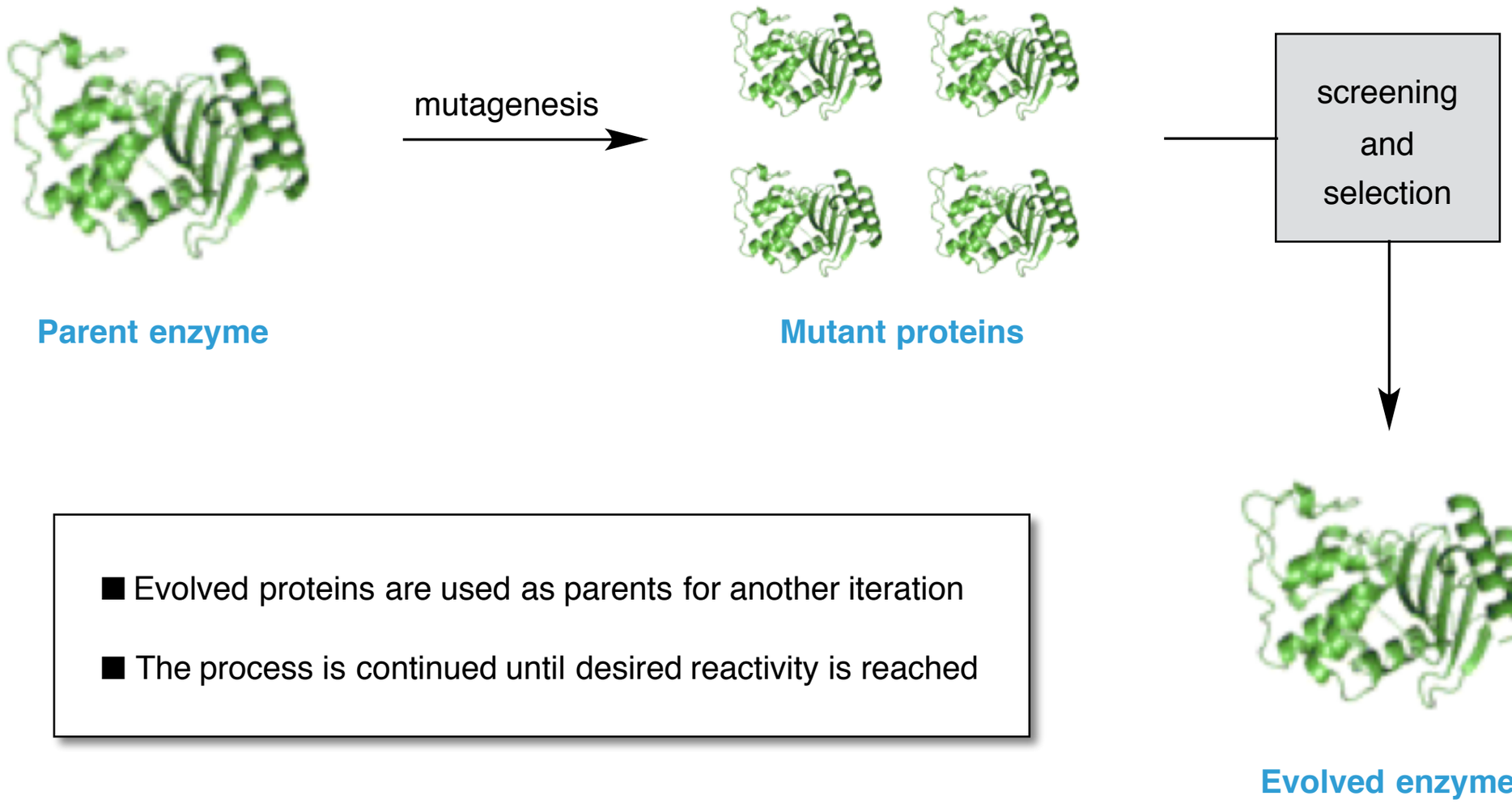
Tolerates process conditions

How do chemists transform natural enzymes into useful biocatalysts?

Protein-Engineered Biocatalysts in Industry

Modification of enzyme catalysts

■ Proteins are modified through iterative mutation and screening



■ Evolved proteins are used as parents for another iteration

■ The process is continued until desired reactivity is reached

Bloom, J. D.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 9995–10000.

Protein-Engineered Biocatalysts in Industry

Generating protein diversity

Methods for mutagenesis

Site-directed mutagenesis

A number of methods are known for specifically substituting individual amino acids in a protein

Requires a lot of structural information to be useful, often a crystal structure and computational modeling

Error-prone PCR

The polymerase chain reaction reaction is naturally error prone

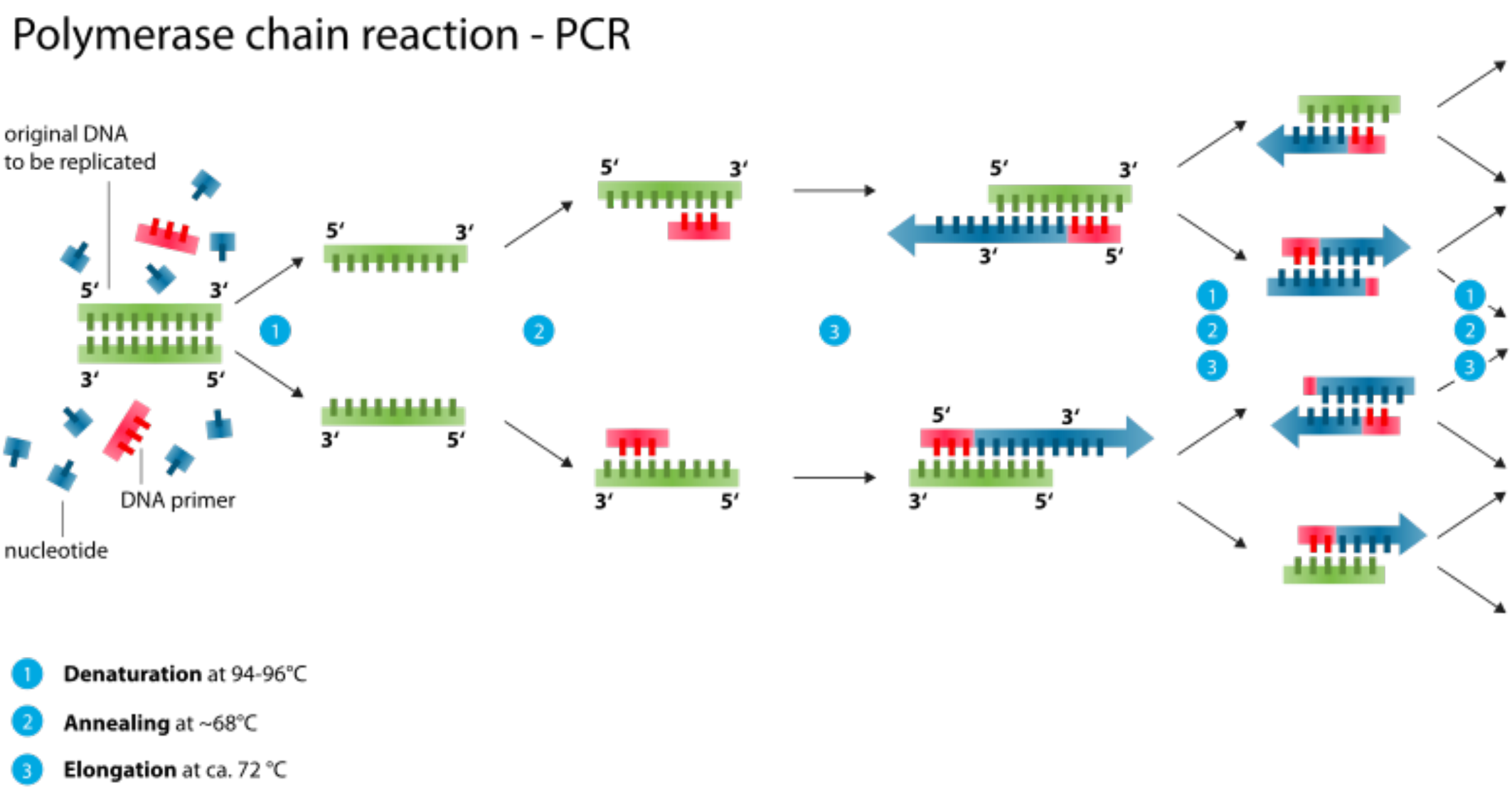
This can be amplified by increasing Mg^{2+}, adding Mn^{2+} and by using unequal nucleotide concentrations

Primrose, S. B.; Twyman, R. In *Principles of Gene Manipulation and Genomics*, 7th ed.; Wiley-Blackwell, 2006; ch. 8

Protein-Engineered Biocatalysts in Industry

Generating protein diversity

■ Methods for mutagenesis



Primrose, S. B.; Twyman, R. In *Principles of Gene Manipulation and Genomics*, 7th ed.; Wiley-Blackwell, 2006; ch. 8

Protein-Engineered Biocatalysts in Industry

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Methods for mutagenesis

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Requires a lot of structural information to be useful, often a crystal structure and computational modeling

Error-prone PCR

The polymerase chain reaction reaction is naturally error prone

This can be amplified by increasing Mg^{2+}, adding Mn^{2+} and by using unequal nucleotide concentrations

Gene shuffling

DNA sequences from several similar proteins are fragmented and randomly recombined

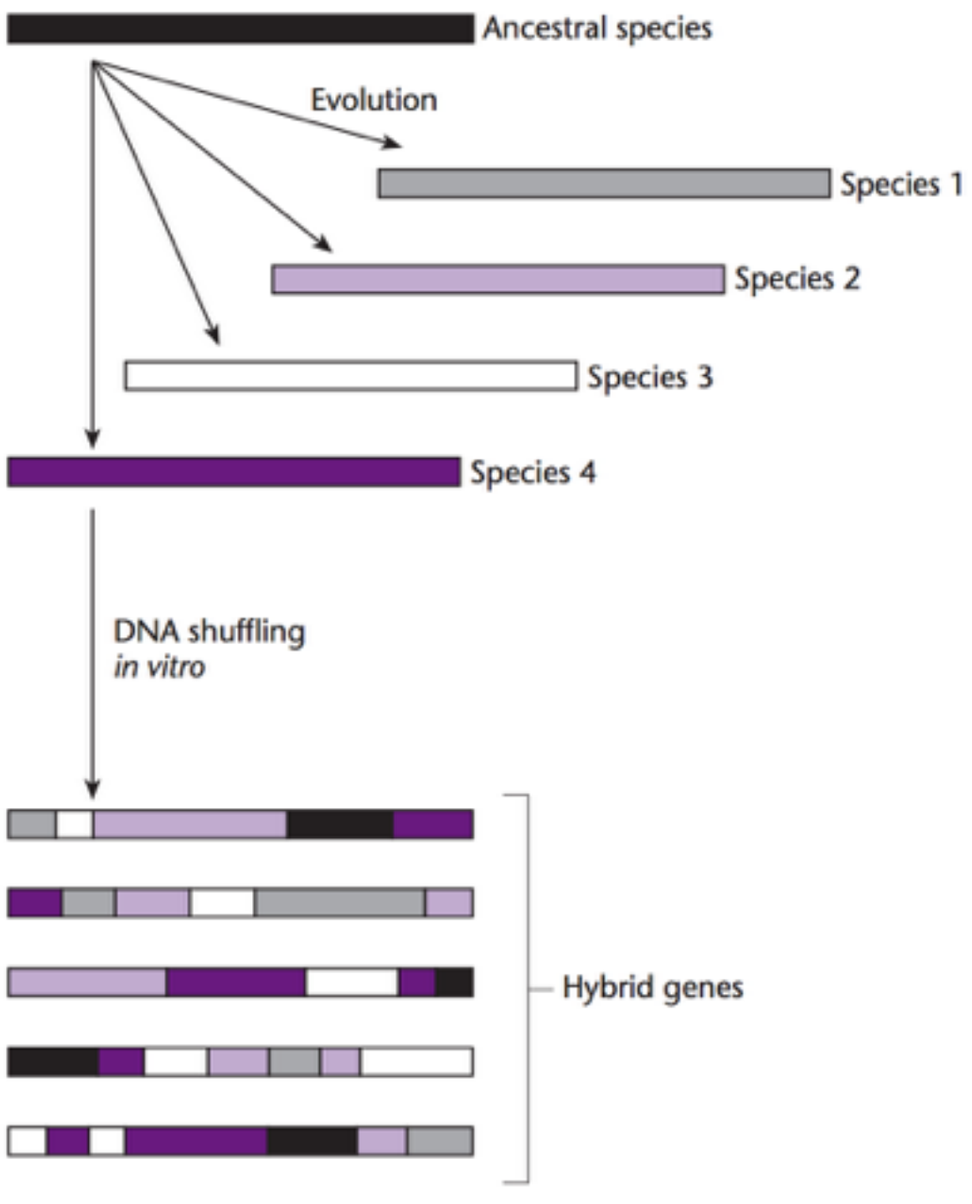
A larger extent of fragmentation results in a greater number of single site mutations

Primrose, S. B.; Twyman, R. In *Principles of Gene Manipulation and Genomics*, 7th ed.; Wiley-Blackwell, 2006; ch. 8

Protein-Engineered Biocatalysts in Industry

Generating protein diversity

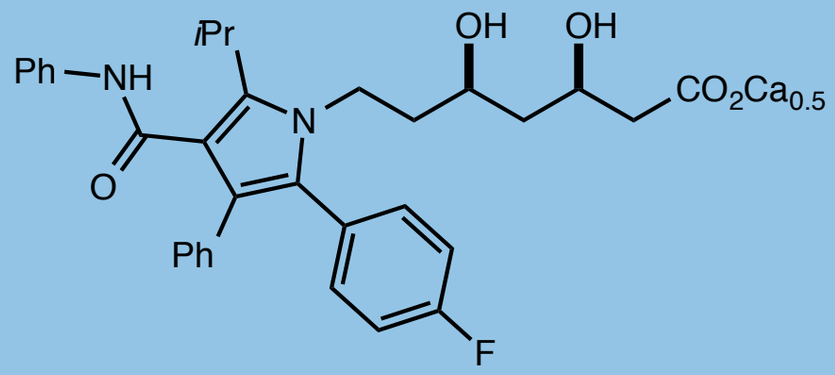
■ Methods for mutagenesis



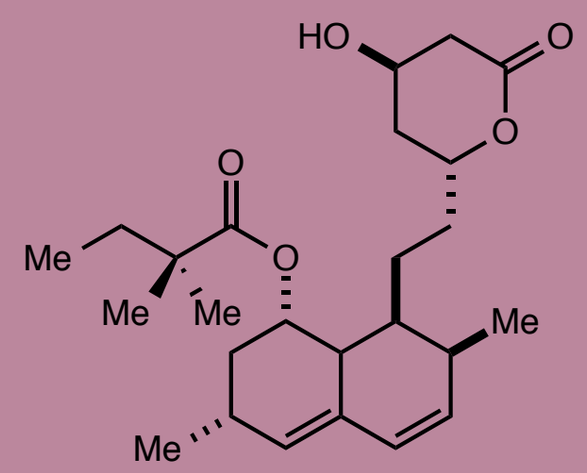
Primrose, S. B.; Twyman, R. In *Principles of Gene Manipulation and Genomics*, 7th ed.; Wiley-Blackwell, 2006; ch. 8

Protein-Engineered Biocatalysts in Industry

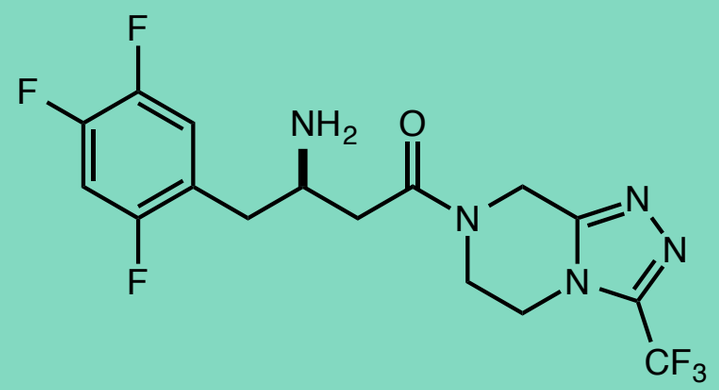
Case studies



Atorvastatin (Lipitor)



