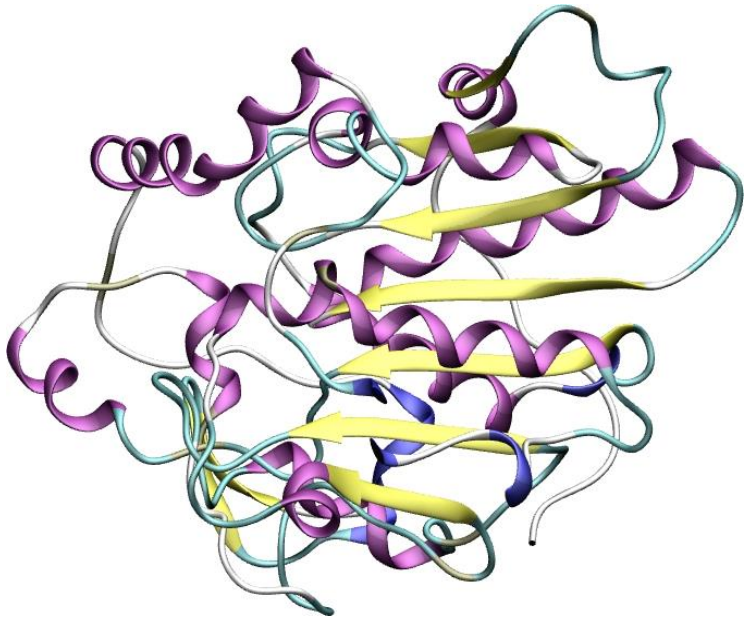
Biocatalysis for pharmaceuticals



Classification of enzymes used in organic synthesis.

Class	Enzyme	Common reaction
1. Oxidoreductases	Dehydrogenases	Oxidation of **alcohols** and aldehydes, reduction of **aldehydes and ketones**; oxidation of **C-C** single bonds, reduction of **C=C** double bonds
	Oxidases	Oxidation of alcohols and amines
	Mono- and dioxygenases	Hydroxylation, sulphoxidation, epoxidation, Baeyer-Villiger oxidation,
	Peroxidases	Oxidation, epoxidation, halohydration
2. Transferases	Kinases	Phosphorylation (ATP-dependent)
	Sulphotransferases	Formation of sulphate esters
	Glycosyltransferases	Glycosidic bond formation
	Transketolases	Ketol (α-hydroxyketones) group transfer

Class	Enzyme	Common reaction
3. Hydrolases	Esterase, lipases	Ester hydrolysis / synthesis
	Amidohydrolases (amidases or acylases)	Amide hydrolysis / synthesis
	Proteases	Peptide bond hydrolysis / synthesis
	Glycosidases	Glycosidic bond formation/hydrolysis
	Nitrilase (nitrile aminohydrolase)	Hydrolysis of nitrile to carboxylate
	Epoxide hydrolases	Hydrolysis of epoxides
	Phosphatases	Hydrolysis of phosphate esters
	Dehalogenases	C-halide hydrolysis

Class	Enzyme	Common reaction
4. Lyases	Aldolases	Aldol reaction (C–C bond)
	Oxynitrilase	Cyanohydrine formation
5. Isomerases	Glucose isomerase	Isomerisation of carbohydrates,
	Mandelate racemase	Racemisation
6. Ligases		Not used at present for practical applications

Main coenzymes required by enzymes used in biocatalysis

Coenzyme	Reaction type
Flavines	Oxygenation
Thiamine pyrophosphate	Decarboxylation, transchetolization
$NAD(P)^+$ /NAD(P)H	Hydrogenation/dehydrogenation
$NAD(P)^+$ /NAD(P)H	Oxygenation
ATP	Phosphorylation
Pyridoxal- phosphate	Modification of aminoacids
Metal-phorphyrin complexes	Peroxidation, oxygenation

Several cofactors can be recycled effectively, including nucleoside triphosphates such as ATP in phosphoryl transfer reactions, (NAD^+/NADH and $NADP^+$/NADPH) in oxidoreductions, acetylCoA in acyl transfer reactions, and sugar nucleotides in glycosyl transfer reactions.

Many cofactor dependent reactions have been applied on preparative or industrial scales.

Nevertheless, hydrolases still remain the most widely employed enzymes due to their large availability and to the fact that they do not need organic coenzymes.

CHIRALITY AND PHARMACEUTICALS

Chirality in biologically active molecules is of natural occurrence. Traditionally, it was common practice for a pharmaceutical company to market a chiral drug as the racemate, and as recently as 1985, more than 75% of chiral drugs were sold as the racemate.

This policy implied that each dose of a drug is contaminated with an equal amount of an isomer, which usually has no therapeutic value but may have the potential to cause unsuspected deleterious side effects.

Review TRENDS in Biotechnology Vol.25 No.2

Biocatalysis for pharmaceutical intermediates: the future is now

David J. Pollard[1] and John M. Woodley[2]

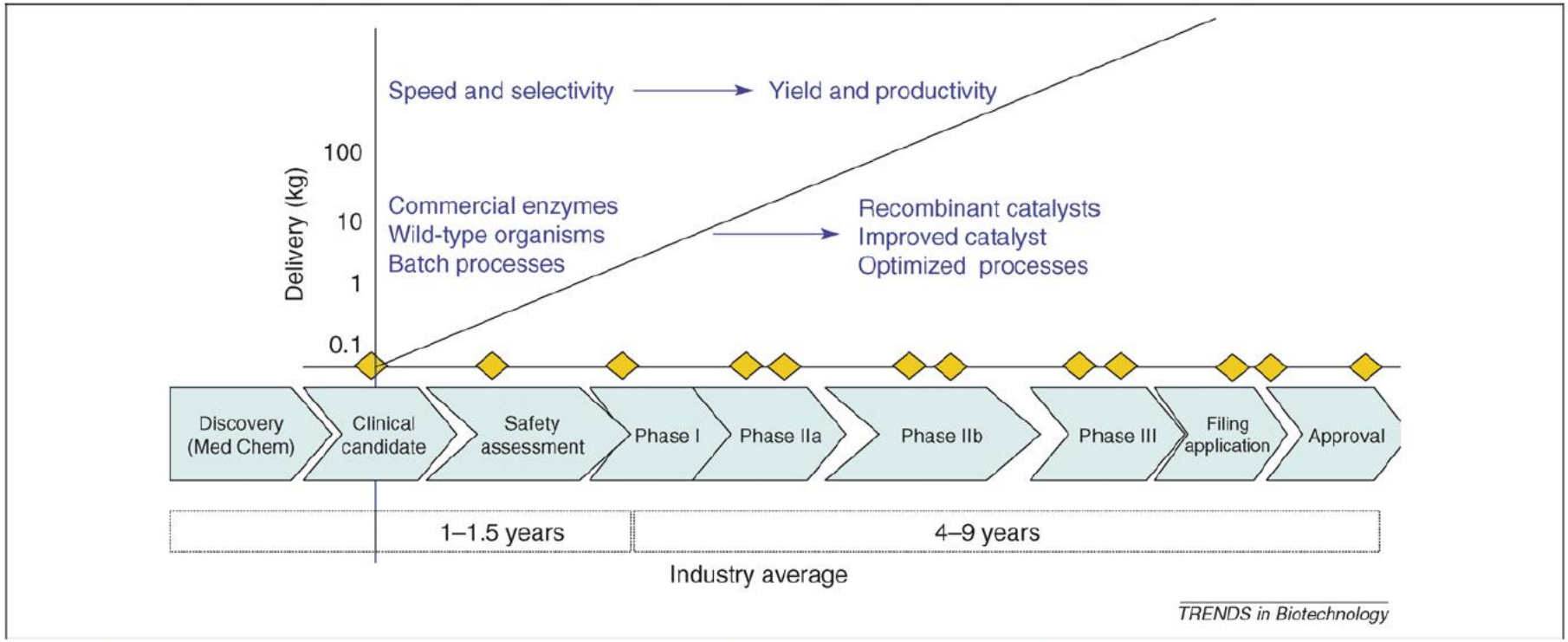
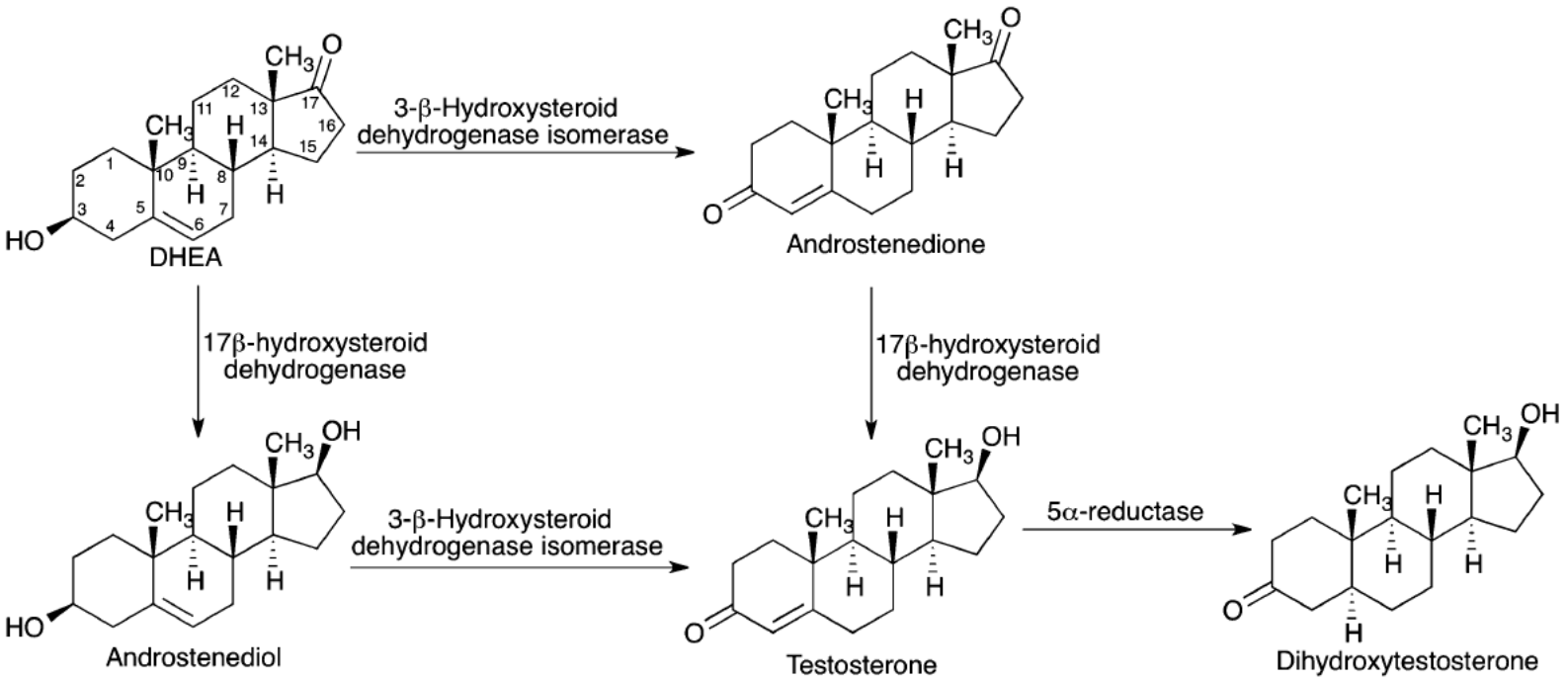


Figure 2. Development timeline for pharmaceutical products and processes. Initial development requires rapid synthesis of compounds to provide material for safety assessment. Later stage enables development time for optimized processes using recombinant catalysts. Abbreviation: Med Chem, medicinal chemistry.

Some classical examples of aplications of biocatalysts in the synthesis of drugs at industrial scale

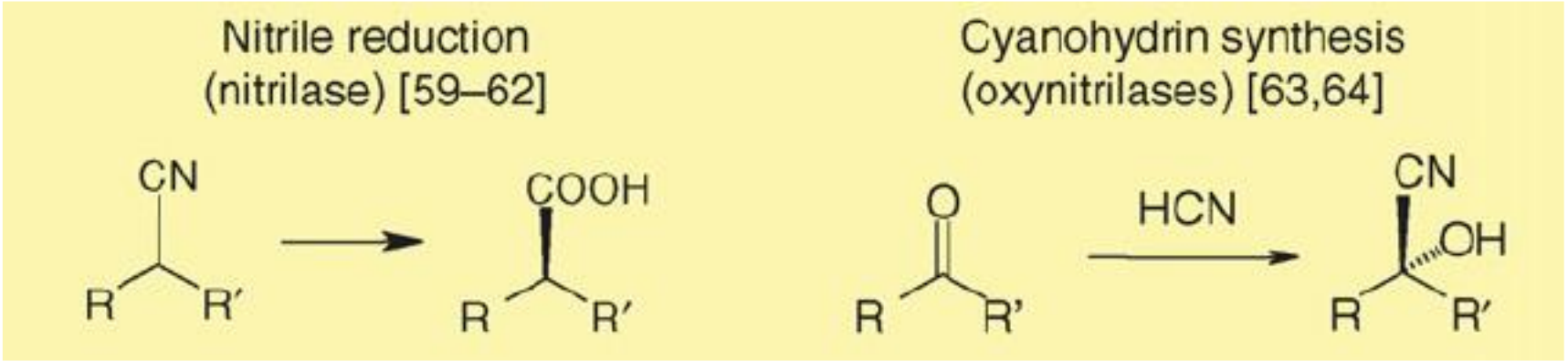
Steroids synthesis

DHEA (deidroepiandrosterone), natural steroid (max in humans 15-20 anni)
Precursor of estrogens and androgens
produced from natural vegetal sources (tropical plant)

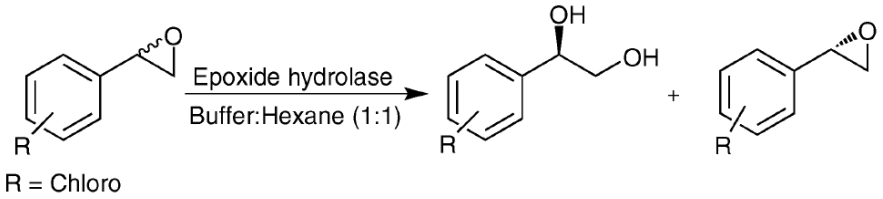


used as a radiation countermeasure: stimulates white cells and platelets production

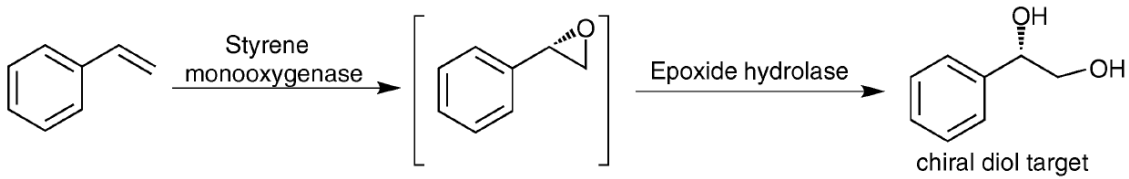
Playing with nitrile group



Playing with epoxides



Scheme 6 Biocatalytic kinetic resolution of racemic 2-, 3-, and 4-chlorostyrene oxides using epoxide hydrolase.

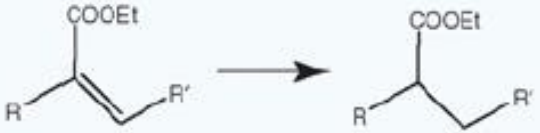


Scheme 7 Asymmetric dihydroxylation of an aryl olefin *via* a tandem monooxygenase and epoxide hydrolase biocatalysis approach.

Emerging chemistries

Transamination (transaminase) [53,65,66]

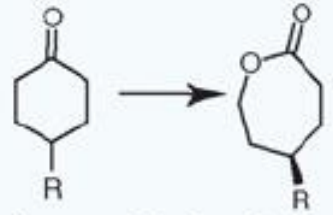
Enoate reduction (enoate reductase) [67–69]



Hydroxylation (cytochrome P450) [70,71]

Dihdroxylation (cytochrome P450) [72,73]

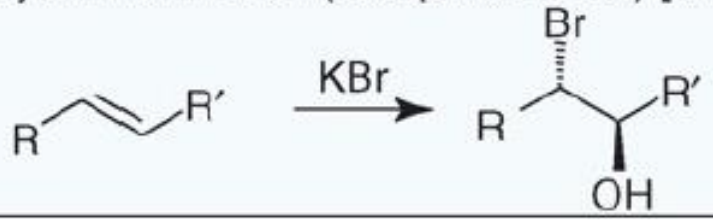
Baeyer-Villiger (monooxygenase) [74]



Hydrolysis (epoxide hydrolase) [75–77]

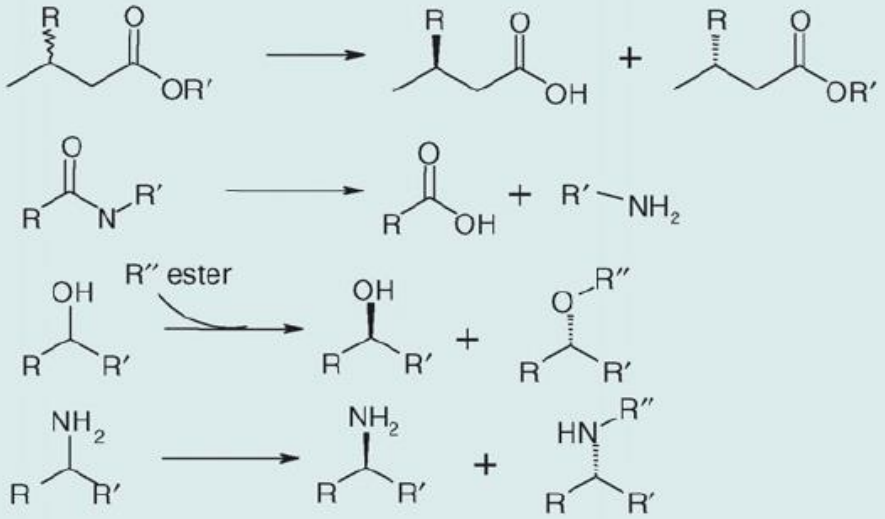
Epoxidation (haloperoxidase, cytochrome P450) [78–80]

Halohydrin formation (haloperoxidase) [81–82]



Established chemistries

Resolution (lipase/protease) [20,21,34,56]



Hydrolysis (lipase/protease) [56,57]

Ketone reduction (ketoreductase) [49–52,58]

Classical resolution in chemistry **is based on formation of diastereomers, by addition of a pure enantiomer (from the chirality pool) to the racemate solution, which can be separated by crystallization.**

To date it has been the most commonly used industrial technique.

B –biotecnol * chyral

§ not chyral

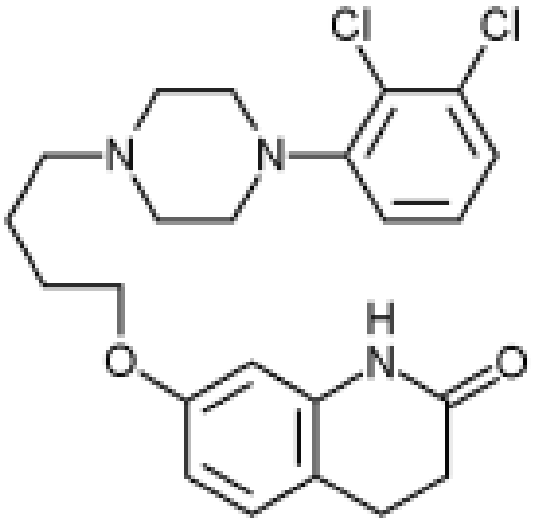
Top drugs 2011-13

2011		2012		2013	
Rank	Drug	Rank	Drug	Rank	Drug
1	*Lipitor* *	1	Nexium *	1	Abilify §
2	*Plavix* *	2	Abilify §	2	Nexium *
3	Nexium *	3	Crestor *	3	Cymbalta *
4	Abilify §	4	Advair Diskus*	4	Humira B
5	Advair Diskus*	5	Cymbalta *	5	Crestor *
6	*Seroquel* B	6	Humira B	6	Advair Diskus *
7	*Singulair* *	7	Enbrel B	7	Enbrel B
8	Crestor *	8	Remicade B	8	Remicade B
9	Cymbalta *	9	Copaxone *	9	Copaxone *
10	Humira B	10	Neulasta B	10	Neulasta B

Abilify (achiral)

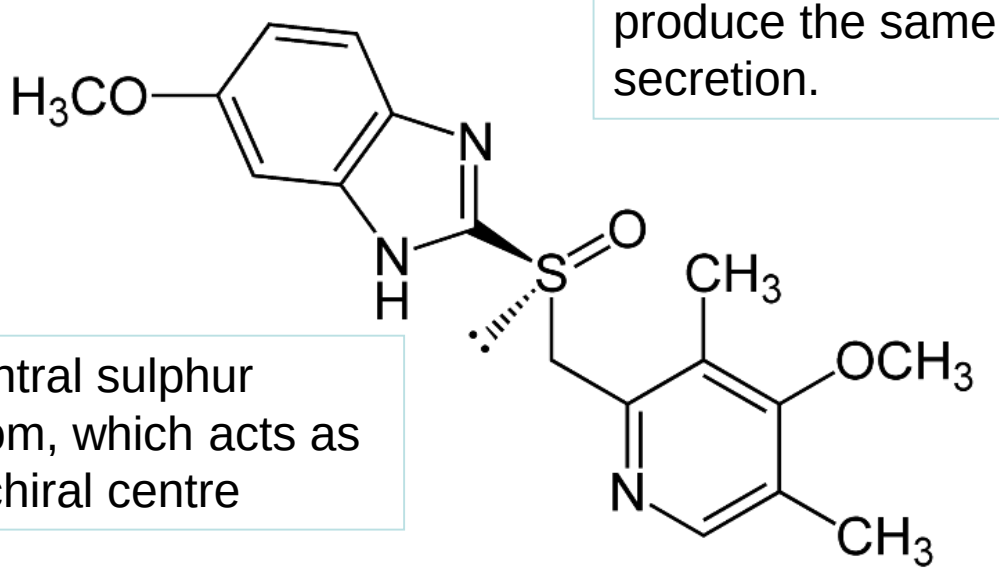
ARIPRIPAZOLE:

atypical antipsychotic
schizophrenia
bipolar disorder
major depressive disorder
irritability associated with autism



Nexium
S-isomer, **esomeprazole**

At the cellular level, both of these isomers convert to the same inhibitor of the H+,K+-ATPase (proton pump inhibition) and produce the same reduction in gastric acid secretion.