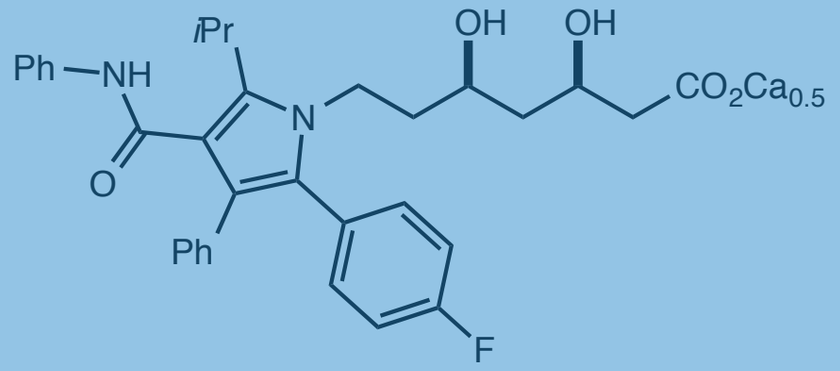
Simvastatin (Zocor)



Sitagliptin (Januvia)

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin



Atorvastatin (Lipitor)

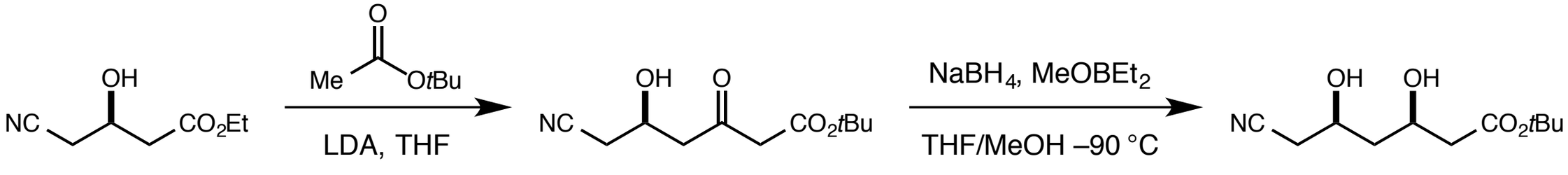
- Lowers blood cholesterol by inhibiting the HMG-CoA reductase enzyme
- Discovered at Parke-Davis, later acquired by Pfizer
- Best-selling drug of all time, with over $125 billion in total sales

LIPITOR
atorvastatin calcium

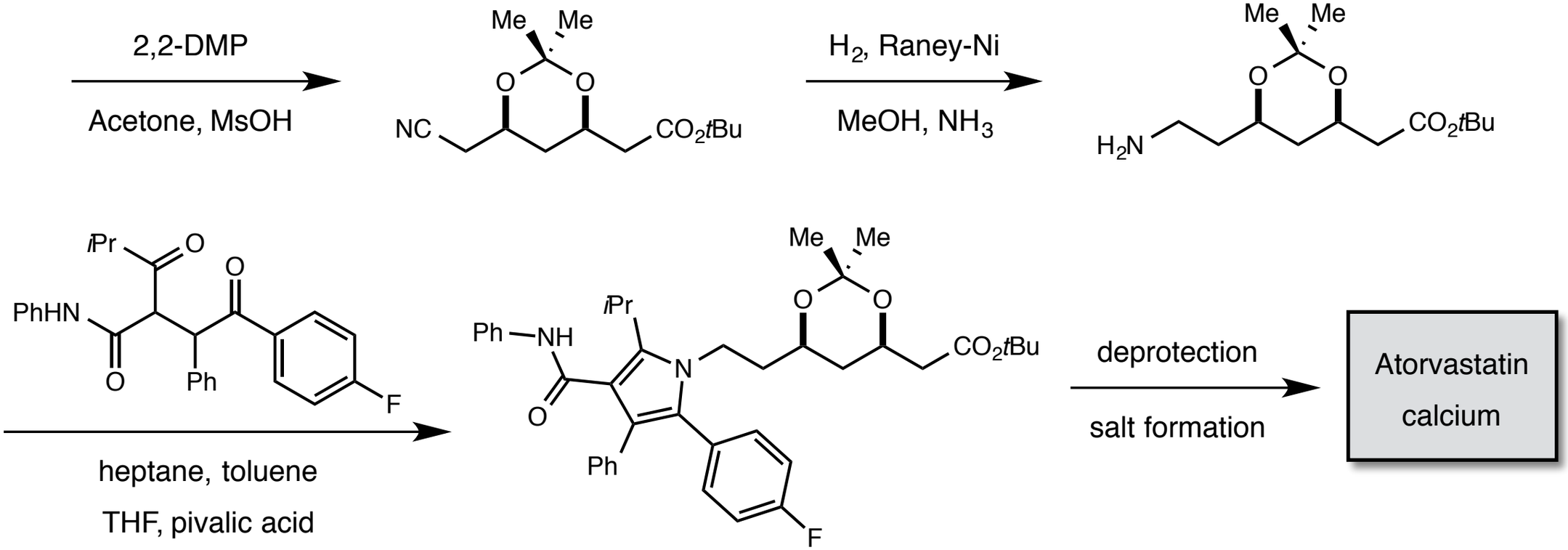
Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Process route



key hydroxynitrile

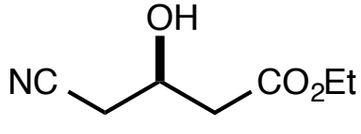


Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

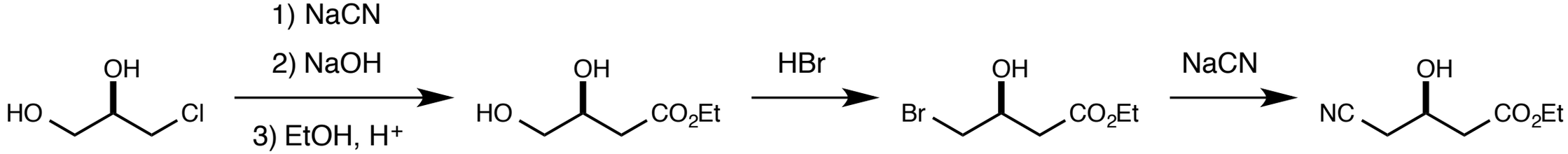
Synthesis of atorvastatin

■ Previous routes to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
- Simplified route that reduces waste is highly desirable



from kinetic resolution

kinetic resolution - max 50% yield

two steps involving the use of cyanide

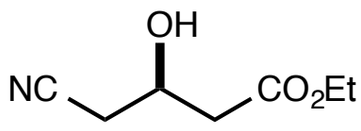
HBr required to form bromohydrin

Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Previous routes to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
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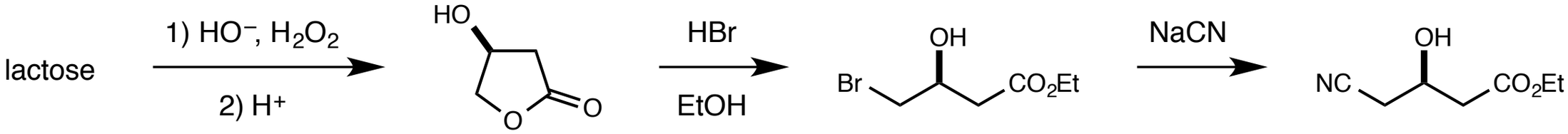


uses chiral pool materials

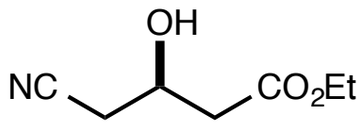
HBr required to form bromohydrin

Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Previous routes to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
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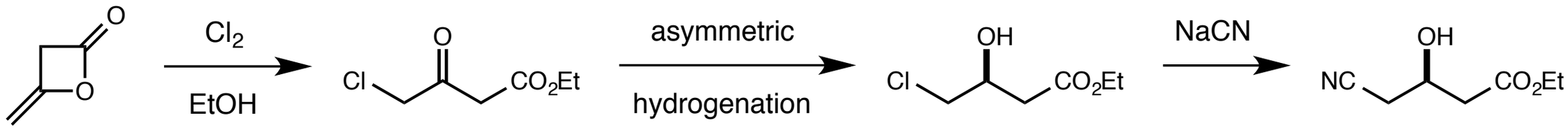


high pressure H_2 for asymmetric reduction

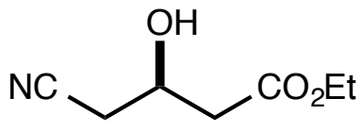
harsh cyanation conditions

Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Previous routes to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
- Simplified route that reduces waste is highly desirable

Issues to be addressed

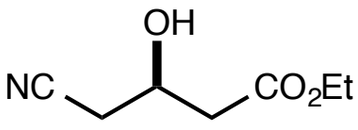
- Requirement of chiral pool materials or high pressure hydrogenation to access alcohol
- Cyanation requires harsh conditions, challenging to separate product from byproducts

Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

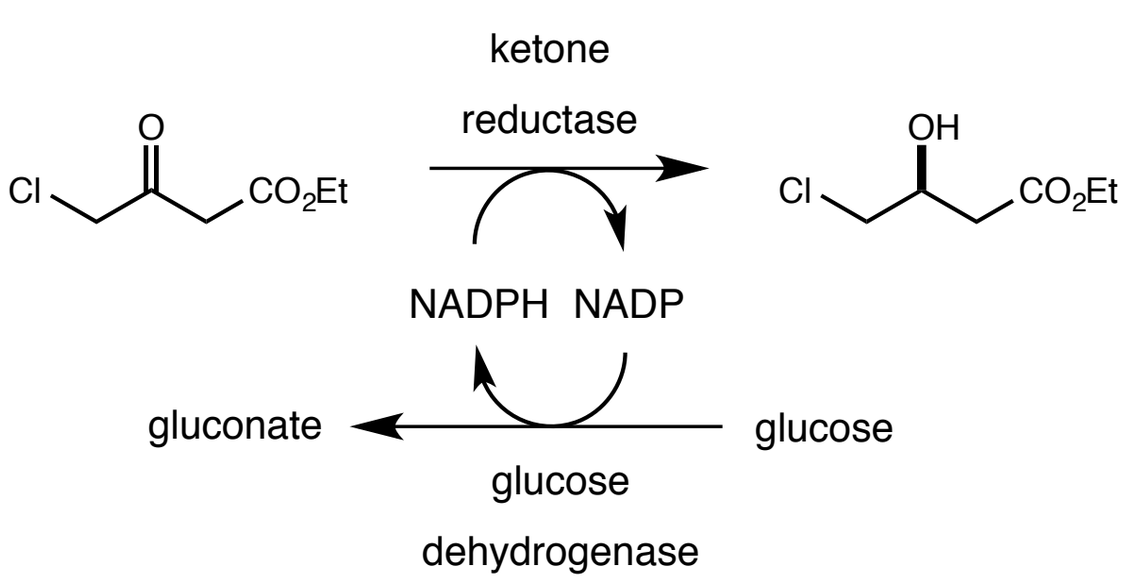
Synthesis of atorvastatin

■ Enzymatic route to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
- Simplified route that reduces waste is highly desirable

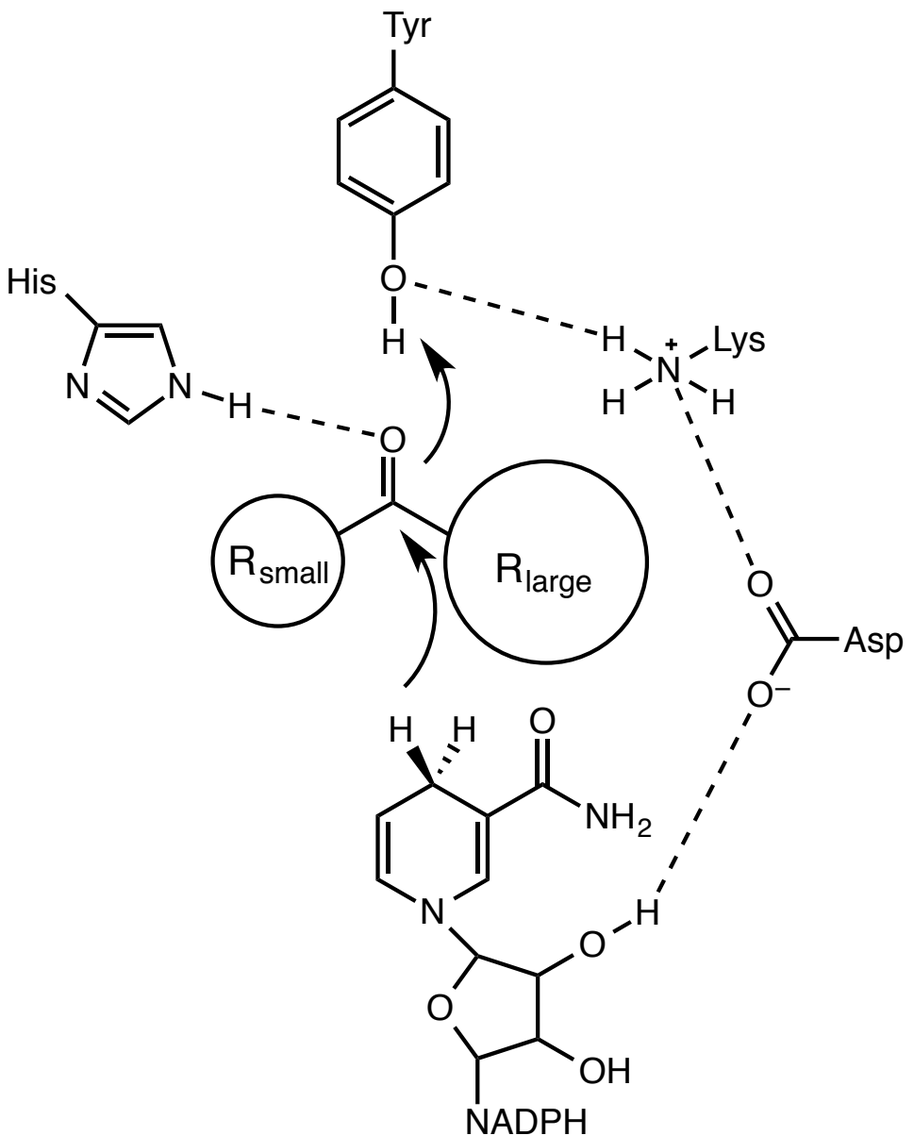


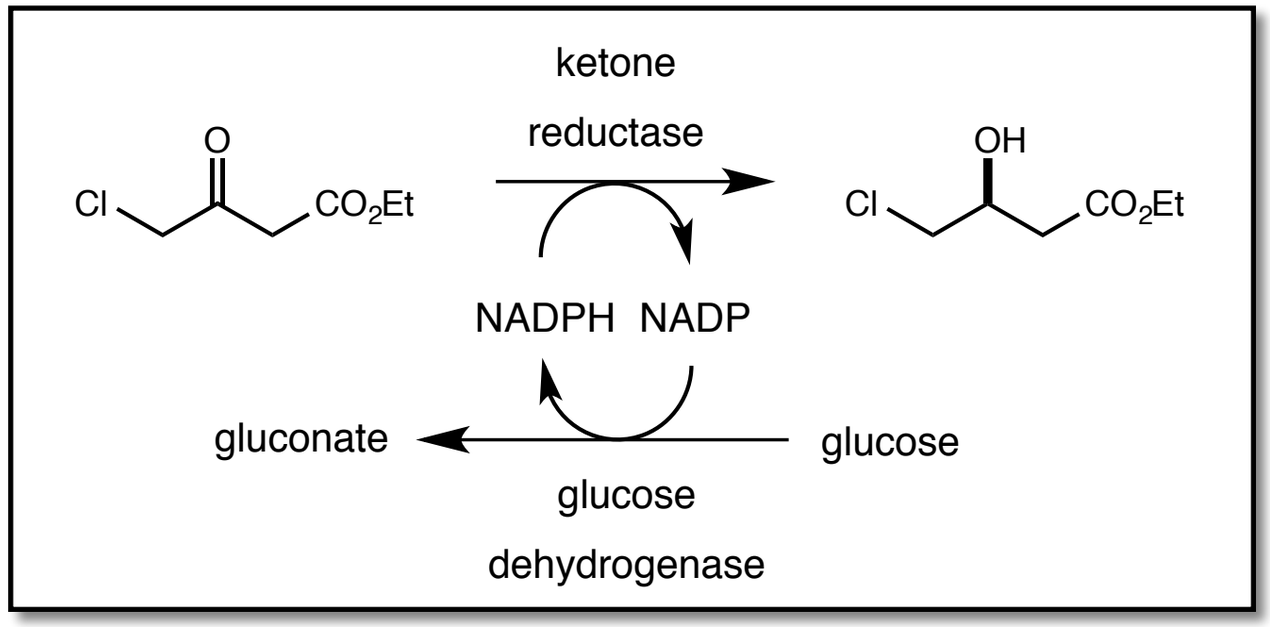
Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Ketone reductase mechanism





■ Hydrogen bond to histidine orients substrate

■ Proton transfer occurs from tyrosine residue

■ Lysine provides electrostatic stabilization

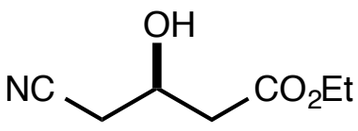
■ NADPH positioned by hydrogen bond to asparagine

Kratzer, R.; Wilson, D. K.; Nidetzky, B. *Life* **2006**, *58*, 499–507.