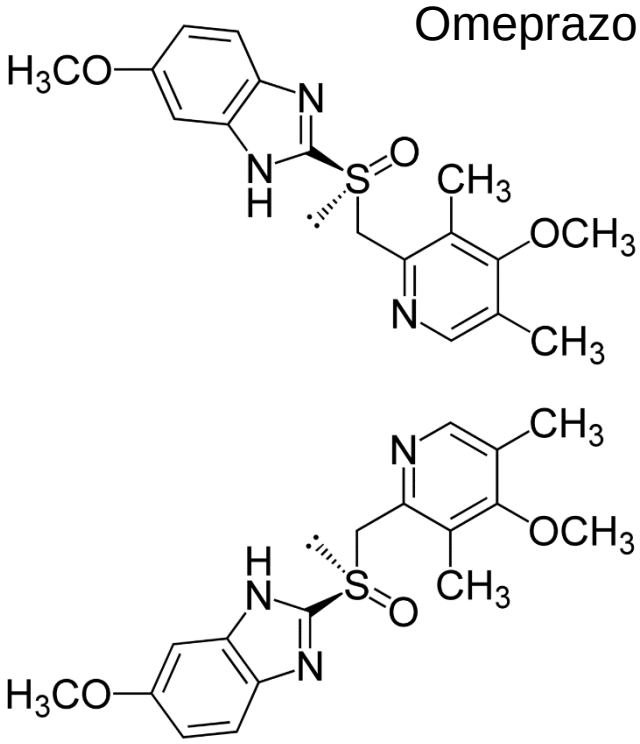


central sulphur atom, which acts as a chiral centre

Obtained via asymmetric oxidation using Ti based catalyst (94% ee)

S-isomer, **esomeprazole,** is metabolized more slowly and reproducibly than the R-isomer and therefore produces higher plasma concentrations for longer and, as a result, inhibits gastric acid production more effectively and for longer. Thus, esomeprazole has the pharmacological properties of a more effective form of treatment for disorders related to gastric acid secretion

Omeprazole is a racemate.

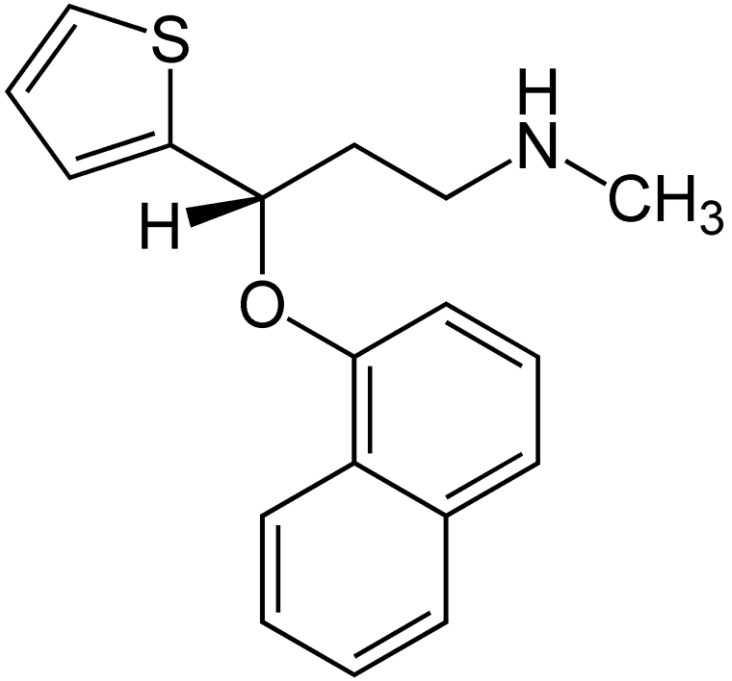


Cymbalta

La duloxetine

prescribed for major depressive disorder, generalized anxiety disorder, fibromyalgia and neuropathic pain

Cymbalta generated sales of nearly $5 billion in 2012 with $4 billion of that in the U.S., but its patent protection terminated January 1, 2014. Lilly received a six-month extension beyond June 30, 2013 after testing for the treatment of depression in adolescents, which may produce $1.5 billion in added sales.

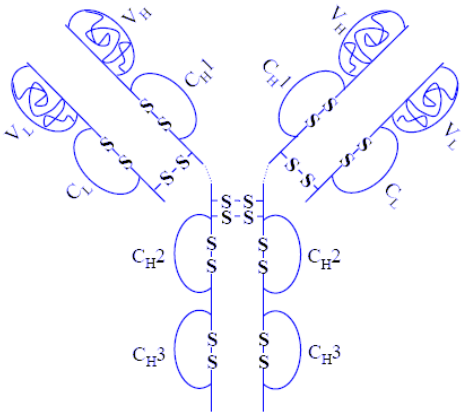


Process for preparation of duloxetine hydrochloride
US 8269023 B2

•An improved process for synthesis of duloxetine hydrochloride (1) having chiral purity greater than 99.9% that is characterized by the following: (i) preparation of racemic condensed compound (RS)—N,N-di methyl-3-(1-naphthyloxy)-3-(2-thienyl)propanamine (4) by reaction of racemic hydroxy compound (2) with 1-fluoronaphthalene (3) in presence of a base such as sodamide, potassium amide or potassium bis(trimethylsilyl)amide (KHDMS) in polar aprotic solvent,
•(ii) optical resolution of racemic condensed compound (5a+5b) with di-benzoyl-L-tartaric acid (7, DBTA, R=H) or di-para-anisoyl-L-tartaric acid (7, DATA, R=OCH_3) to obtain crude (S)—N.N-dimethyl-3-(1-naphthyloxy)-3-(2-thienyl)propanamine dibenzoyl tartarate salt (8a) or (S)—N.N-dimethyl-3-(1-naphthyloxy)-3-(2-thienyl)propanamine di-p-anisoyl tartarate salt (9a) respectively,
•(iii) optionally purification of crude tartarate salts (8a or 9a) by crystallization,
•(iv) optionally purification of duloxetine hydrochloride (1) by crystallization and
•(v) racemization of undesired (R)—N,N-di methyl-3-(1-naphthyloxy)-3-(2-thienyl)propanamine (5b) by treatment with base potassium bis(trimethylsilyl)amide (KHDMS) to obtain racemic mixture of condensed compounds (5a and 5b).

Humira

Adalimumab is a monoclonal antibody used to treat the symptoms and prevent the progression of active rheumatoid arthritis and ankylosing spondylitis. It is used in children 2 years of age and older for juvenile idiopathic arthritis. This medicine is also used to treat psoriatic arthritis, which is a type of arthritis that causes pain and swelling

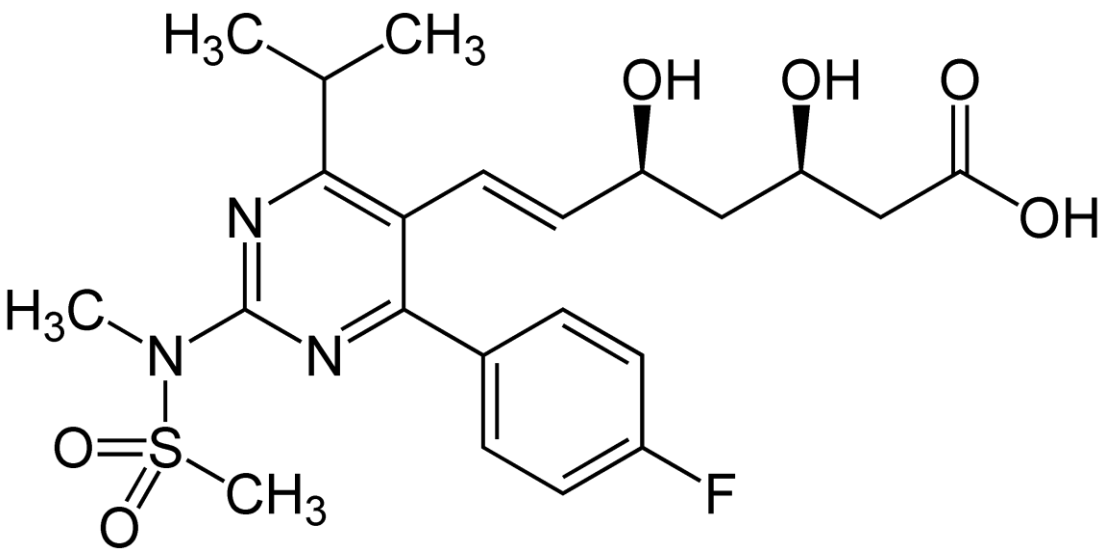


It binds to tumor necrosis factor-alpha (TNFα), which normally binds to TNFα receptors, leading to the inflammatory response of autoimmune diseases. By binding to TNFα, adalimumab reduces this inflammatory response. Because TNFα is also part of the immune system, which protects the body from infection, treatment with adalimumab may increase the risk of infections.

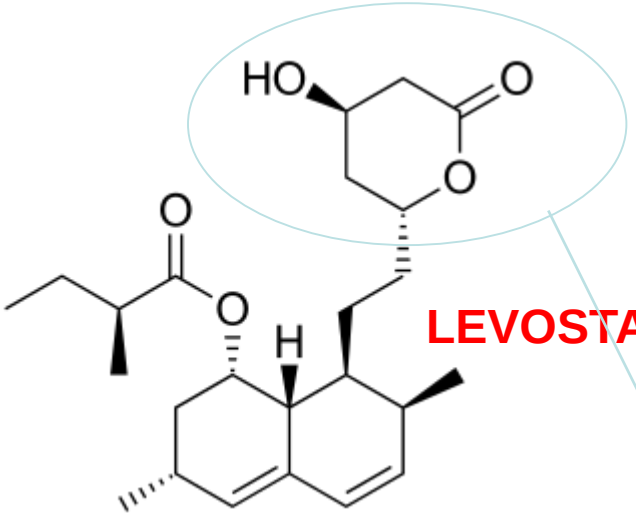
Humira costs approximately $4370 per month (2017). From 2012 until the US patent expiry in 2016, Humira led the list of top-selling pharmaceutical products, and in 2016, it had $16 billion of global sales. In 2014, in India, the first adalimumab biosimilar came to market at a price of $200.

Crestor

Rosuvastatina



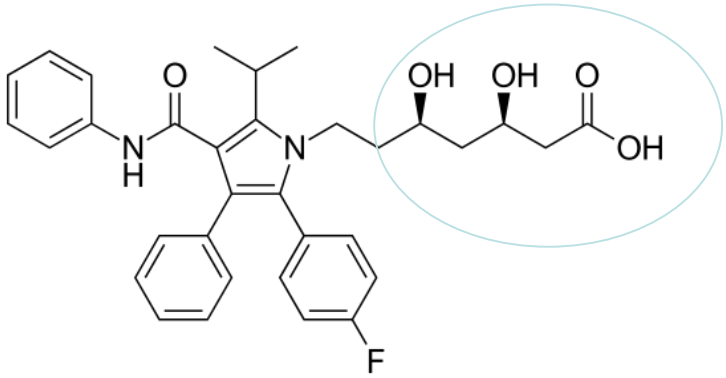
Statins
Inhibitors of cholesterol synthesis



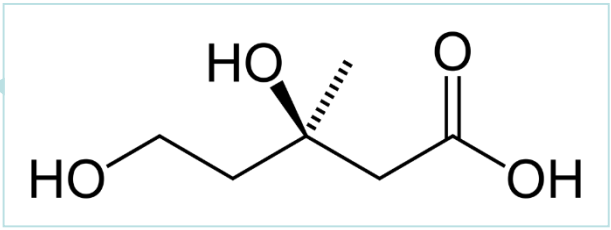
LEVOSTATIN

isolated from *Aspergillus terreus*, was the first statin to be marketed.

In 2005, sales were estimated at US$ 18.7 billion in the United States.
The best-selling statin is atorvastatin, which in 2003 became the best-selling DRUG in history.
The manufacturer Pfizer reported sales of US$ 12.4 billion in 2008.
Due to patent expirations, several statins are now available as less expensive generics.



ATORVASTATIN (Lipitor)



(R) – mevalonic acid (intermediate in cholesterol synthesis)

Biosintesi del colesterolo

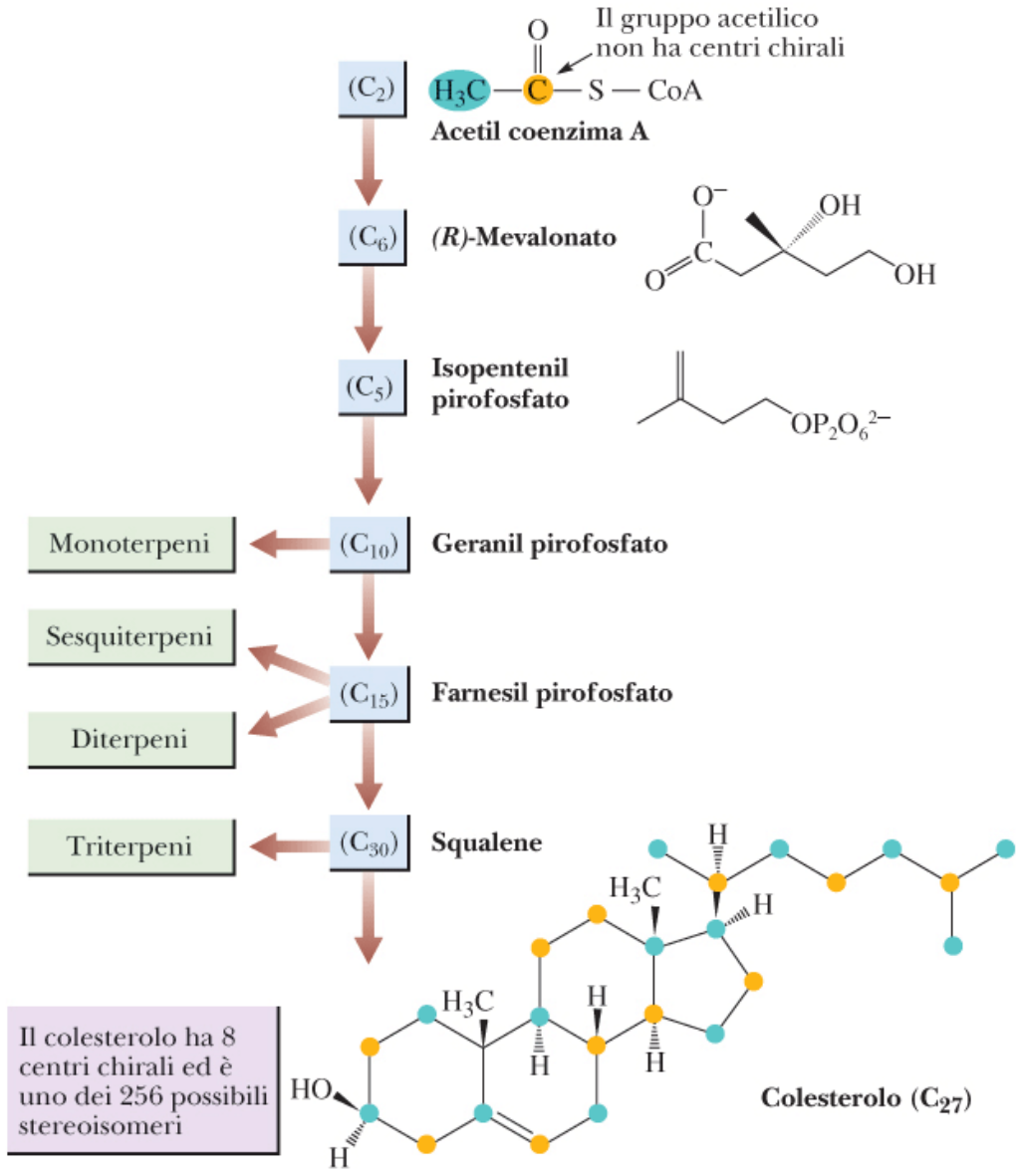
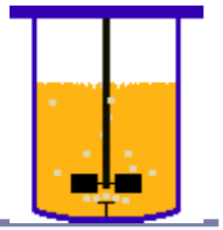


Figura 26.10
Alcuni intermedi chiave nella sintesi del colesterolo a partire del gruppo acetilico dell'acetil-CoA. Sono necessarie otto molecole di acetil-CoA per la sintesi di una mole di colesterolo.

Brown, Foote
Chimica Organica, IV Ed.
EdiSES

Biotechnological production of statins

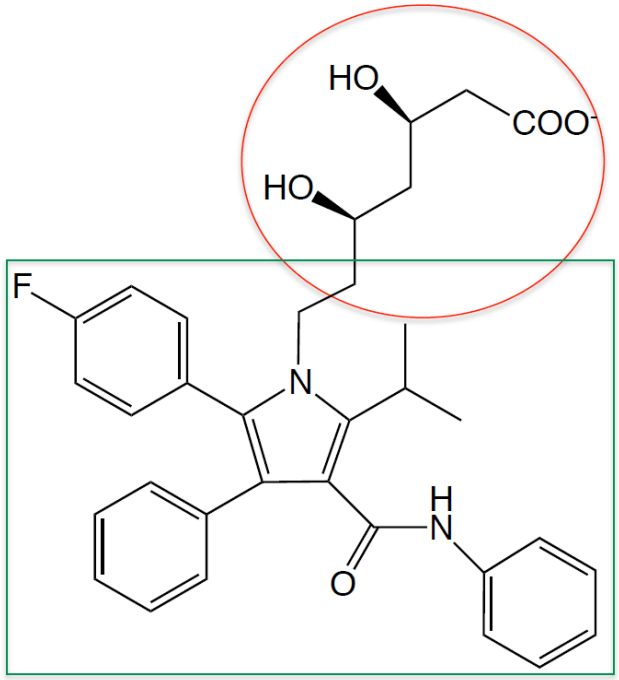


In 2006 Forbes reported an annual sale of statins in the US of 17.4 billion $

The main statins are coming off patent and competition with generic versions is getting rough so only the most efficient production processes will prevail

Name	Brand Name in U.S.	Production Method
Lovastatin	Mevacor	Fermentation
Simvastatin	Zocor	Fermenatation-modified
Pravastatin	Pravachol	Fermentation and biocatalysis
Fluvastatin	Lescol	Synthesis
Atorvastatin	Lipitor	Synthesis and biocatalysis
Rosuvastatin	Crestor	Synthesis
Cerivastatin	Baycol	Synthesis

A case study: preparation of Lipitor® (Atorvastatin calcium)