Protein-Engineered Biocatalysts in Industry

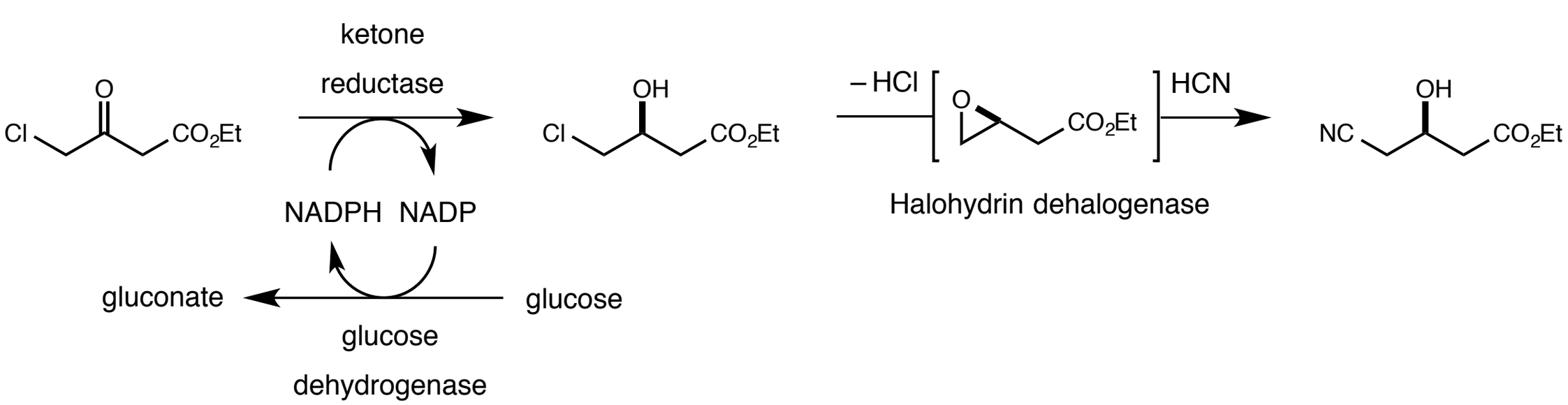
Synthesis of atorvastatin

■ Enzymatic route to hydroxynitrile starting material



key hydroxynitrile

- Estimated demand 100 mT per year
- Simplified route that reduces waste is highly desirable

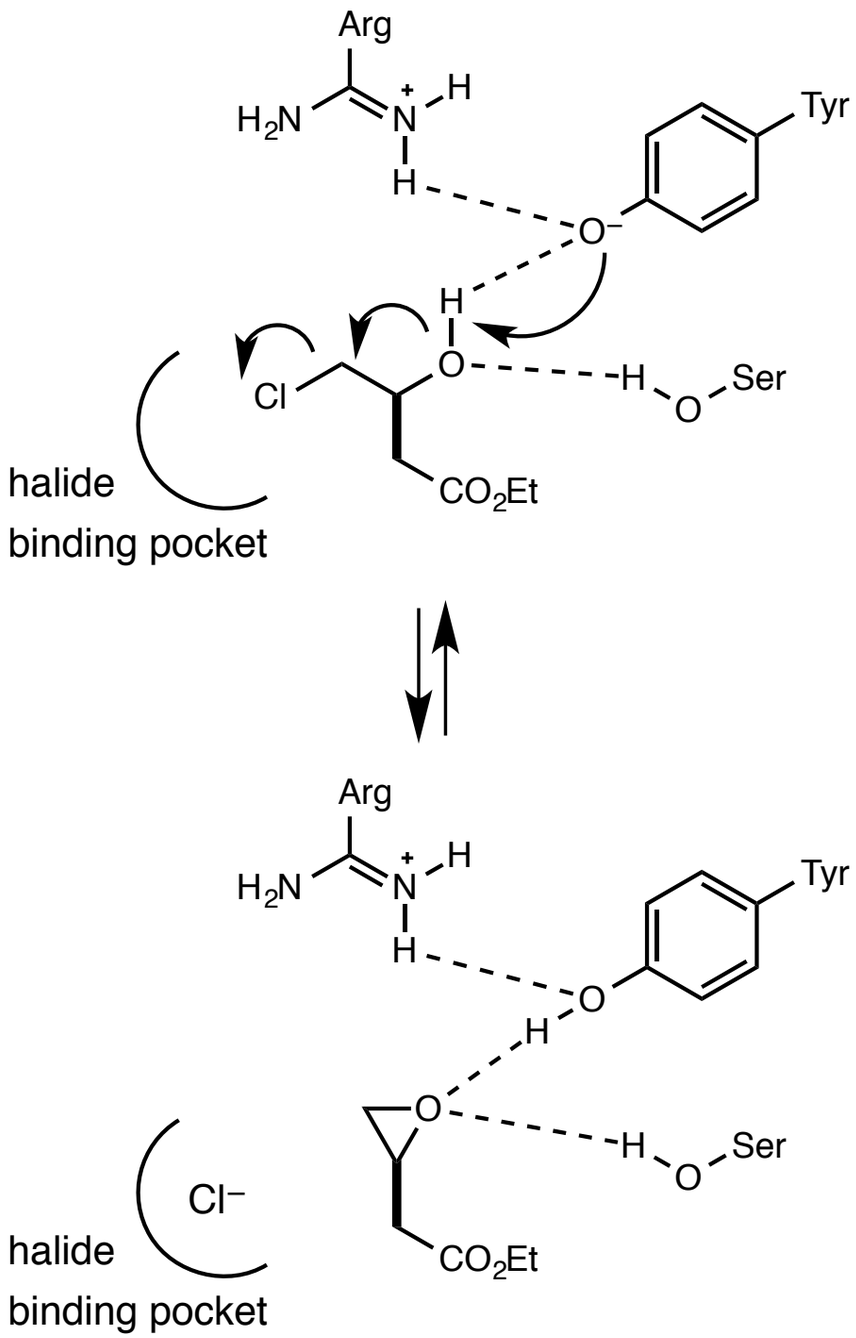


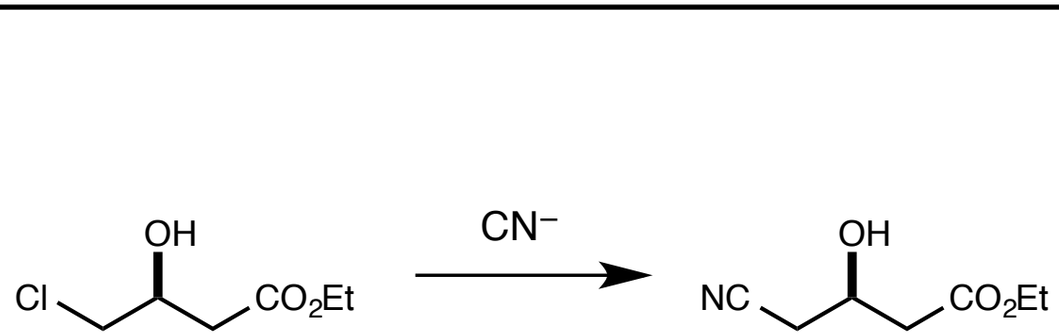
Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

Halohydrin dehalogenase mechanism





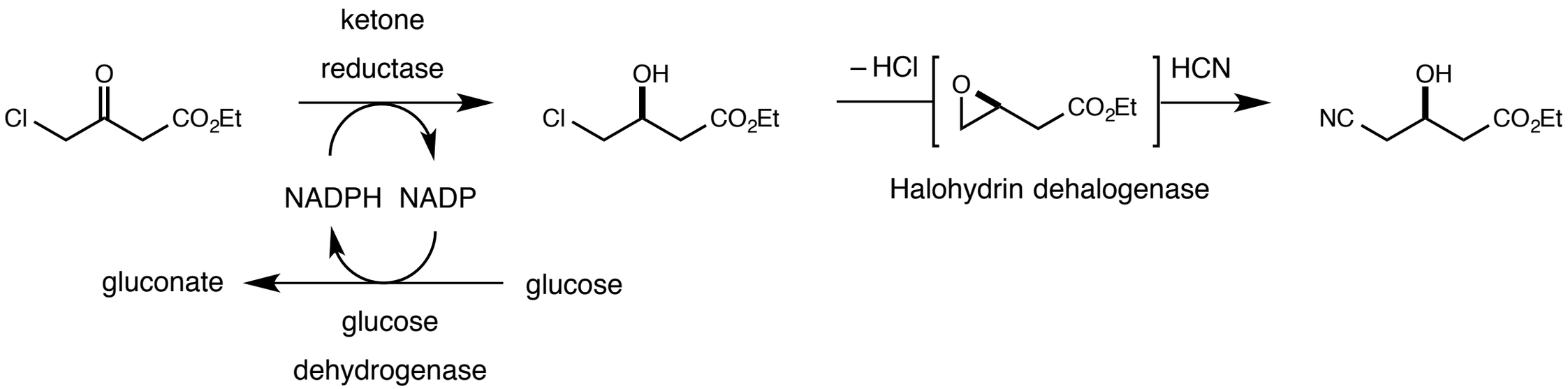
- Deprotonation by tyrosine residue
- Hydrogen bonding catalysis from serine
- Binding pocket for departing halide

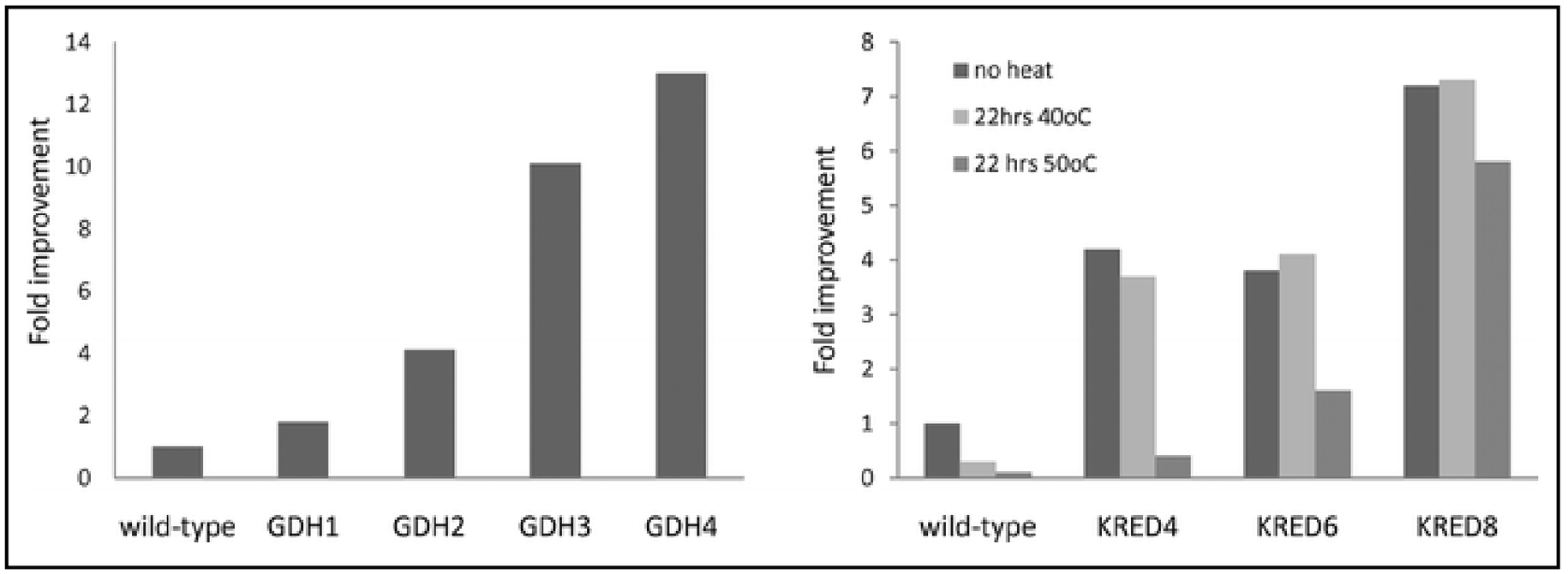
Janssen, D. B. et. al. *J. Bacteriol.* **2001**, *183*, 5058–5066.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Directed evolution of KRED and GDH enzymes using DNA shuffling



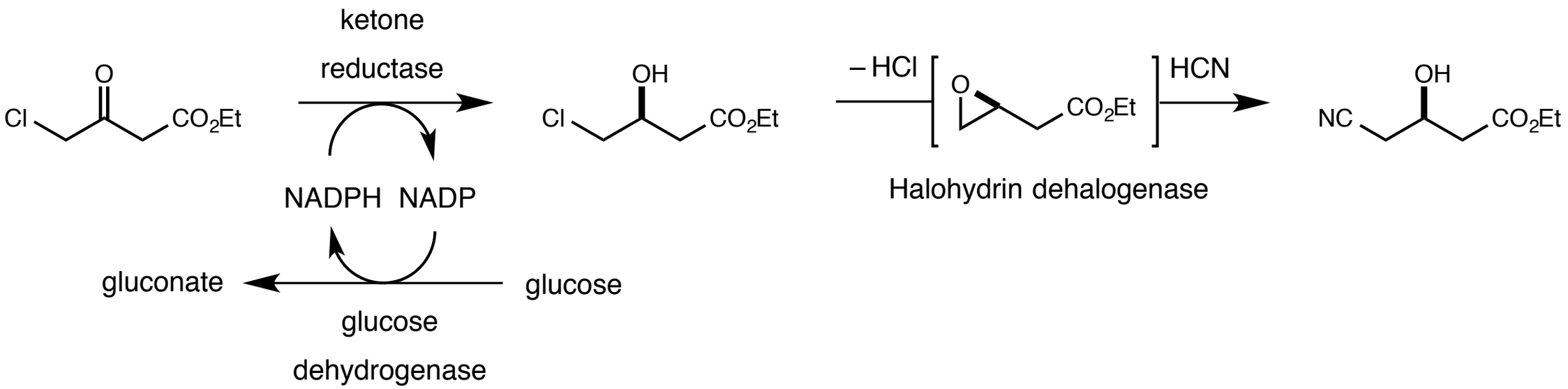


Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Directed evolution of KRED and GDH enzymes using DNA shuffling



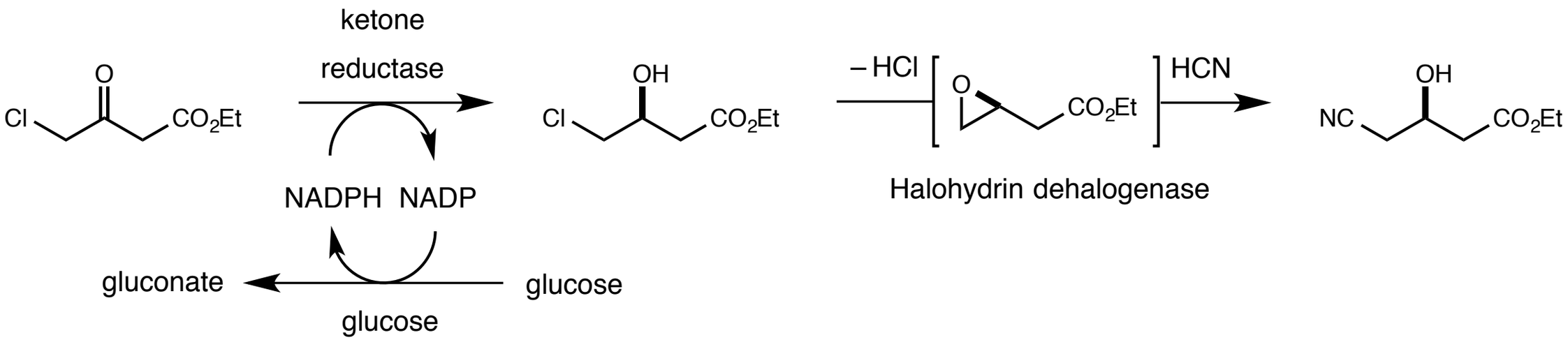
	Initial process (wild-type enzymes)	Final process (evolved enzymes)
Substrate (g/L)	80	160
Reaction time (h)	24	8
Enzyme loading (g/L)	9	0.9
Isolated yield (%)	85	95
Product ee (%)	> 99.5	> 99.9