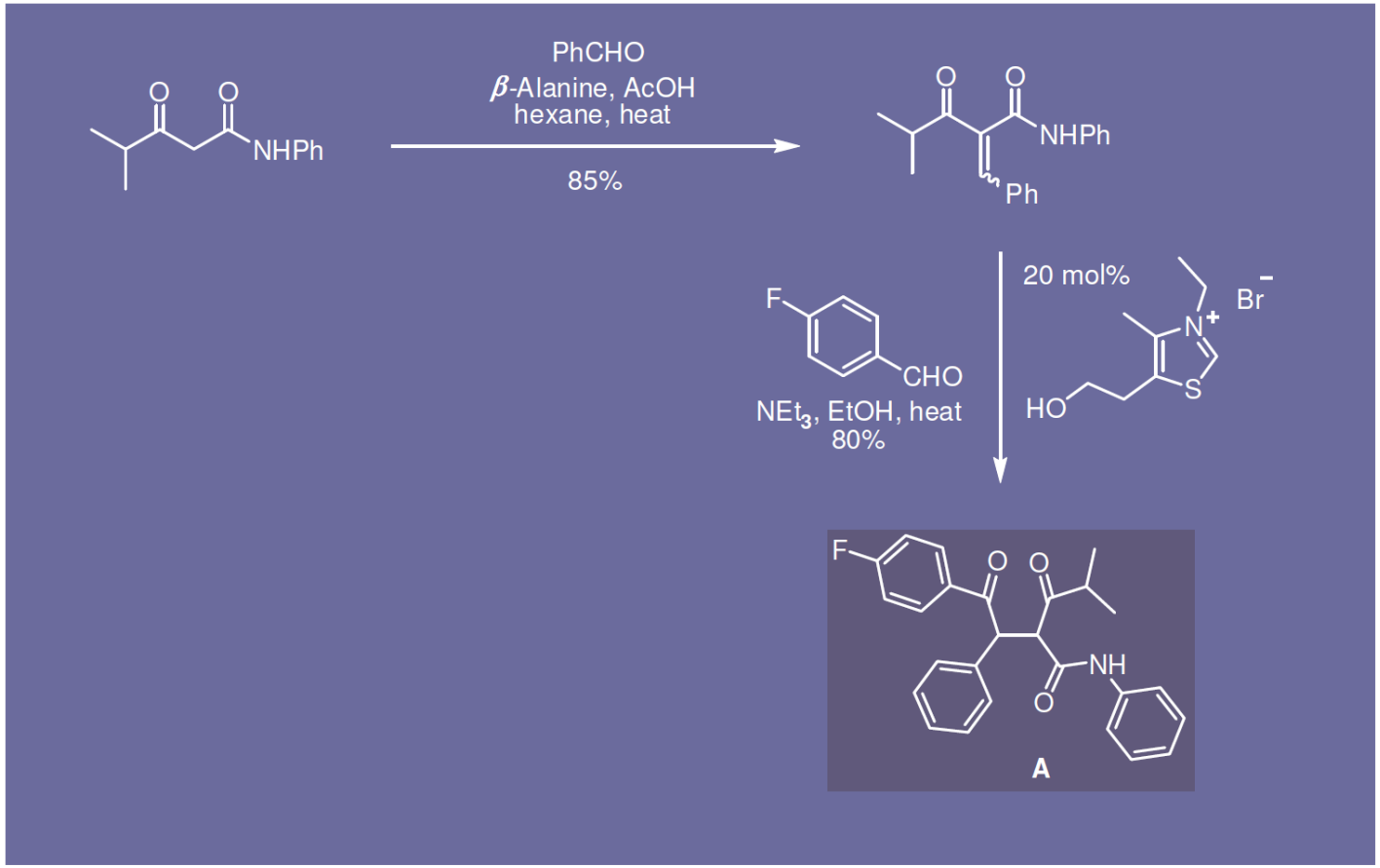


Chiral side chain (fragment B)