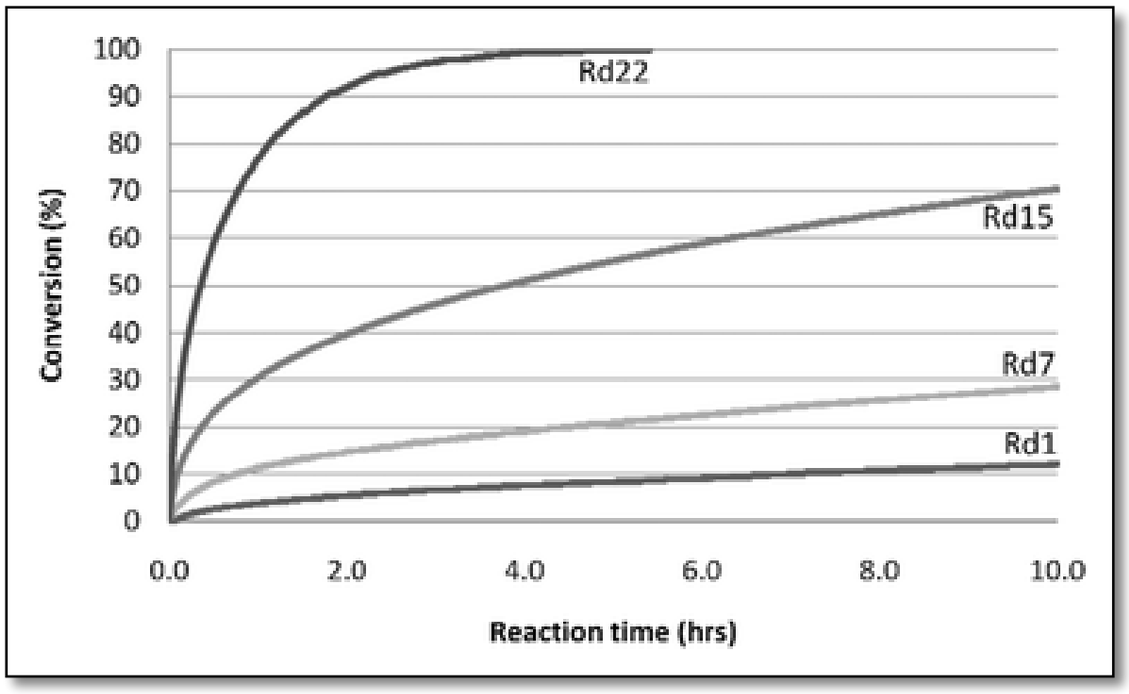
Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Directed evolution of HDDH enzyme using DNA shuffling



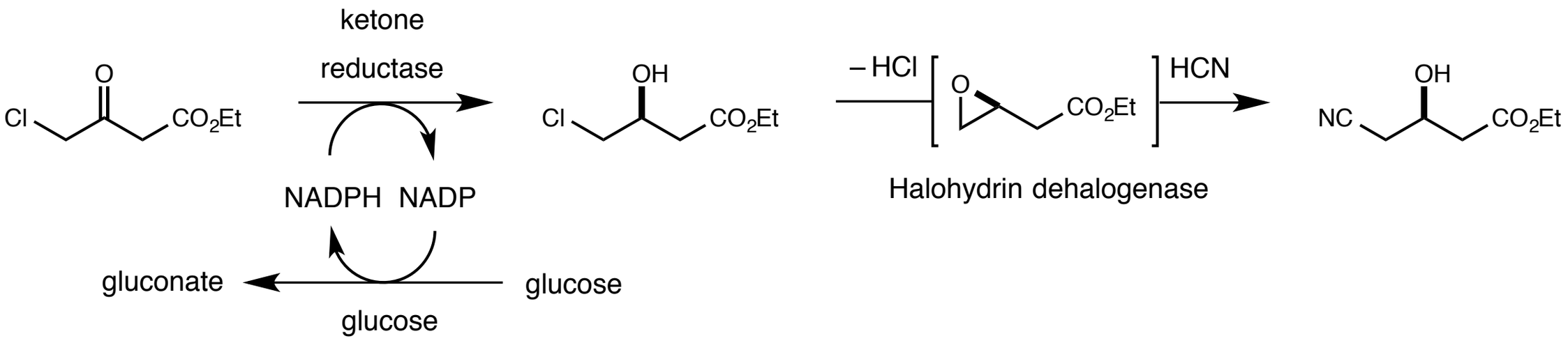


Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of atorvastatin

■ Directed evolution of HDDH enzyme using DNA shuffling

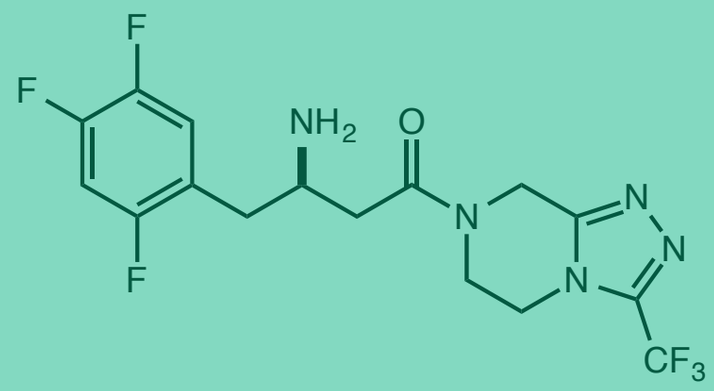


	Inital process (wild-type enzyme)	Final process (evolved enzyme)
Substrate (g/L)	20	140
Reaction time (h)	72	5
Enzyme loading (g/L)	30	1.2
Isolated yield (%)	67	92
Product ee (%)	> 99.5	> 99.5

Ma, S. K. et. al. *Green Chem.* **2012**, *12*, 81–86.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin



Sitagliptin (Januvia)

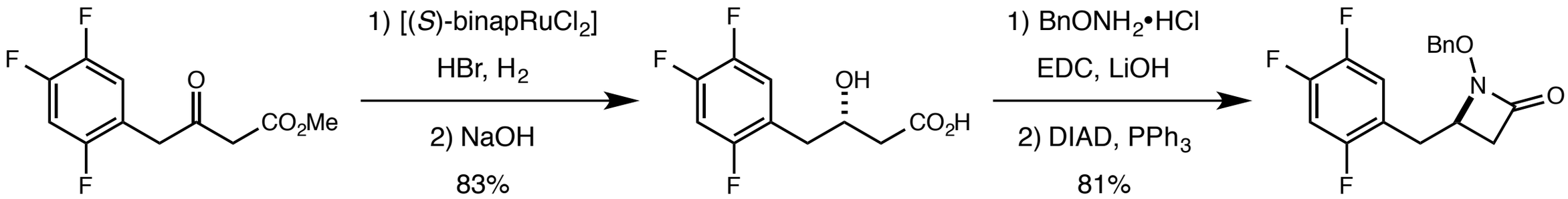
- Antidiabetic dipeptidyl peptidase-4 inhibitor
- Developed and marketed by Merck
- Often used as a combination therapy with other medicines

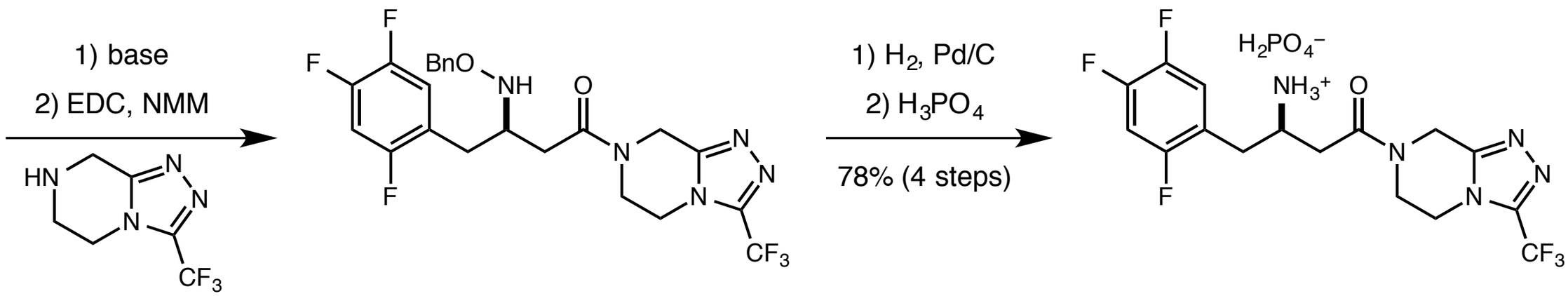
Januvia
(sitagliptin)
25 mg, 50 mg, 100 mg tablets

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

■ First generation process route



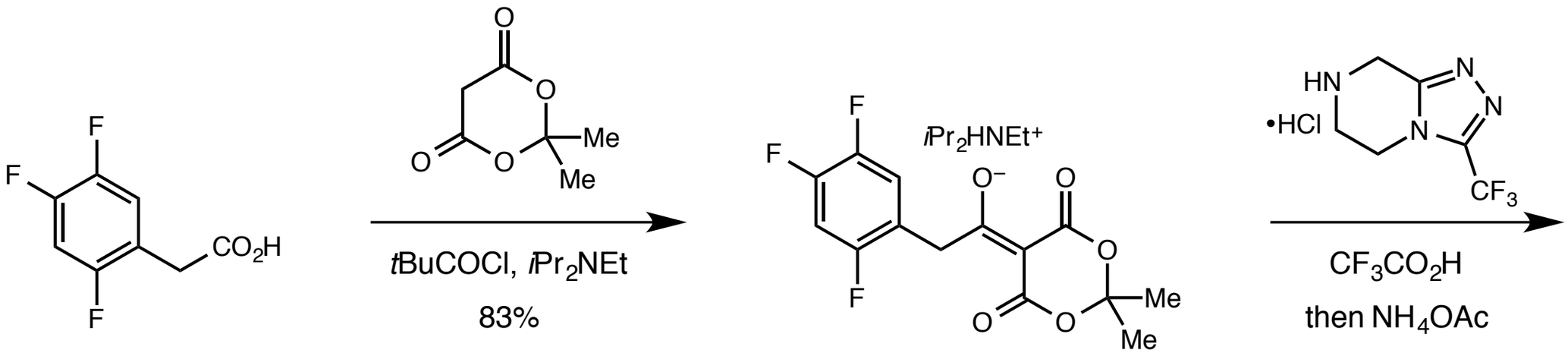


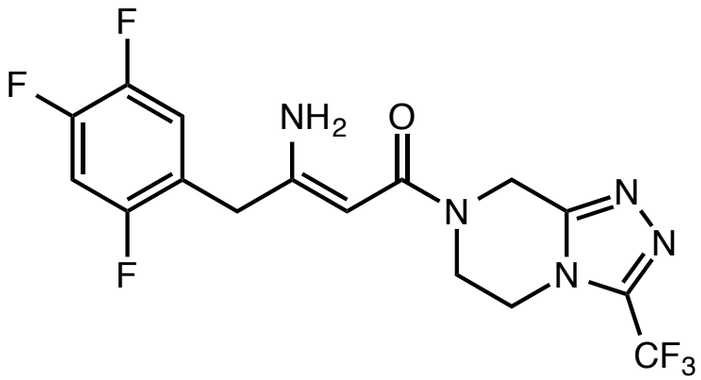
Desai, A. A. *Angew. Chem. Int. Ed.* **2011**, *50*, 1974–1976.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

■ Second generation process route



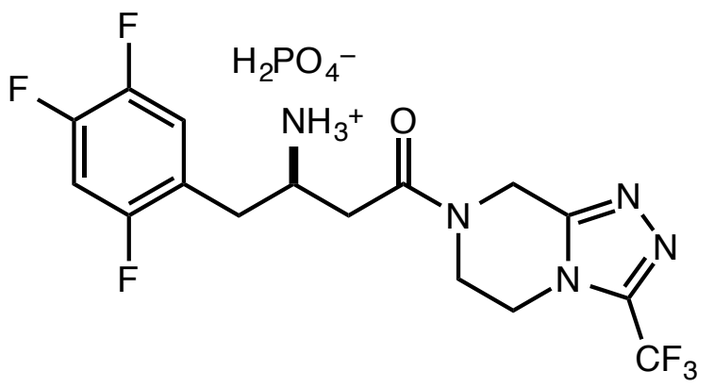


one pot, 82% yield

1) $[\{Rh(cod)Cl\}_2]$, NH_4Cl,
*t*Bu-josiphos, H_2

2) heptane/*i*PrOH

3) H_3PO_4



81% yield

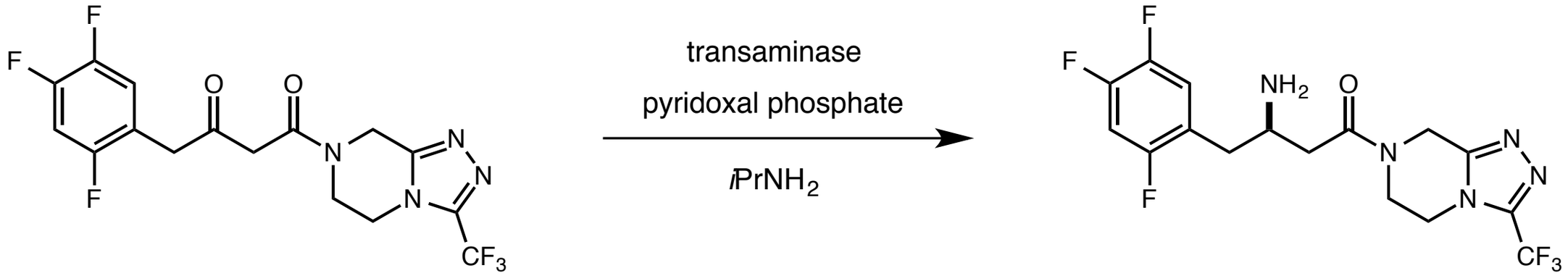
> 99.9% ee

Desai, A. A. *Angew. Chem. Int. Ed.* **2011**, *50*, 1974–1976.

Protein-Engineered Biocatalysts in Industry

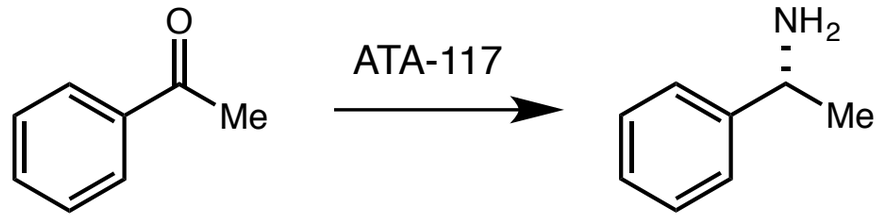
Synthesis of sitagliptin

■ Third generation process route



■ Transition metal catalyzed hydrogenation required carbon treatment to remove Rh as well as ee upgrade through recrystallization

■ Biocatalysis presents an opportunity to attain excellent yield and near perfect selectivity under mild conditions



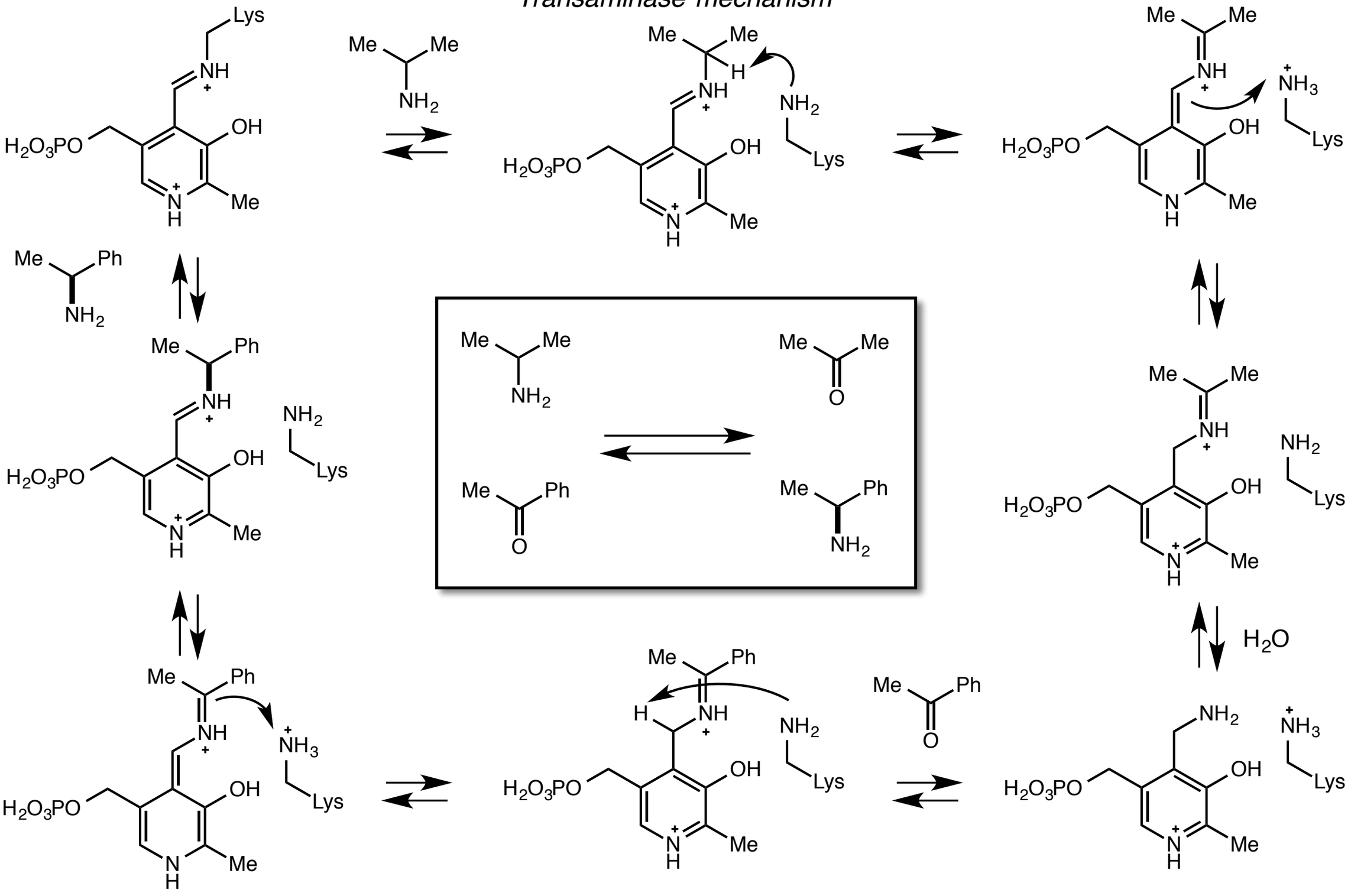
> 99.9% ee

Chem. Commun. **2009**, 2127–2129.

Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Transaminase mechanism

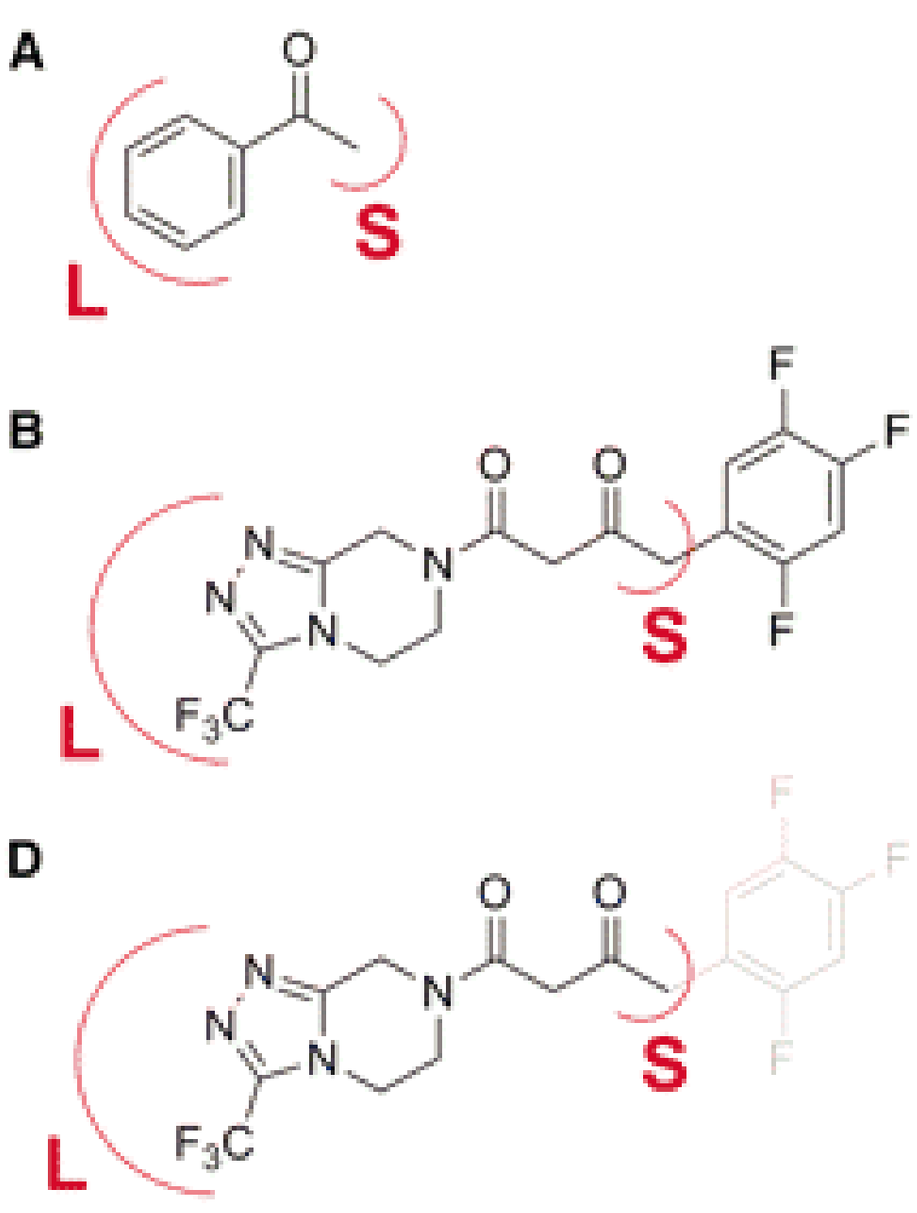


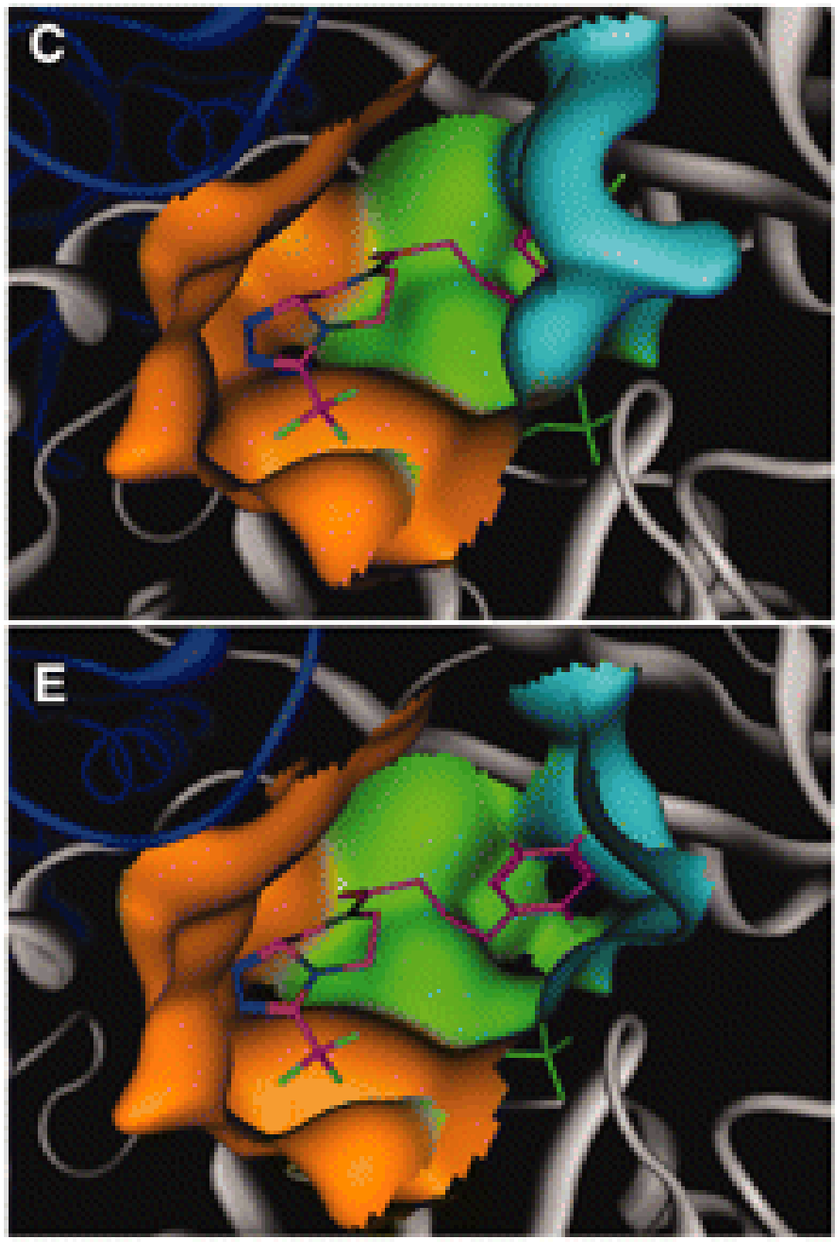
Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

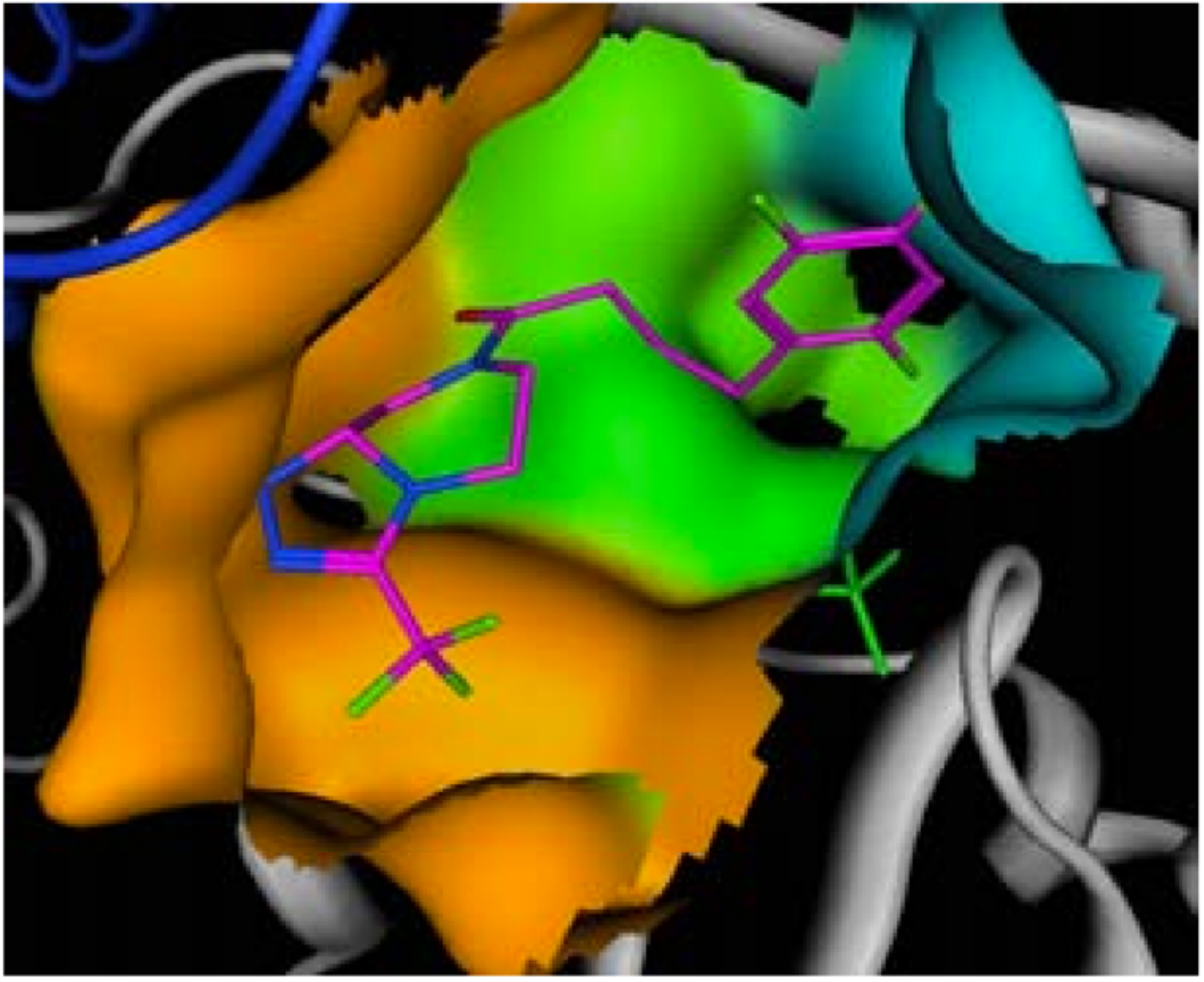
■ Design of a transaminase catalyst - site saturation libraries in small and large pockets





Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry



Orange: Large pocket

Green: Catalytic residues

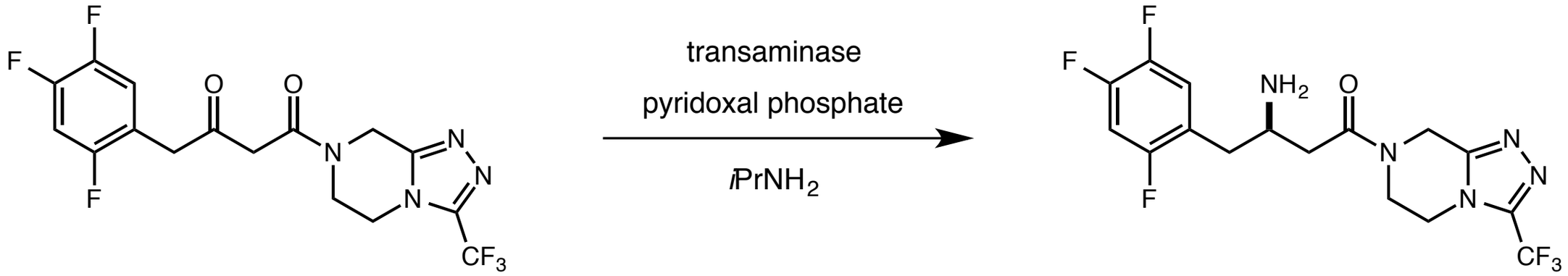
Teal: Small Pocket

Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

■ Third generation process route



■ Site saturation and combinatorial libraries of binding pocket screened for activity

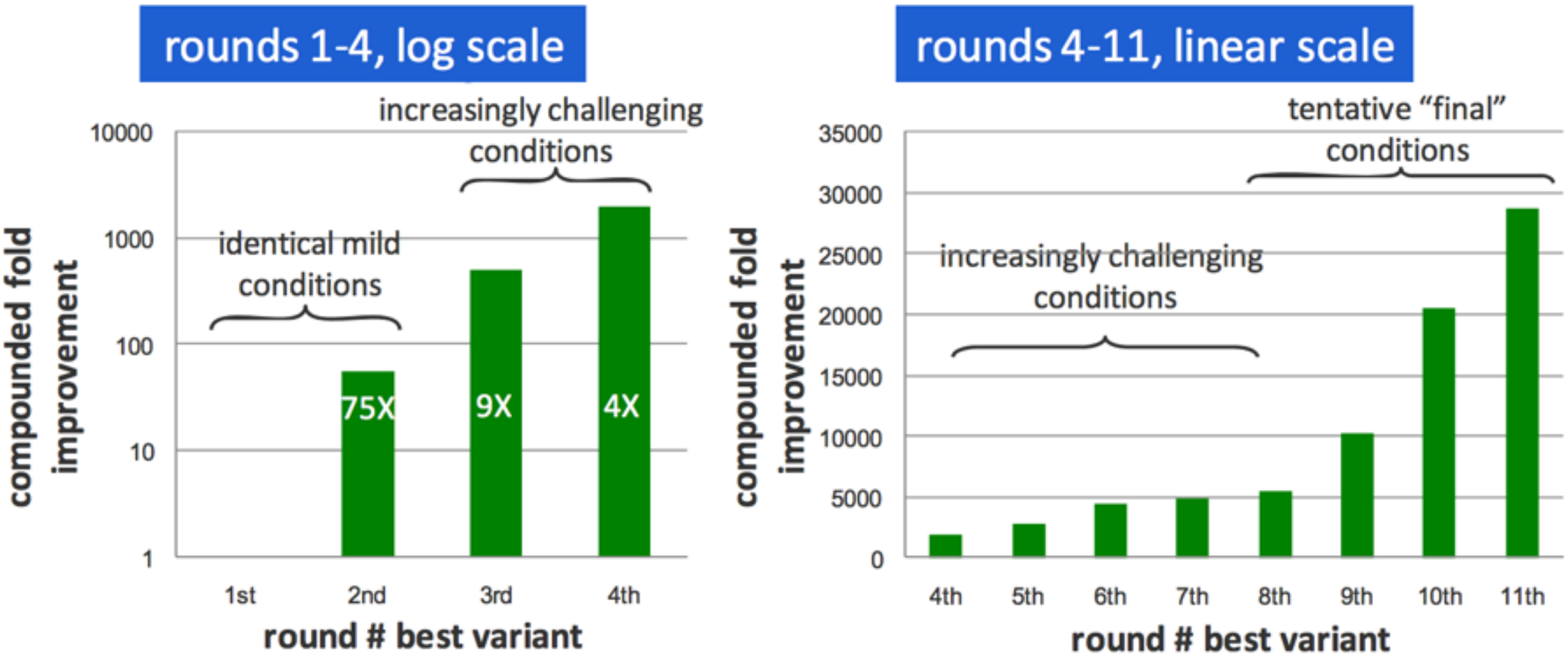
■ Most active mutant subjected to directed evolution for activity and stability under process conditions

Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

Figure S1. Compounded fold improvements identified in high-throughput screening.