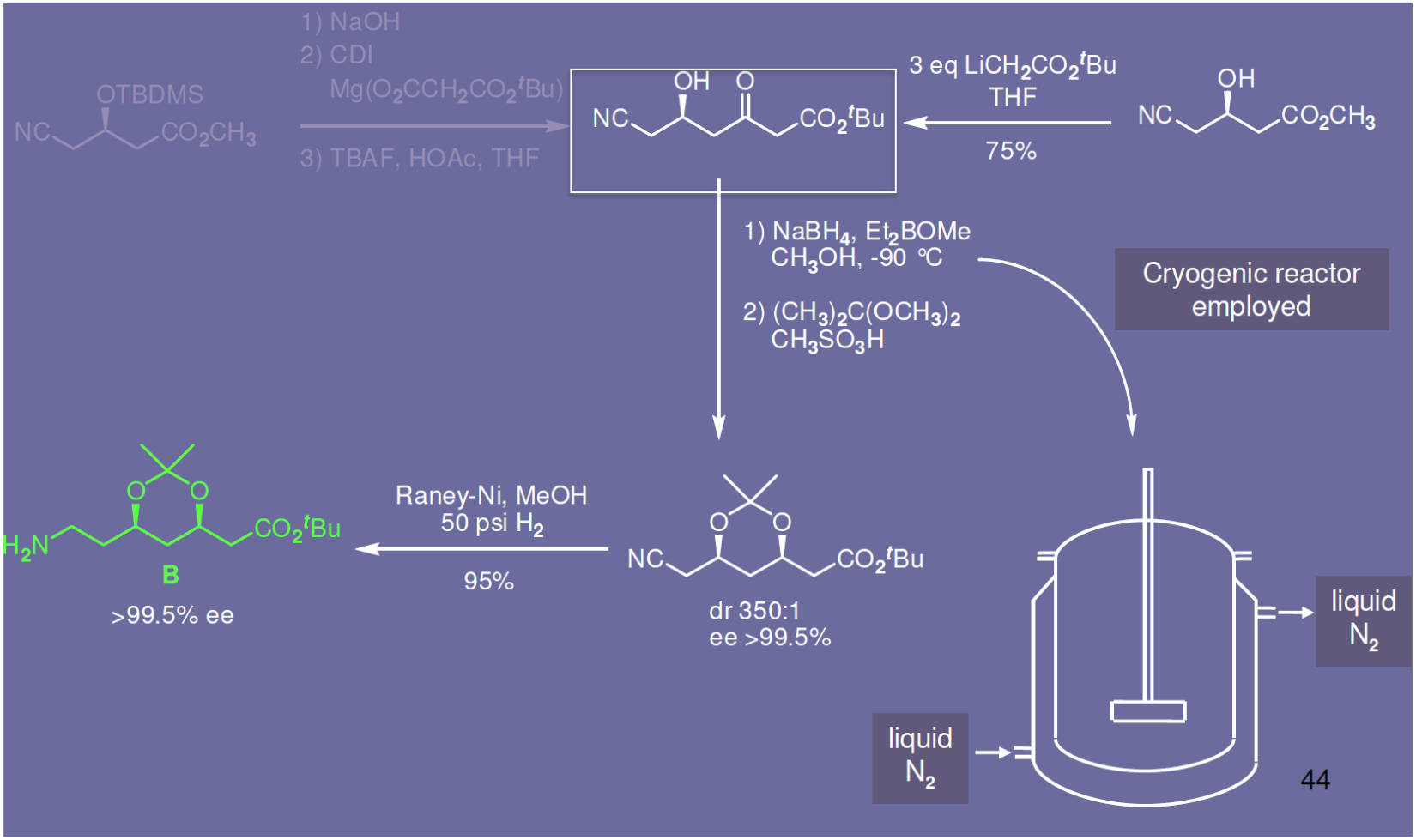
Fragment A

LIPITOR® – $12 billion/year sales (2005)– 220 ton/year

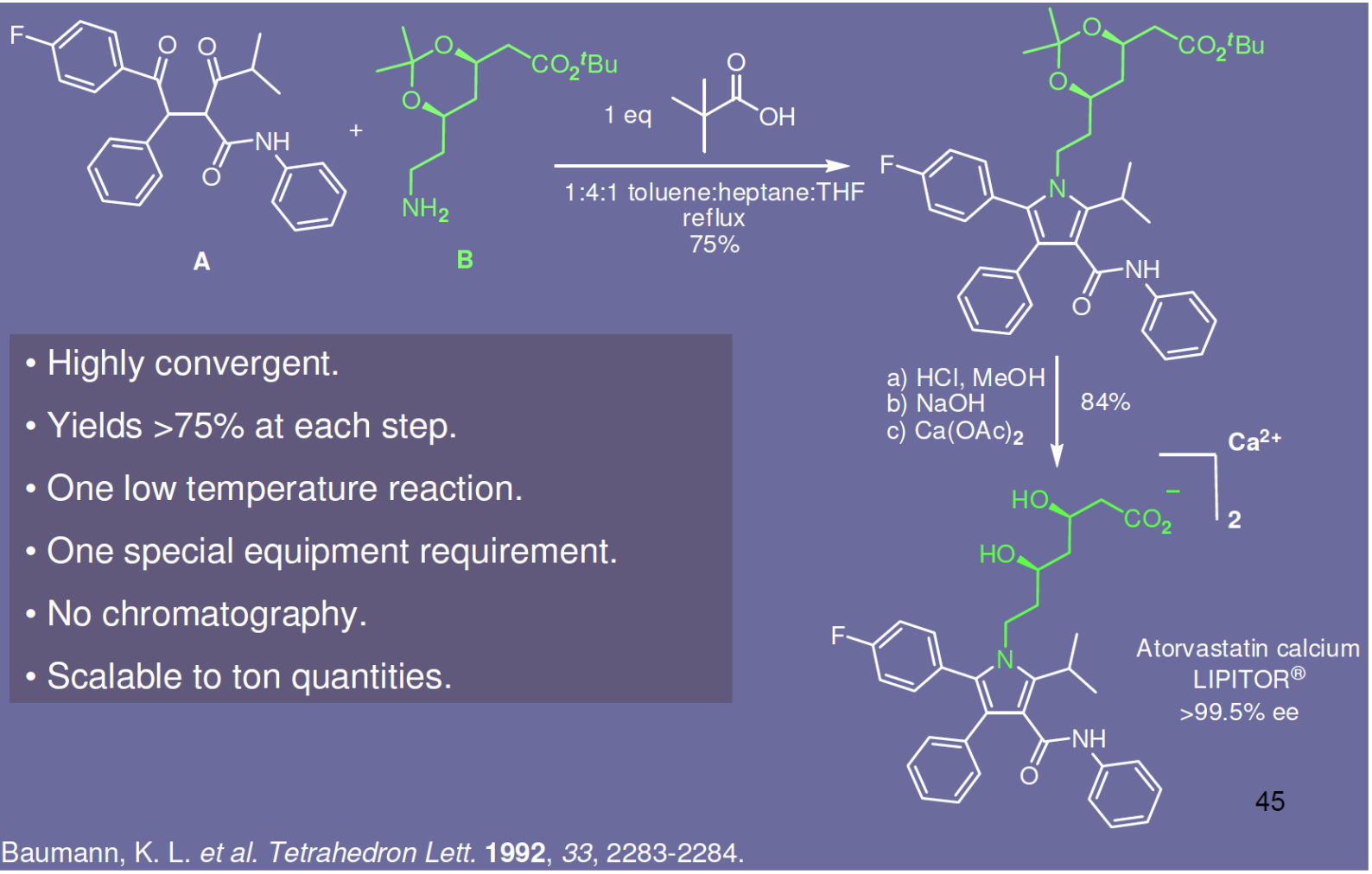
Synthesis of Lipitor®: fragment A



Synthesis of Lipitor®: fragment B, chiral side chain



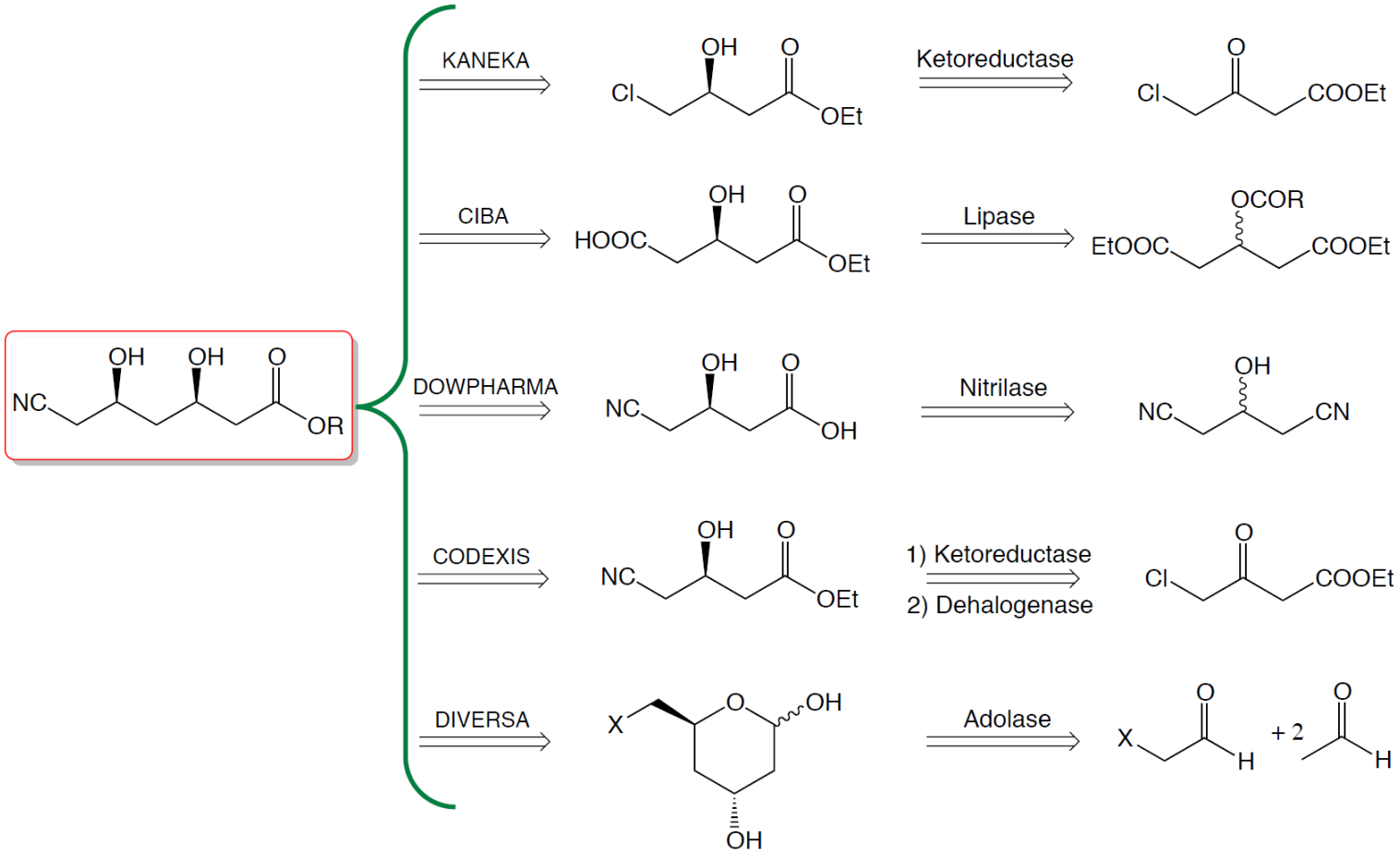
Synthesis of Lipitor®: coupling of fragment A and B



- Highly convergent.
- Yields >75% at each step.
- One low temperature reaction.
- One special equipment requirement.
- No chromatography.
- Scalable to ton quantities.

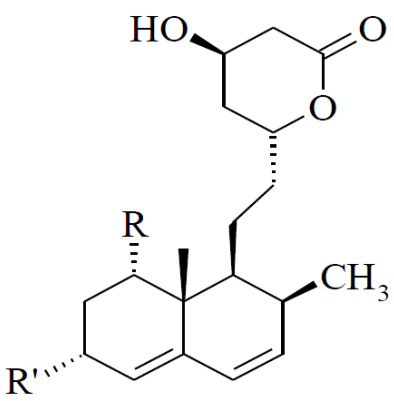
Baumann, K. L. *et al. Tetrahedron Lett.* **1992**, *33*, 2283-2284.

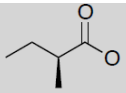
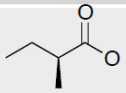
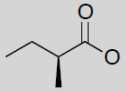
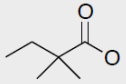
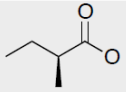
Synthesis of Lipitor®: biocatalytic alternatives for chiral side chain



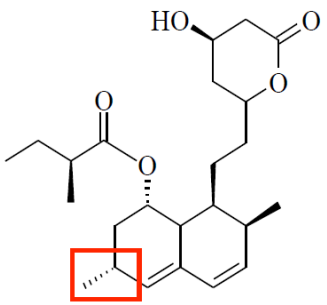
Fermentation-derived statins

Statins were firstly discovered as secondary metabolites from different filamentous fungi (*Monascus ruber, Aspergillus terreus, Penicillium citrinum, Penicillium brevicompactum*)



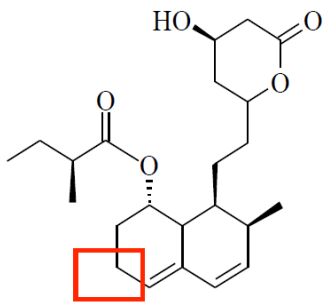
Name	R	R′
Lovastatin		CH_3
Mevastatin		H
Pravastatin		OH
Simvastatin		CH_3
Monacolin J	OH	CH_3
Monacolin X		CH_3

Fermentative production of lovastatin



Lovastatin

(Merck, approved in 1987)



Mevastatin

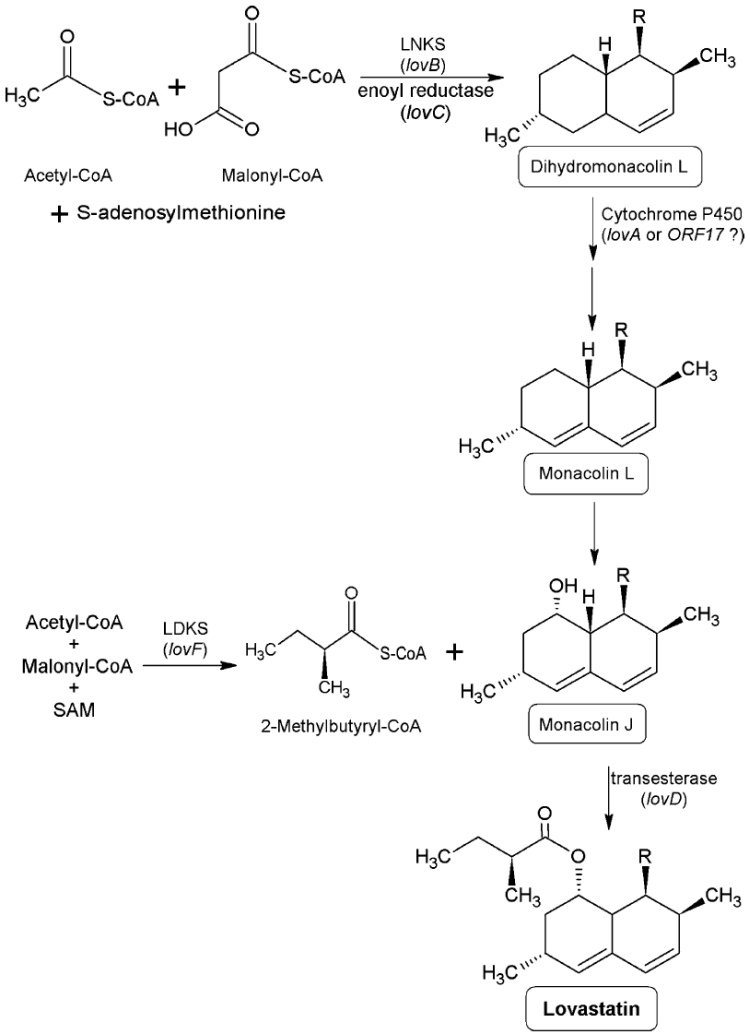
(Sankio)

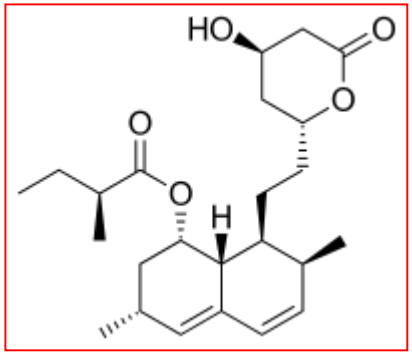
did not pass clinical tests

A.terreus fermentations for producing lovastatin are typically carried out at 28 °C and pH 5.8–6.3, and the dissolved oxygen level is controlled at ≥ 40% of air saturation (Kumar et al. 2000). Batch fermentations generally run for approximately 10 days.

Metkinen produces from 2000 lovastatin with 7-8 g/l yields

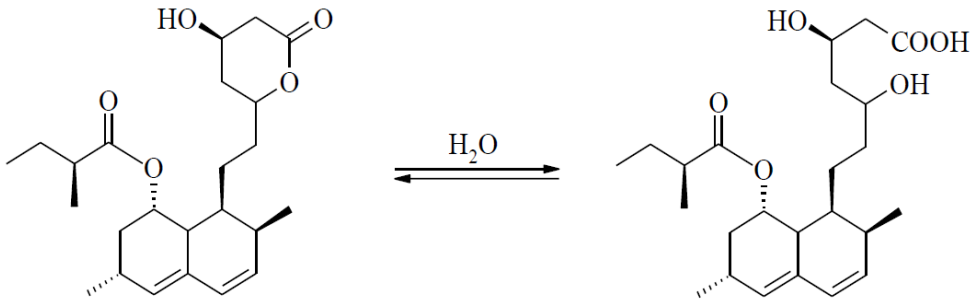
Biosynthesis of lovastatin



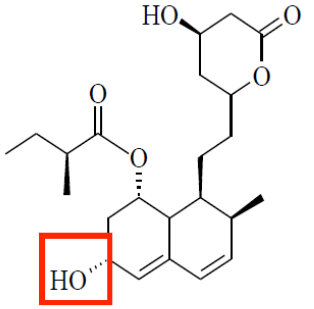


Recovery of lovastatin

At the end of the fermentation process, the broth is acidified for favouring the equilibrium towards lactone formation:

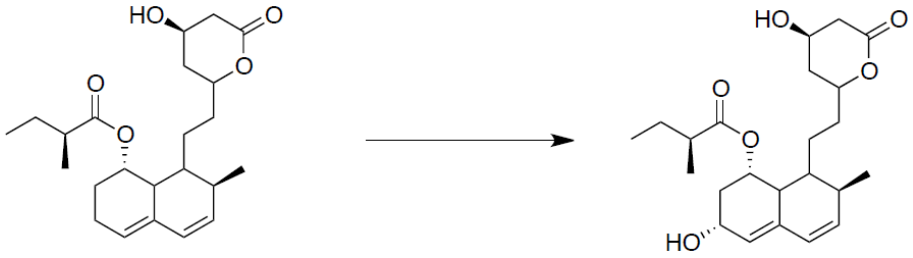


Production of pravastatin (Sankio and Bristol-Myers Squibb)



Preparation of pravastatin is carried out by a two-step process:

1) Fermentation for producing mevastatin with *P. citrinum* or *P. brevicompactum*
2) Biotransformation with a a regio- and stereo-selective monooxygenase



The best performing monooxygenases are *Streptomyces carbophilus* e da *Actinomadura* sp. furnishing pure pravastatin with 78-82% yields

Alternative routes to Pravastatina

Asymmetric synthesis for the production of pravastatin hydroxynitrile intermediate (1.)