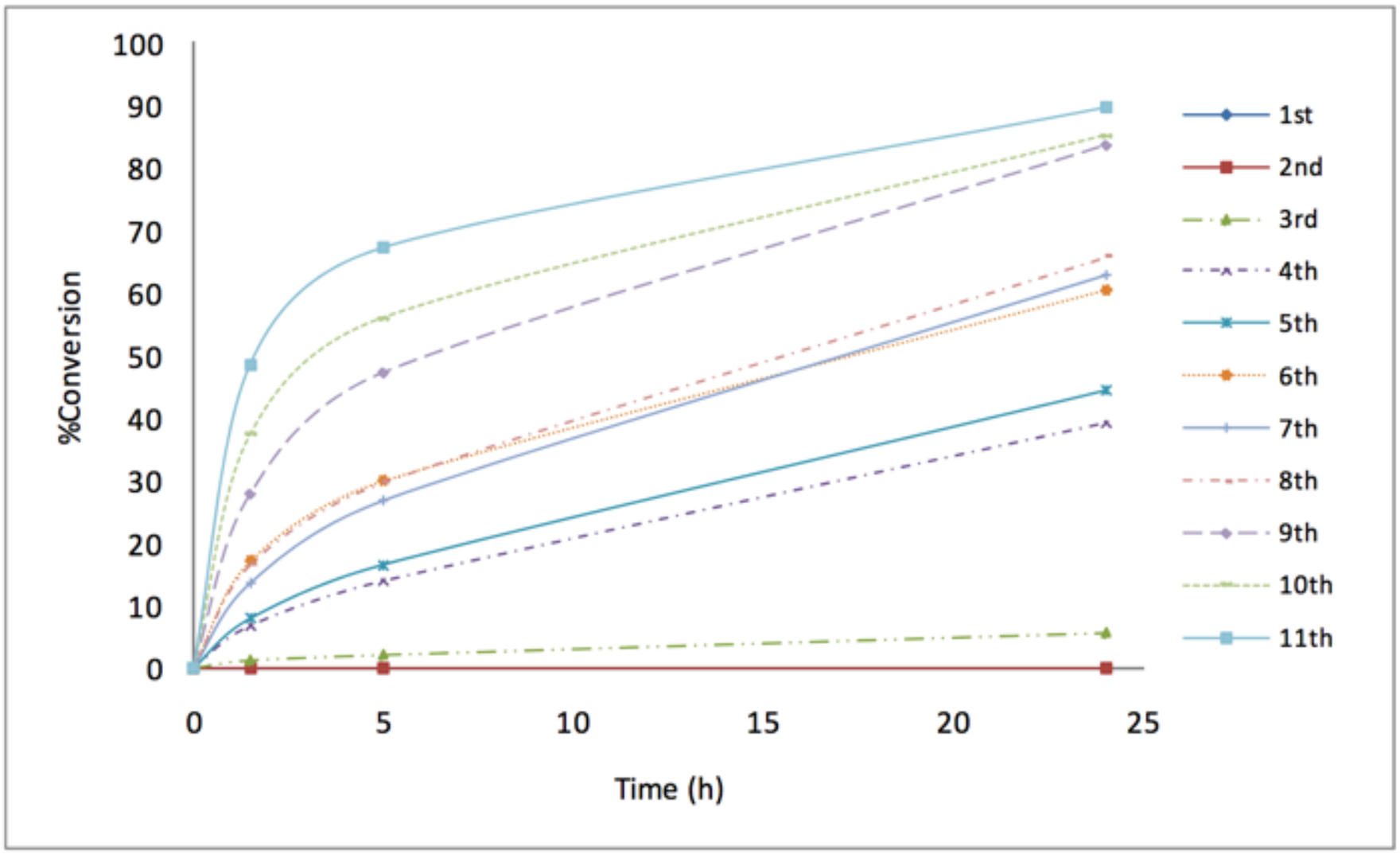


Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

Figure S2. Head-to-head comparison of the top variants from each round of evolution under process-like conditions. The top variants from each round of evolution were compared under identical reaction conditions: 5 g/L enzyme, 50 g/L substrate, 1 M *i*-$PrNH_2$, 1 mM PLP, 50% DMSO in 100 mM triethanolamine, pH 8.5 at 45°C for 24 h without acetone removal or additional pH control.

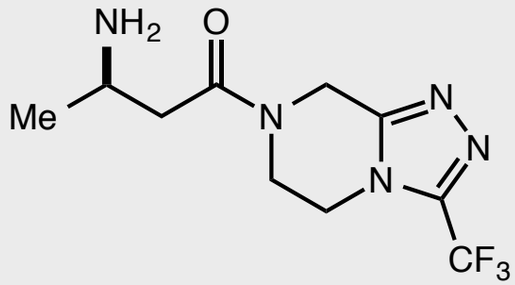


Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

■ Process optimization



Activity on model substrate

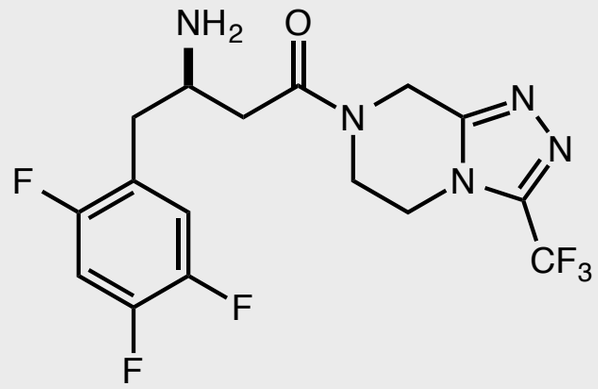
38% conversion

10 g/L enzyme

2 g/L substrate

0.5 M *i*PrNH$_2$

5% DMSO / triethanolamine



Activity on sitagliptin ketone

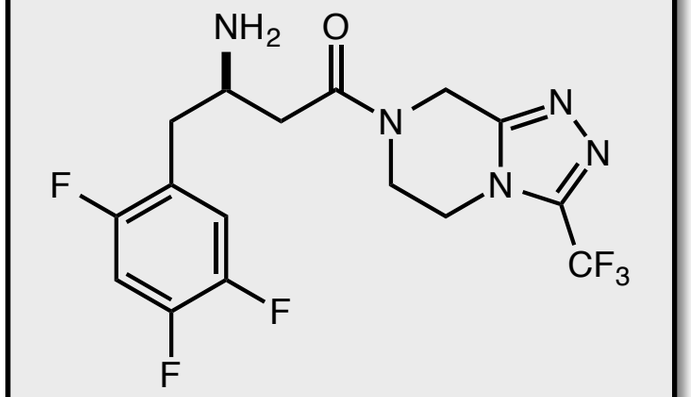
76% conversion

10 g/L enzyme

2 g/L substrate

0.5 M *i*PrNH$_2$

5% DMSO / triethanolamine



Optimized process

92% yield, > 99.9% ee

0.6 g/L enzyme

200 g/L substrate

1 M *i*PrNH$_2$

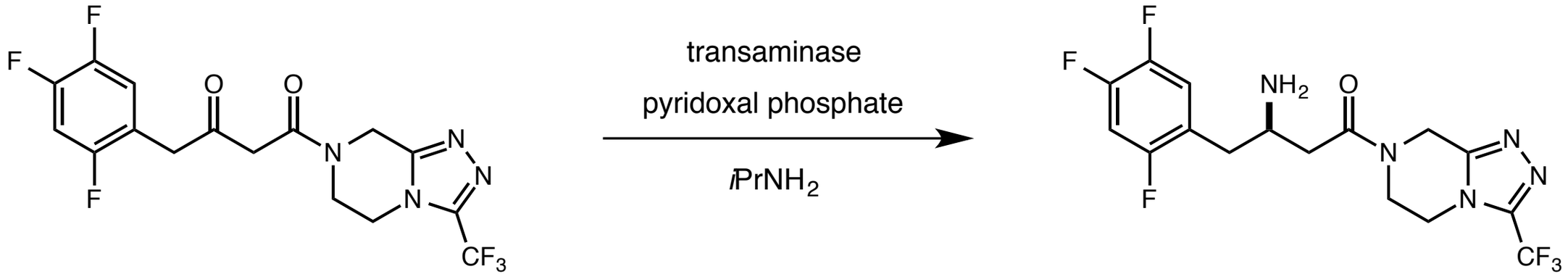
50% DMSO / triethanolamine

Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of sitagliptin

■ Third generation process route



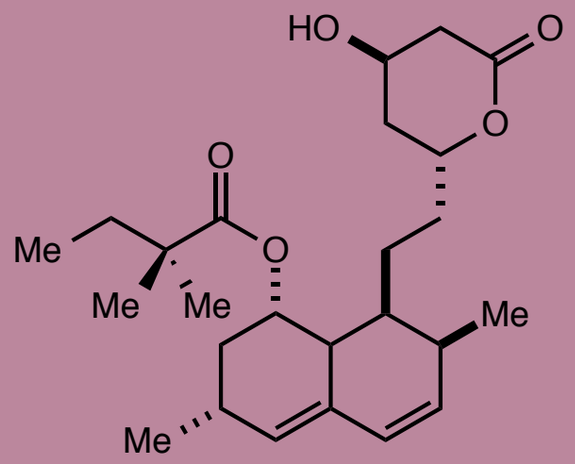
■ Increase in overall yield relative to second generation process: 13%

■ Waste reduction relative to second generation process: 19%

Savile, C. K. et. al. *Science* **2010**, *329*, 305–309.

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin



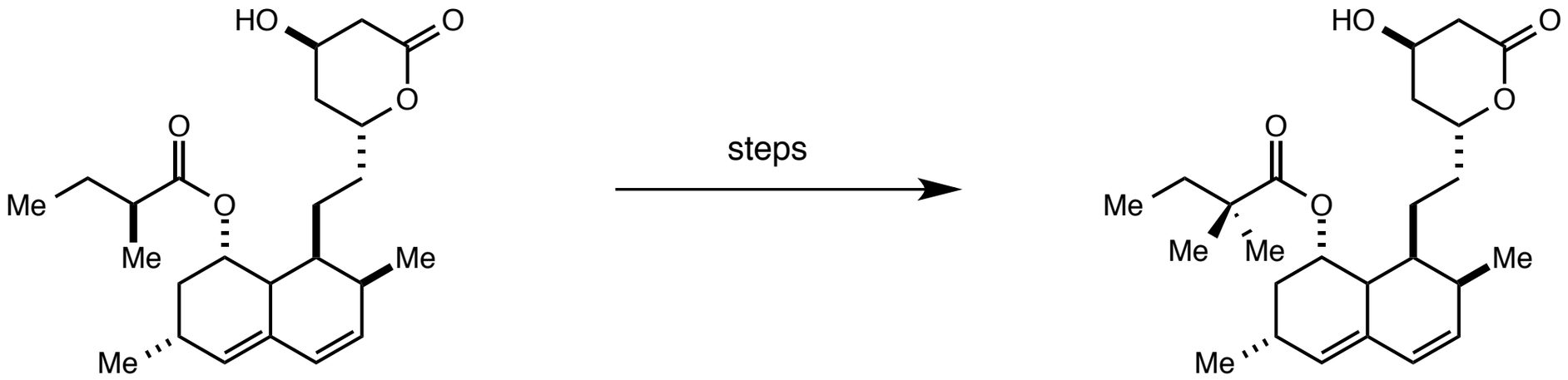
Simvastatin (Zocor)

- Lipid lowering medication - HMG CoA reductase inhibitor
- Developed and marketed by Merck
- Produced by semisynthesis from lovastatin

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

Produced by semisynthesis



Lovastatin

Isolated from *Aspergillus terreus*

Hydroxymethylglutaryl CoA reductase inhibitor

Simvastatin

Increased inhibitory properties

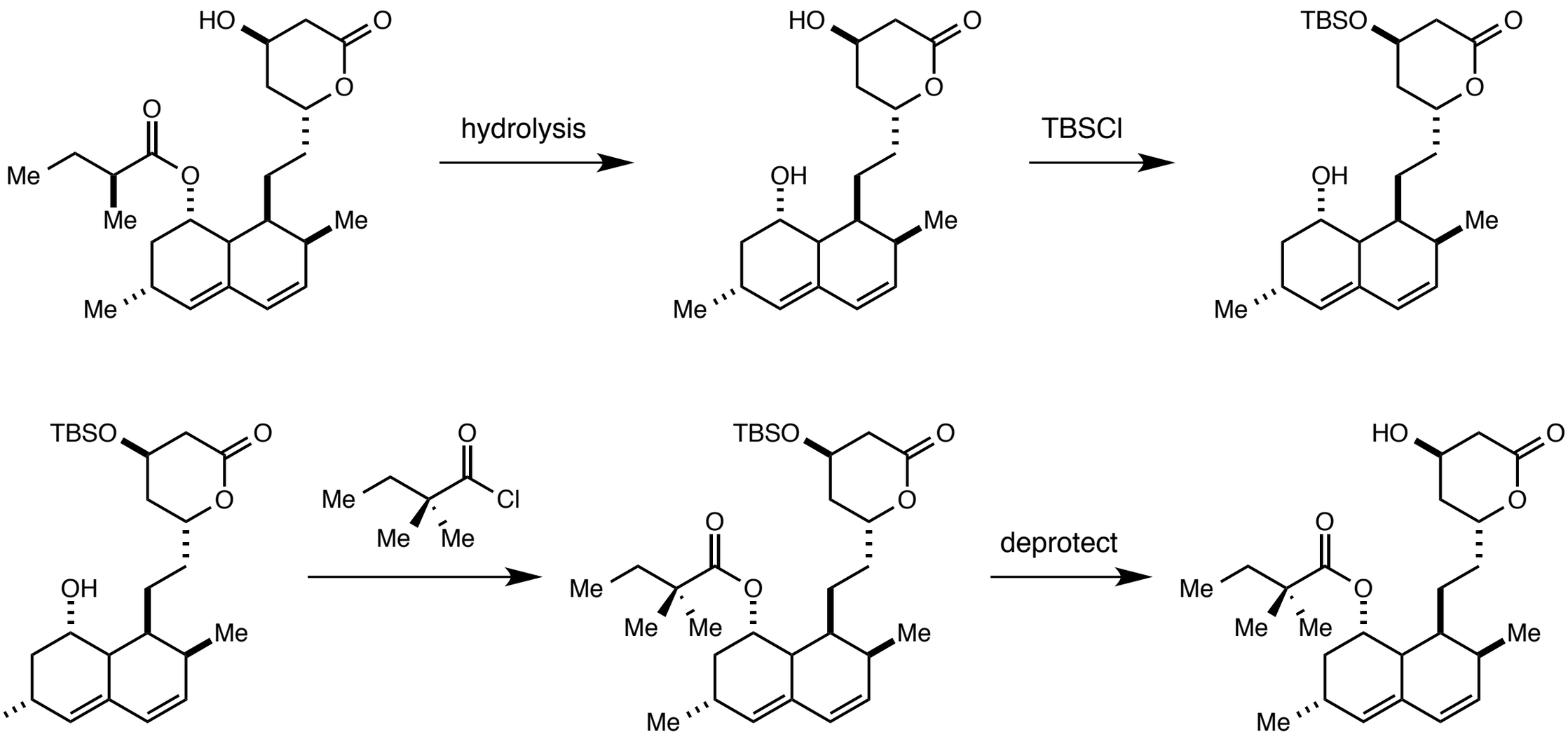
Lower undesirable side effects

Xie, X.; Watanabe, K.; Wojcicki, W. A.; Wang, C. C. C.; Tang, Y. *Chem. Biol.* **2006**, *13*, 1161–1169.