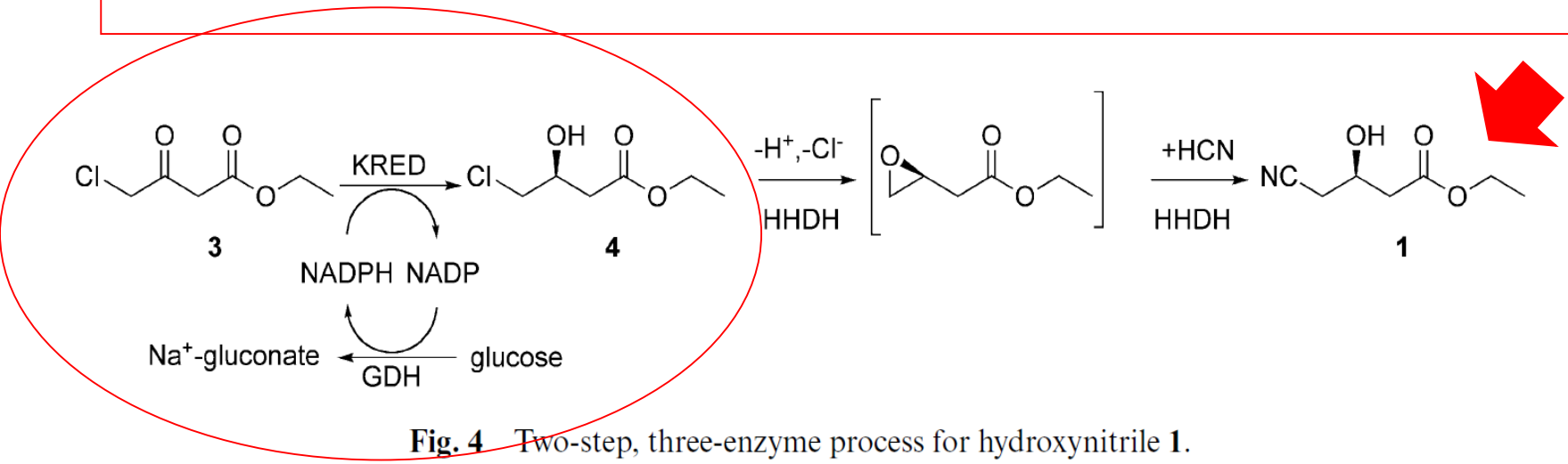


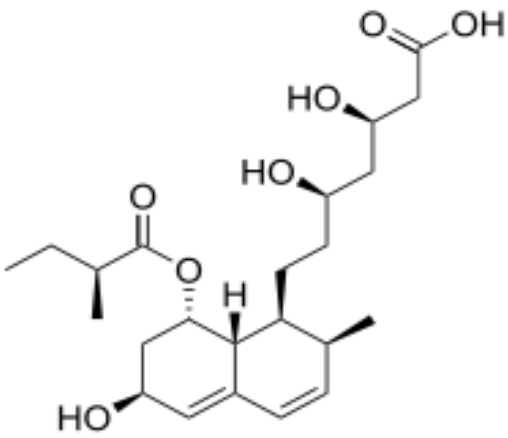
Fig. 4 Two-step, three-enzyme process for hydroxynitrile **1**.

1.

Ketoreductase

+

Glucose dehydrogenase for cofactor recycling



pravastatina

Asymmetric synthesis for the production of statin intermediates (2.)

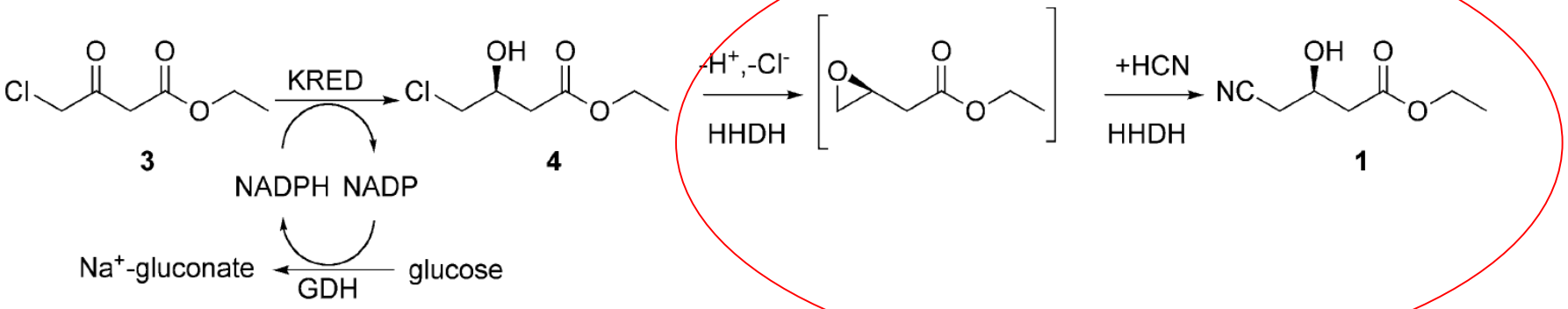


Fig. 4 Two-step, three-enzyme process for hydroxynitrile **1**.

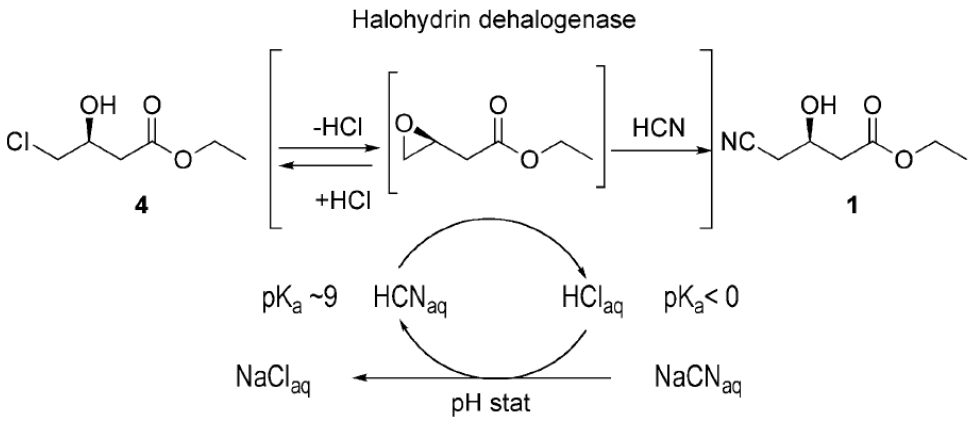
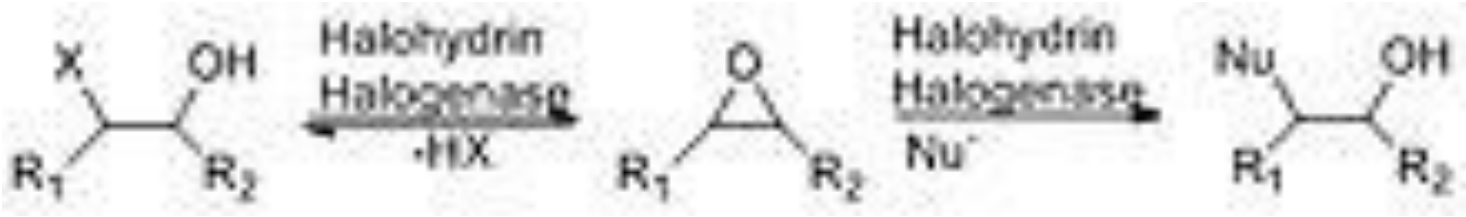


Fig. 6 HHDH-catalyzed cyanation of **4**.

2.
The halohydrin dehalogenase (HHDH) is employed to catalyse the replacement of the chloro substituent with cyano by reaction with HCN at neutral pH and ambient temperature

Halohydrin dehalogenases mechanistically cleaves the carbon-halogen bond through the formation of an epoxide from a vicinal hydroxyl group

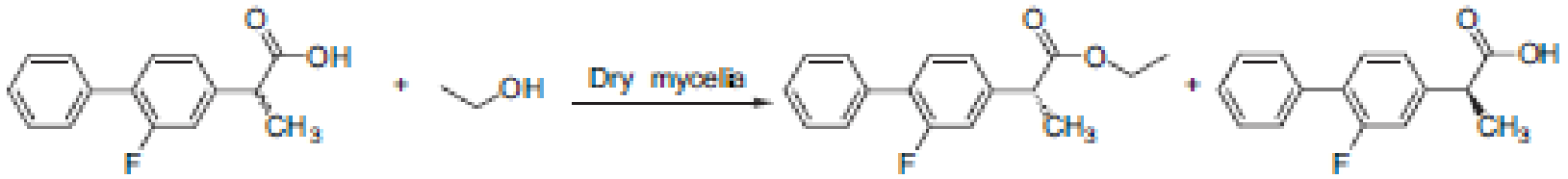


Halohydrin dehalogenase is involved in the degradation of vicinal halohydrines. In several species of bacteria, it catalyses the dehalogenation of halohydrins to produce the corresponding epoxides.