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Typical semisynthetic route

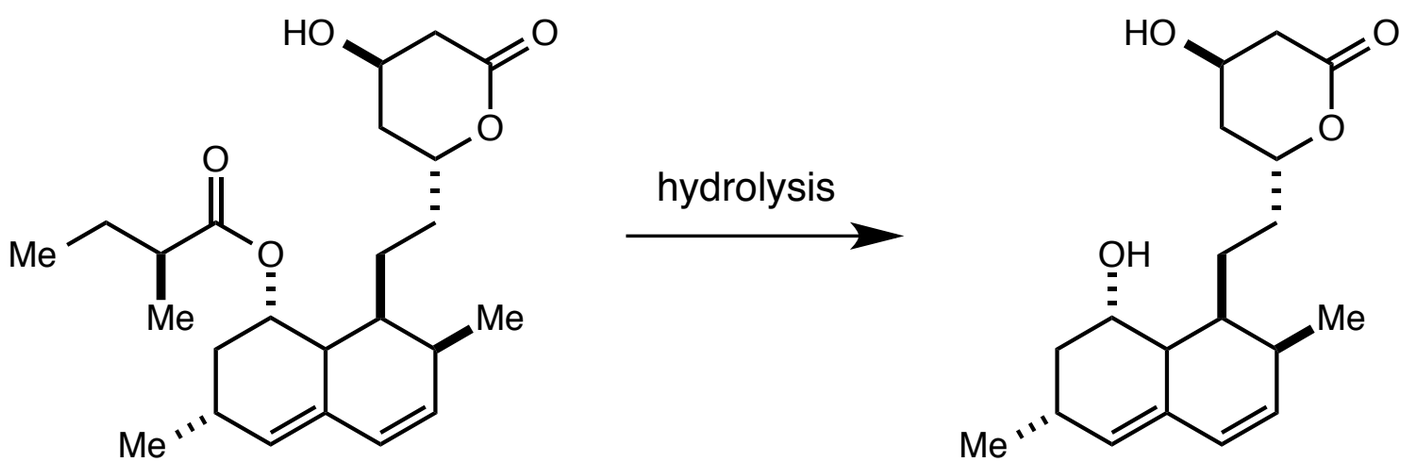


Xie, X.; Watanabe, K.; Wojcicki, W. A.; Wang, C. C. C.; Tang, Y. *Chem. Biol.* **2006**, *13*, 1161–1169.

Protein-Engineered Biocatalysts in Industry

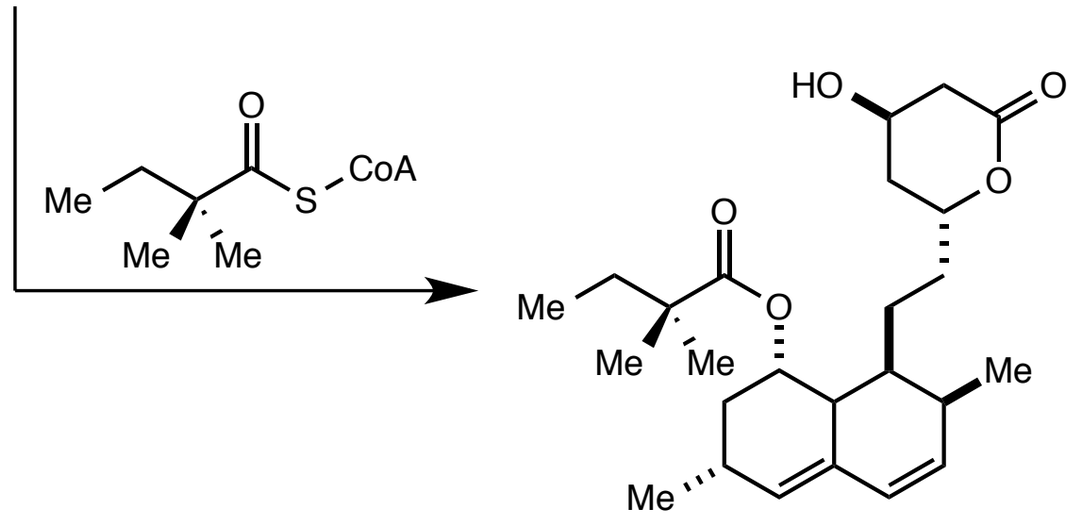
Synthesis of simvastatin

■ Potential synthesis utilizing biocatalysis



Direct enzymatic route:

- *Higher yielding process, cost savings*
- *Remove need for protecting groups*

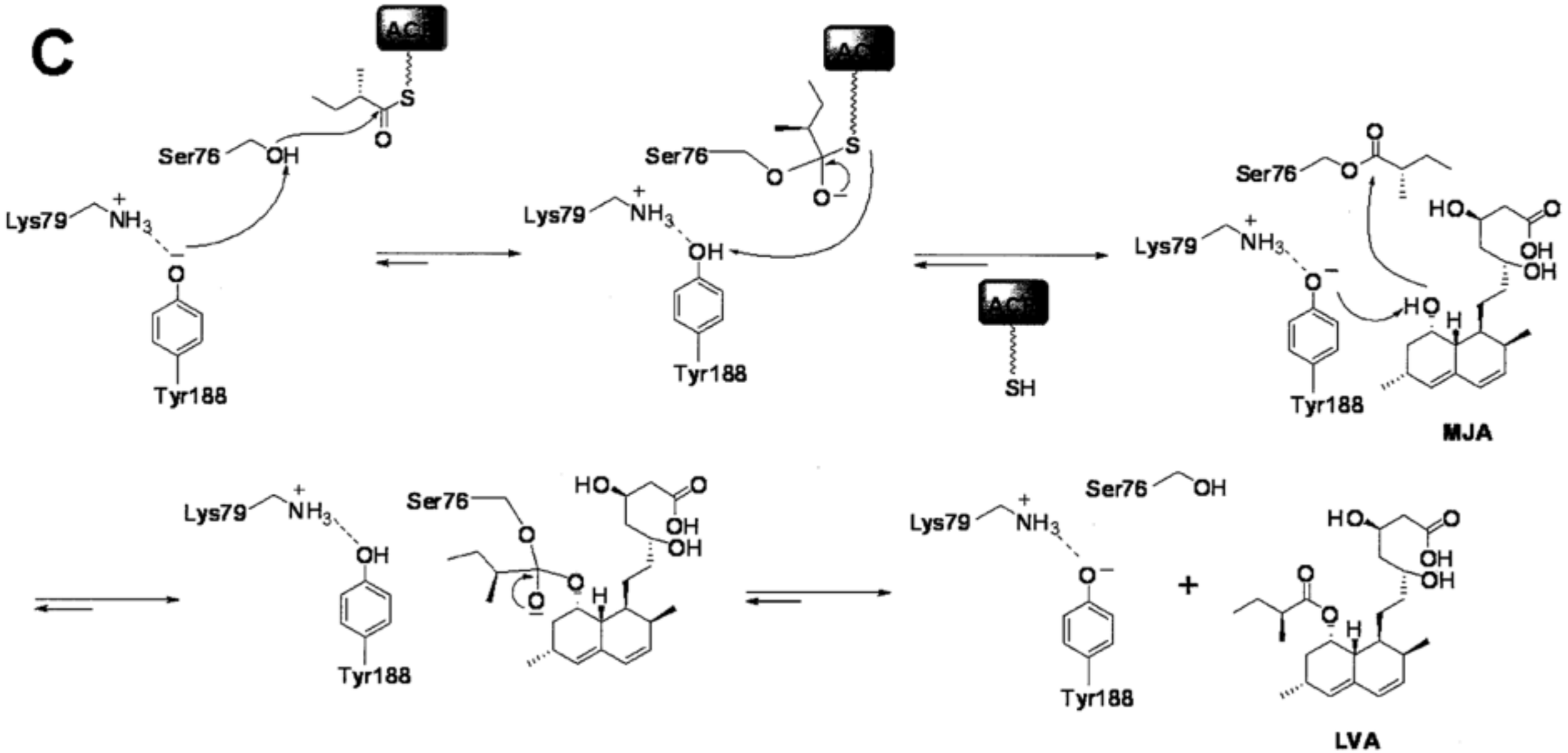


Xie, X.; Watanabe, K.; Wojcicki, W. A.; Wang, C. C. C.; Tang, Y. *Chem. Biol.* **2006**, *13*, 1161–1169.

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Selective biocatalytic acylation reaction

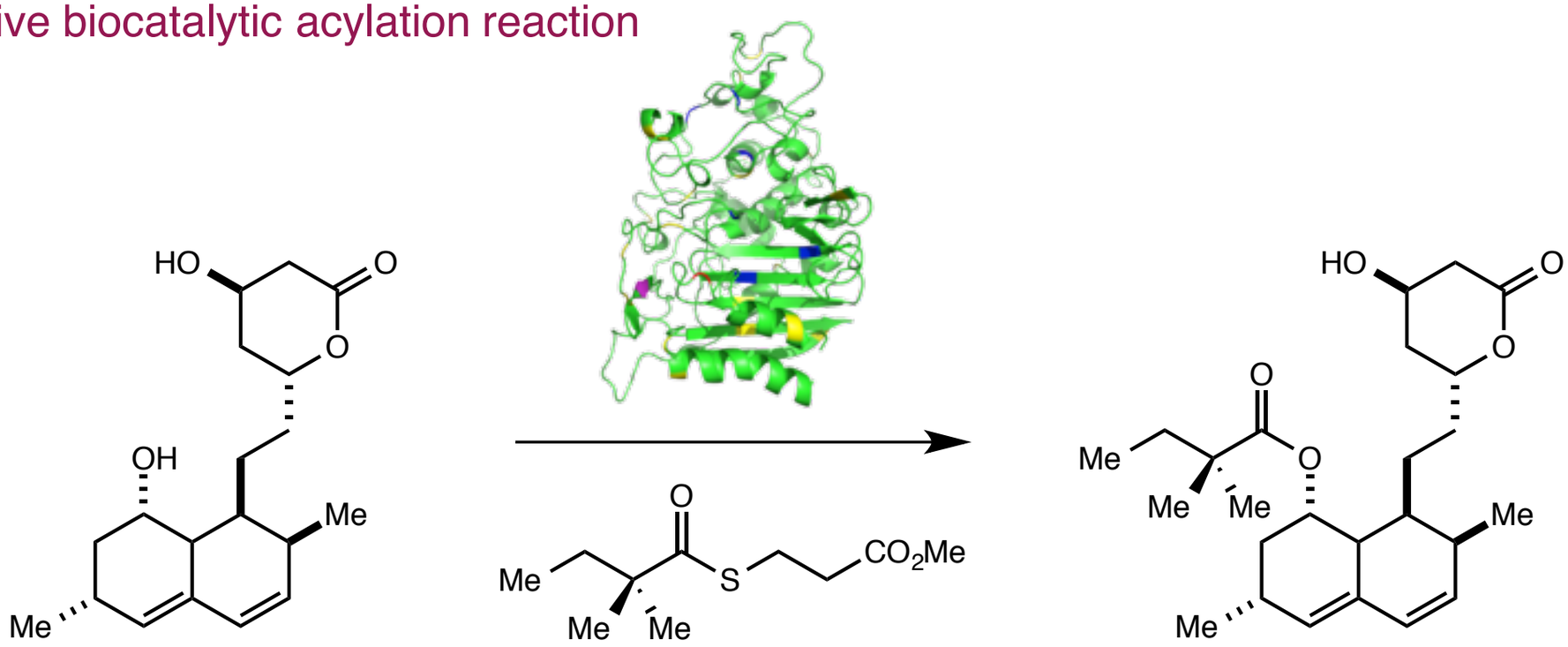


US Patent WO/2011/041231

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Selective biocatalytic acylation reaction



Directed evolution	saturation mutagenesis → error-prone PCR	***Improved thermal stability*** ***Improved acyltransferase activity*** ***Use of a small molecule acyl donor***

Xie, X.; Watanabe, K.; Wojcicki, W. A.; Wang, C. C. C.; Tang, Y. *Chem. Biol.* **2006**, *13*, 1161–1169.

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Directed evolution of acyltransferase

FIG. 13: . Amino acid substitutions and characterization of LovD variants

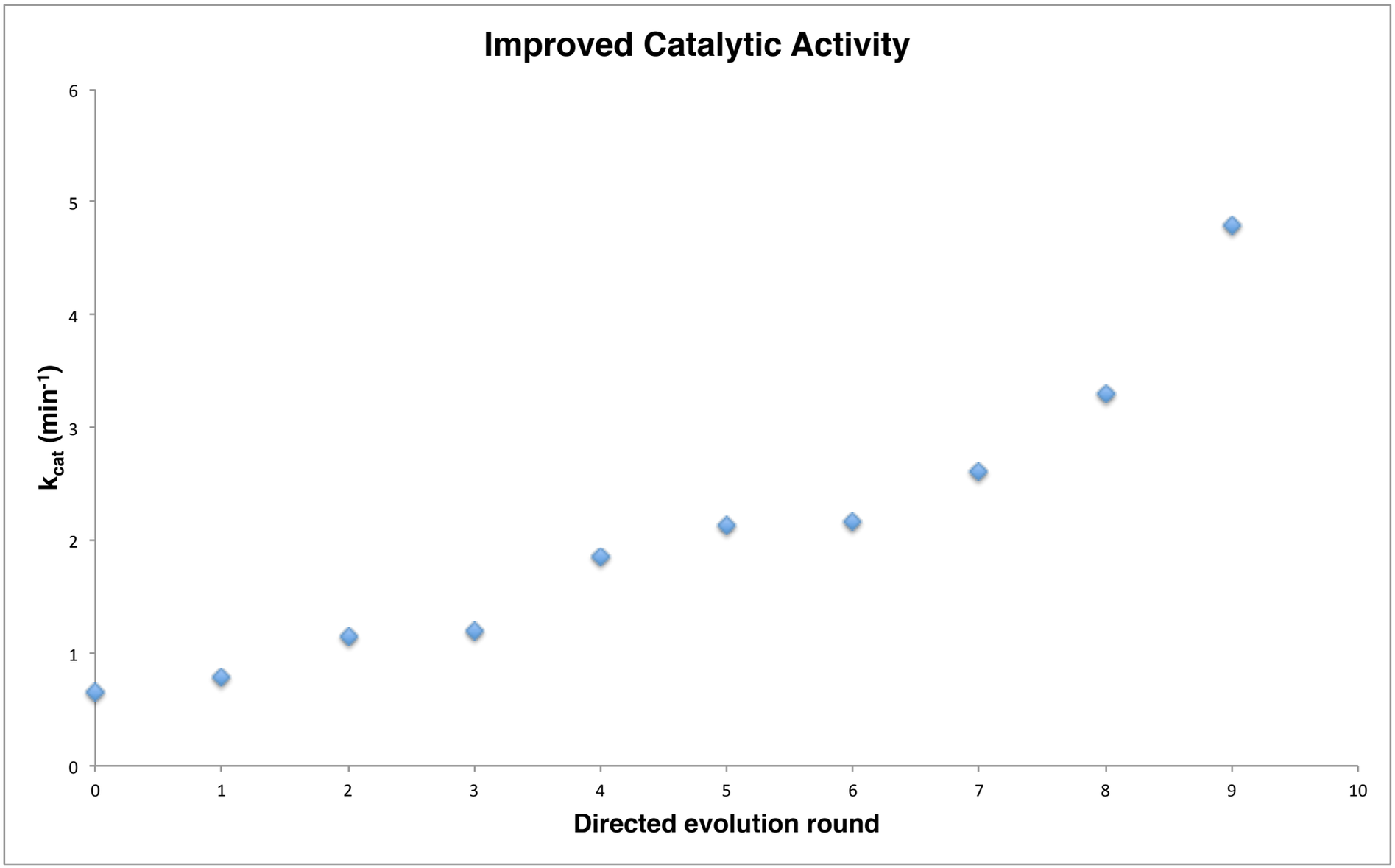
	Mutations	Whole cell Activity*	k_{cat} (min^{-1})	K_M of MJA (mM) †	K_M of DMB-SMMP (mM^{-1})‡	Soluble protein (mg/L)§	T_m(°C) ¶
G0		1	0.66±0.03	0.77±0.17	0.67±0.12	138±11	39.5±0.4
G1	A86V	1.2	0.79±0.03	0.74±0.16	0.66±0.19	140±5.4	41±0.7
G2.1	A86V D12G G275S	1.9	1.14±0.03	0.91±0.17	0.62±0.10	184±8.7	40.5±0.4
G2.2	A86V A190T	1.8	1.20±0.09	0.74±0.21	0.69±0.17	168±17	41±0.4
G3	A86V D12G A190T G275S	3.6	1.86±0.09	0.77±0.11	0.70±0.19	205±23	41±0.4
G4.1	A86V D12G A190T G275S A10V K26E	4.8	2.13±0.03	0.70±0.24	0.66±0.16	183±18	43.5±0.7
G4.2	A86V D12G A190T G275S H161Y K227R	5.2	2.16±0.12	0.80±0.24	0.64±0.16	221±9.3	42.5±1.9
G5	A86V D12G A190T G275S K26E H161Y	6.4	2.61±0.03	0.74±0.03	0.69±0.14	206±5.7	46.5±0.4
G6	A86V D12G A190T G275S K26E H161Y V334D L361M	9.3	3.30±0.06	0.70±0.07	0.63±0.15	212±3.9	47±0.1
G7	A86V D12G A190T G275S K26E H161Y V334F	11.2	4.80±0.06	0.70±0.04	0.69±0.17	214±6.3	48.5±0.7

US Patent WO/2011/041231

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

- Directed evolution of acyltransferase

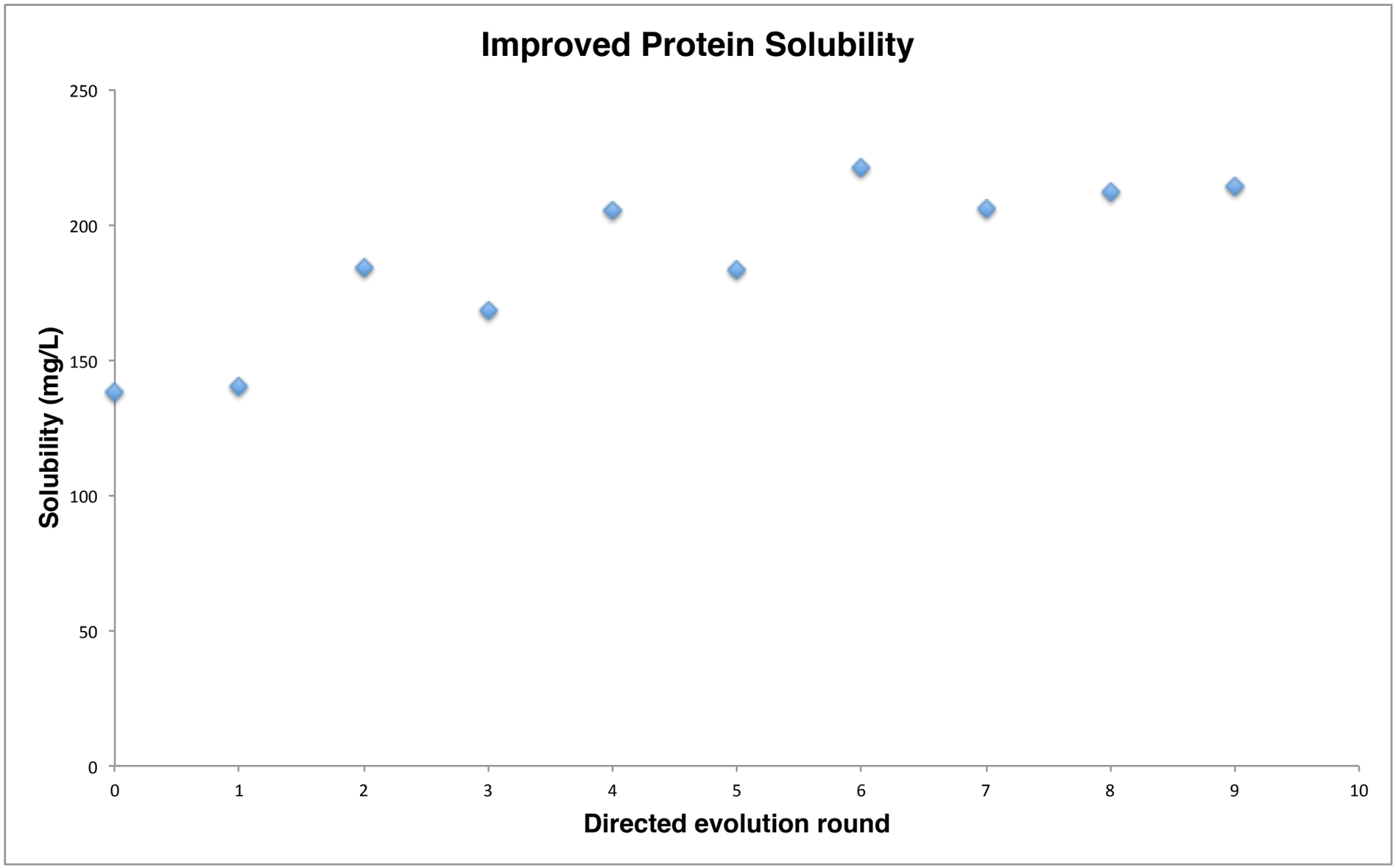


US Patent WO/2011/041231

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Directed evolution of acyltransferase

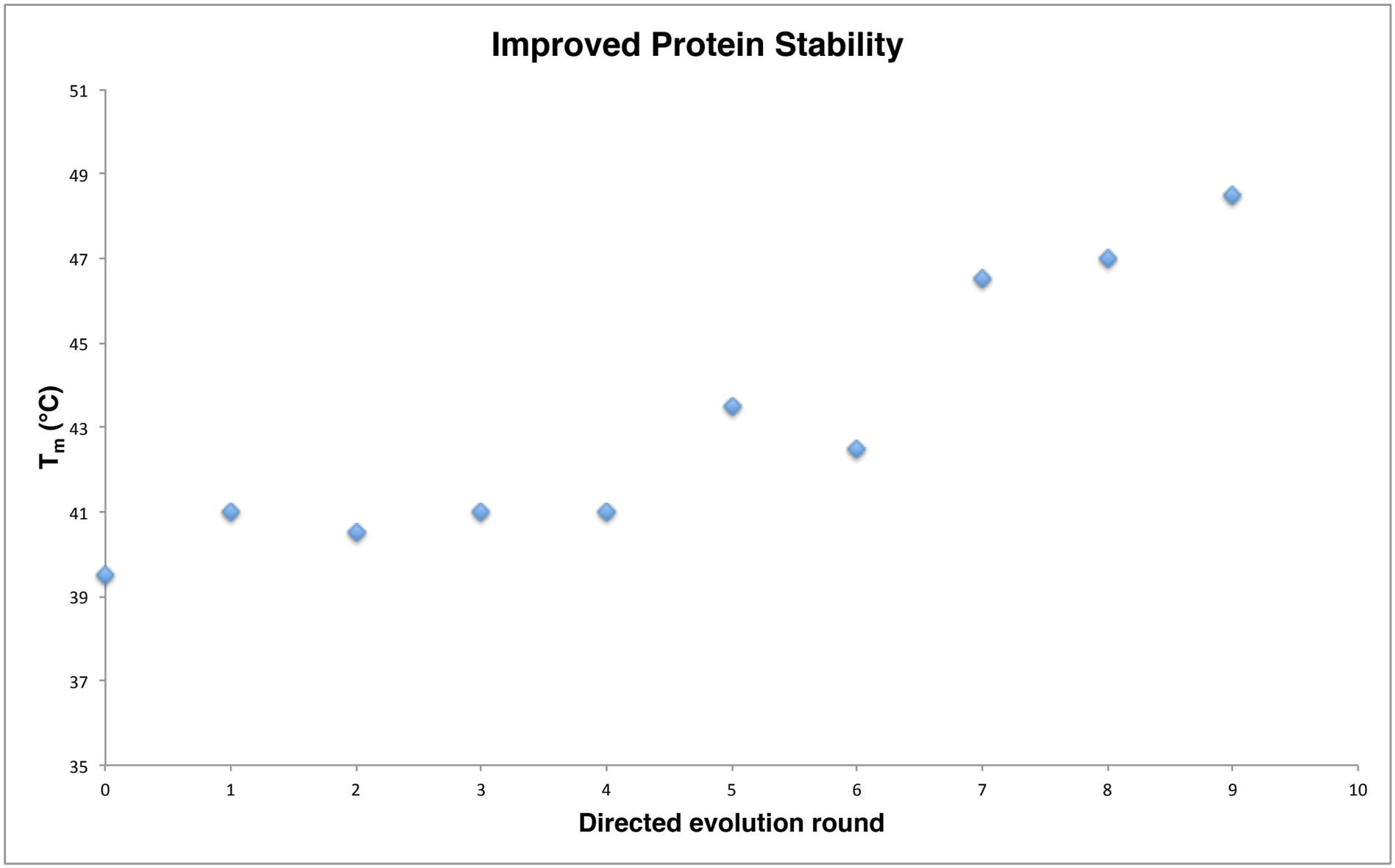


US Patent WO/2011/041231

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Directed evolution of acyltransferase

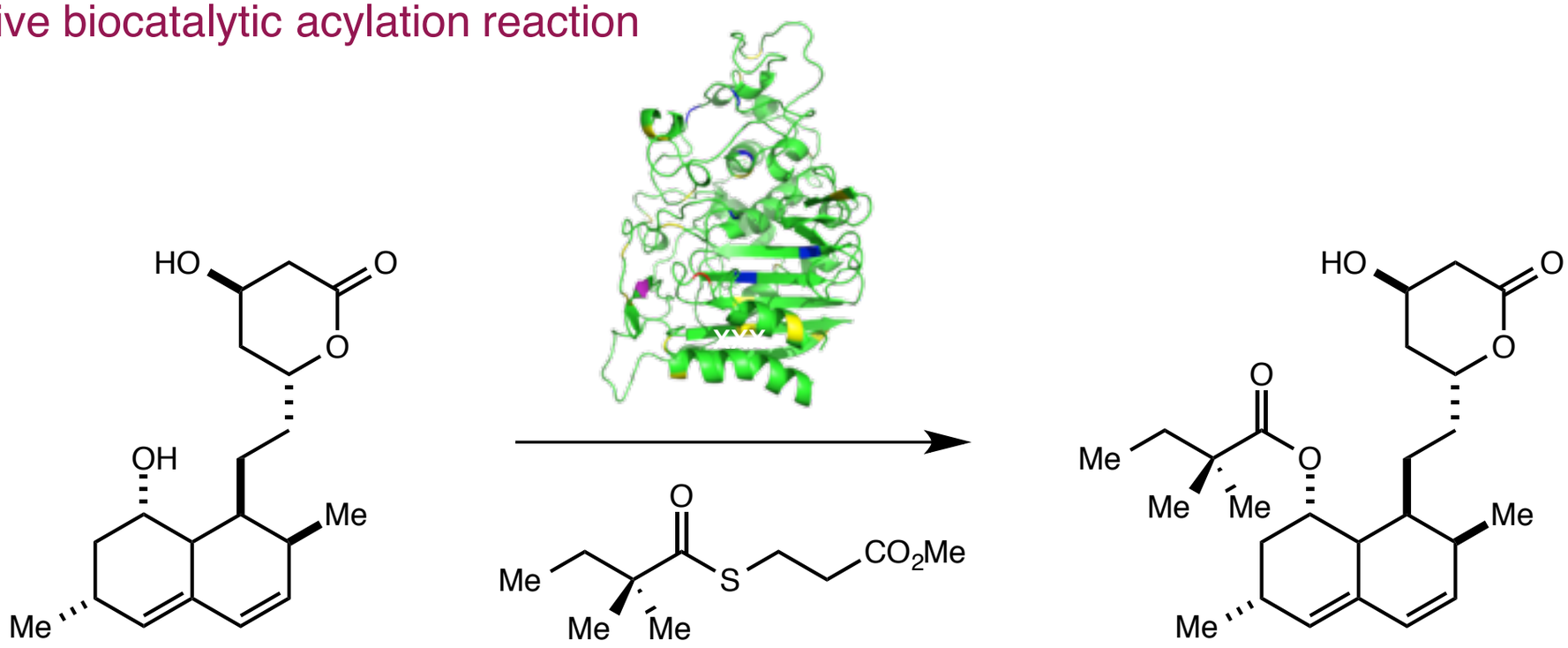


US Patent WO/2011/041231

Protein-Engineered Biocatalysts in Industry

Synthesis of simvastatin

■ Selective biocatalytic acylation reaction



■ Reduced use of hazardous substances and solvents in synthesis

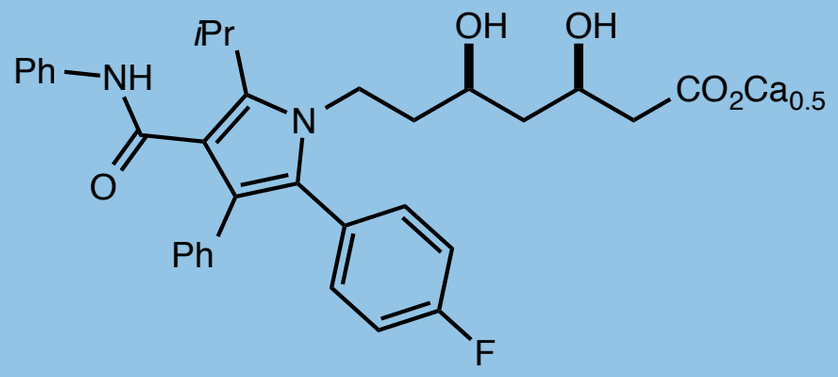
■ Catalyst from renewable feedstocks, byproducts all recycled

■ Produced in 97% yield compared to < 70% for previous processes

Xie, X.; Watanabe, K.; Wojcicki, W. A.; Wang, C. C. C.; Tang, Y. *Chem. Biol.* **2006**, *13*, 1161–1169.

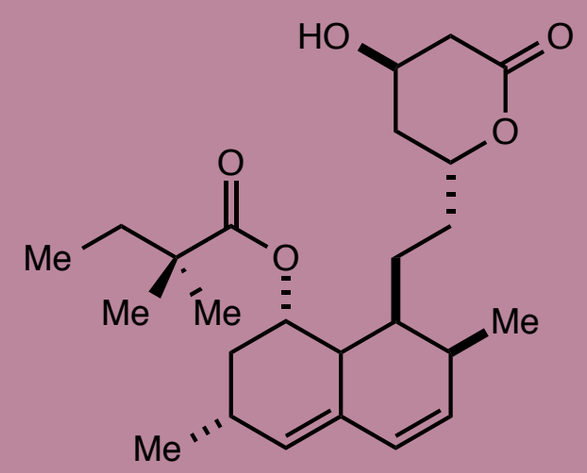
Protein-Engineered Biocatalysts in Industry

Case studies



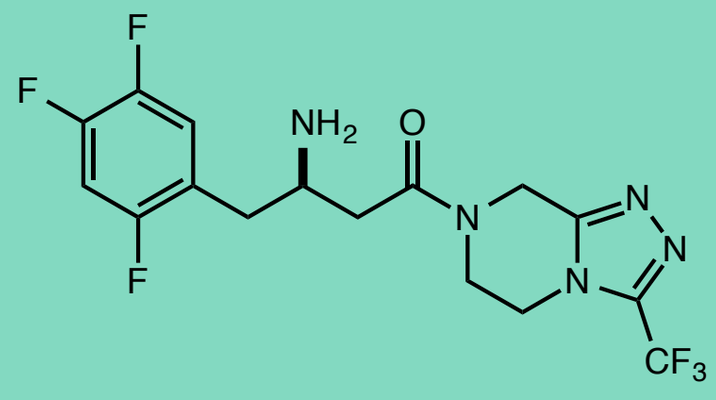
Atorvastatin (Lipitor)

EPA Presidential Green Chemistry Award 2006



Simvastatin (Zocor)

EPA Presidential Green Chemistry Award 2012



Sitagliptin (Januvia)

EPA Presidential Green Chemistry Award 2010