Selectivity of enzymes is not always the key factor for selecting a synthetic route

Resolution of flurbirprofen: esterification catalyzed by lipase (in dry mycelia) in organic solvents

P. Spizzo et al. / Tetrahedron 63 (2007) 11005–11010



(*R,S*)-Flurbiprofen 1 + Ethanol 2 → (*R*)-Flurbiprofen ethyl ester 3 + (*S*)-Flurbiprofen 1-S

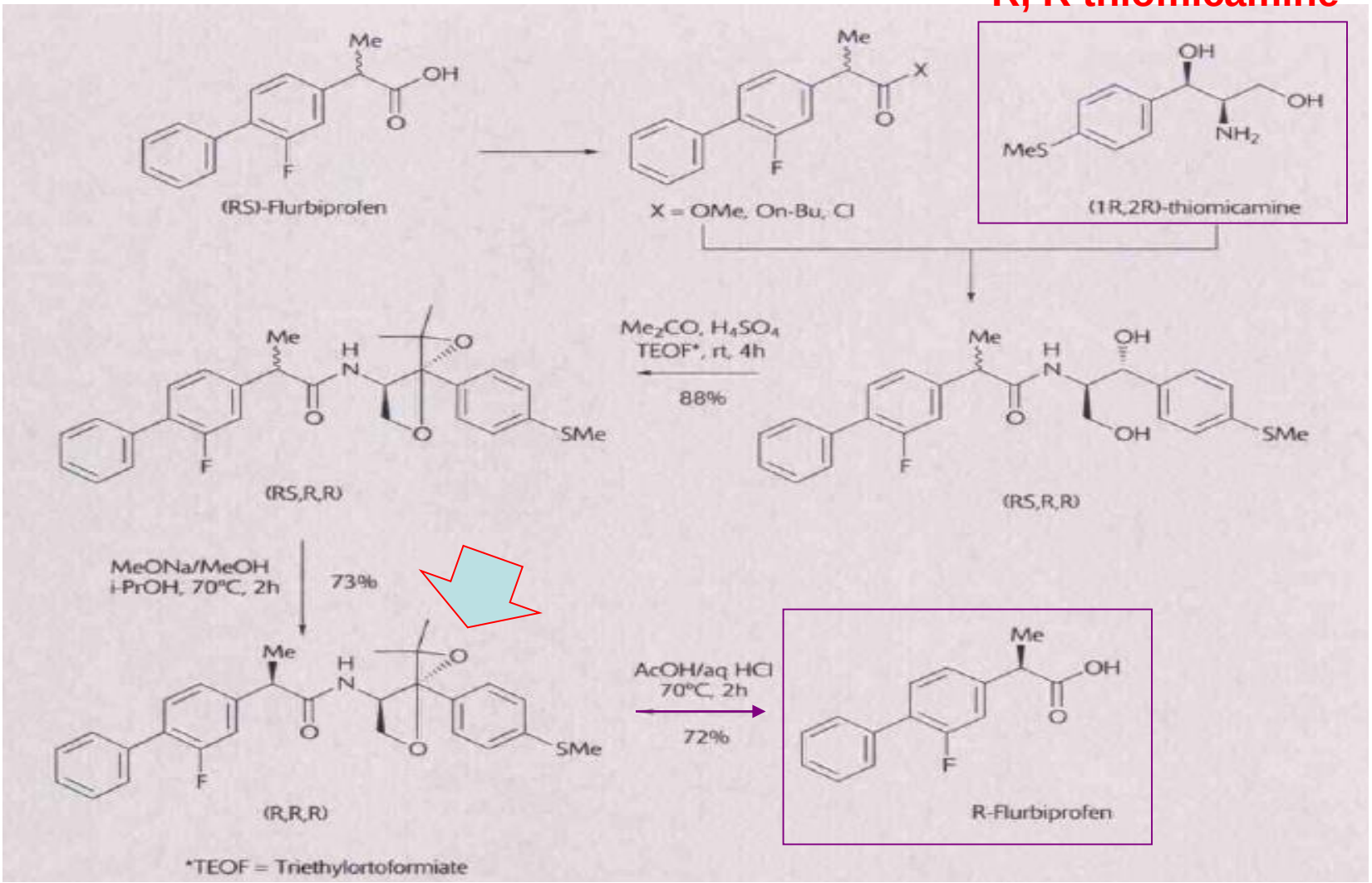
S: anti-inflammatory

R: anti-Alzheimer

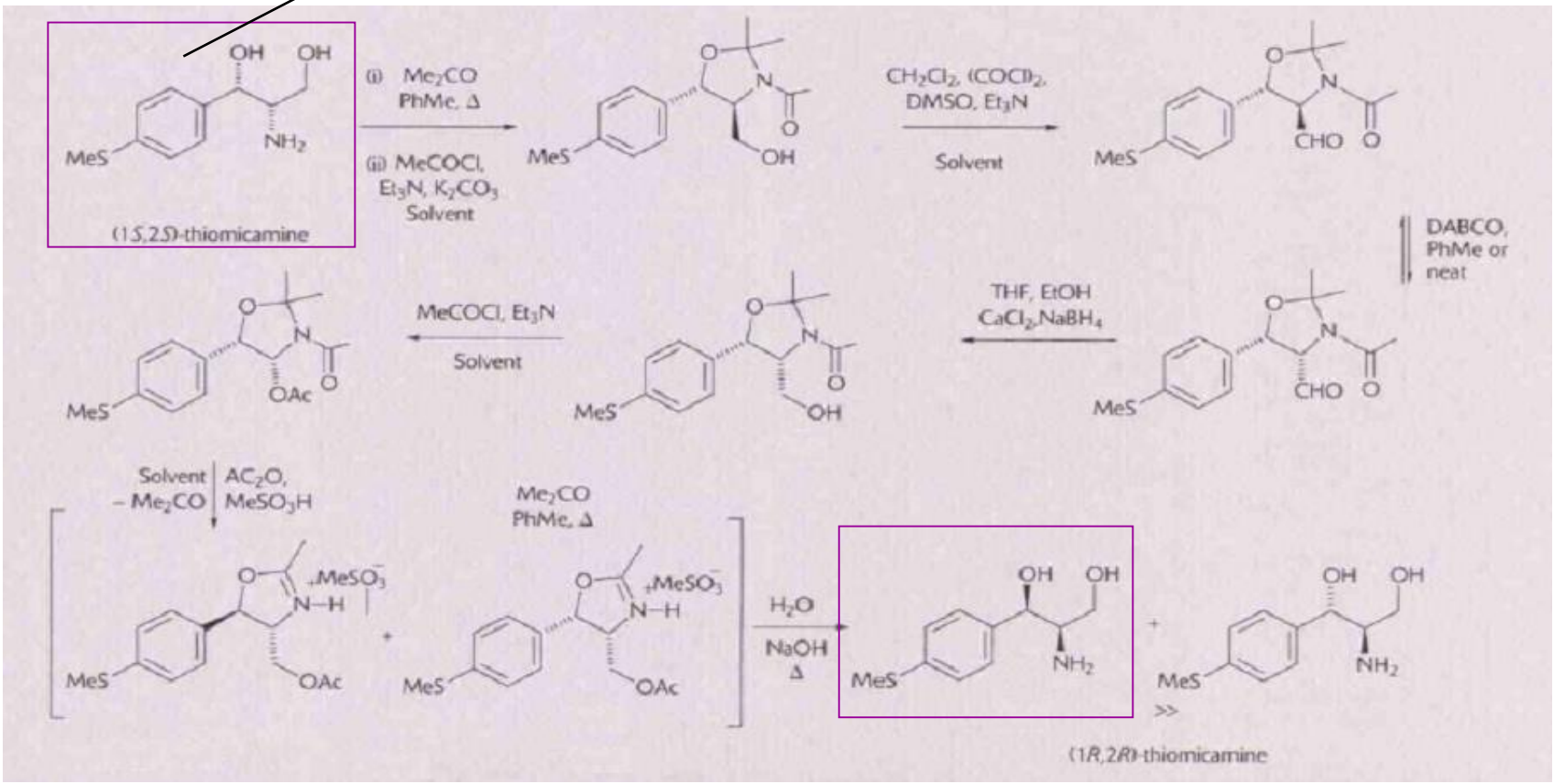
Dry mycelia of *Aspergillus oryzae* in toluene

Chemical industrial resolution Zambon Group S.p.A

R, R thiomicamine



S, S: Intermediate in the production of tiamfenicolo



ZAMBON GROUP SpA

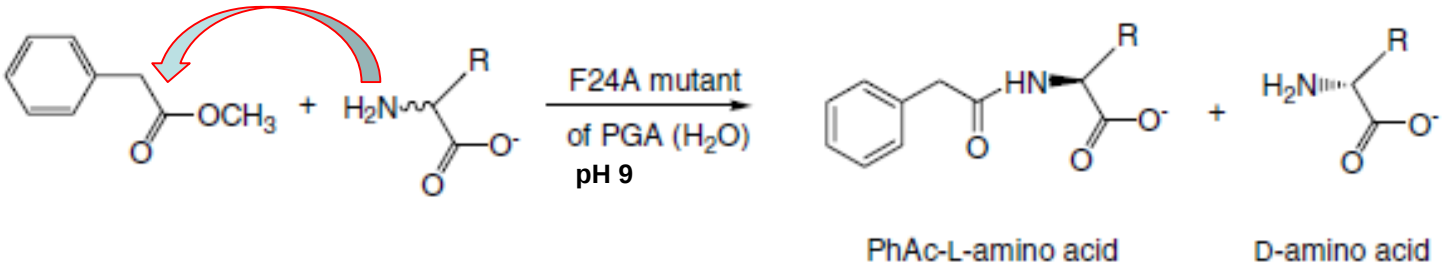
TIAMFENICOLO GLICINATO ACETILCISTEINATO

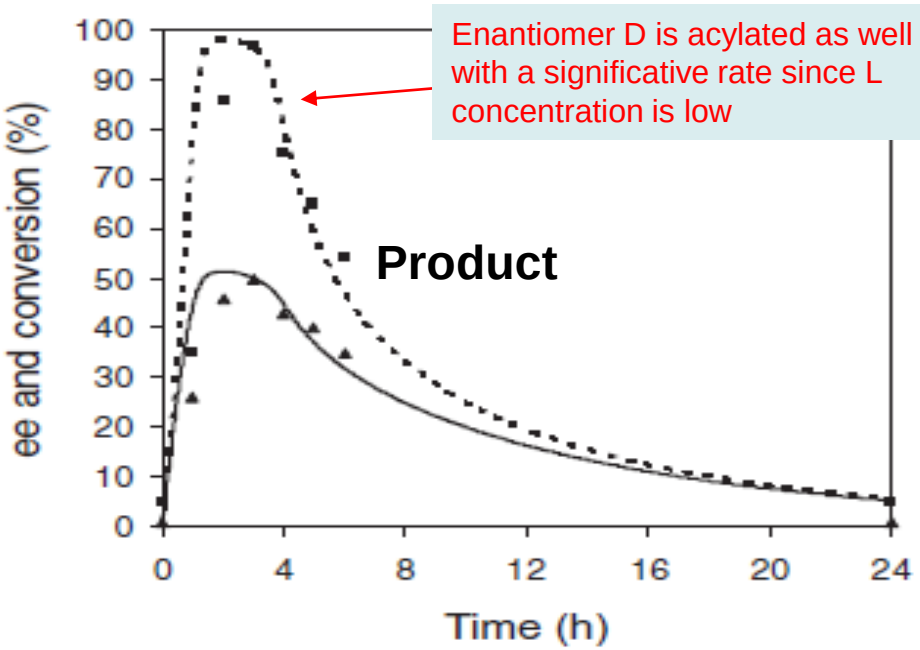
€ 43,38

Chirality and biocatalysis: quantitative description of enantioselectivity

«Enantiomeric Ratio»
«E»

Exampl: Biocatalysis for the production of D-aminoacids



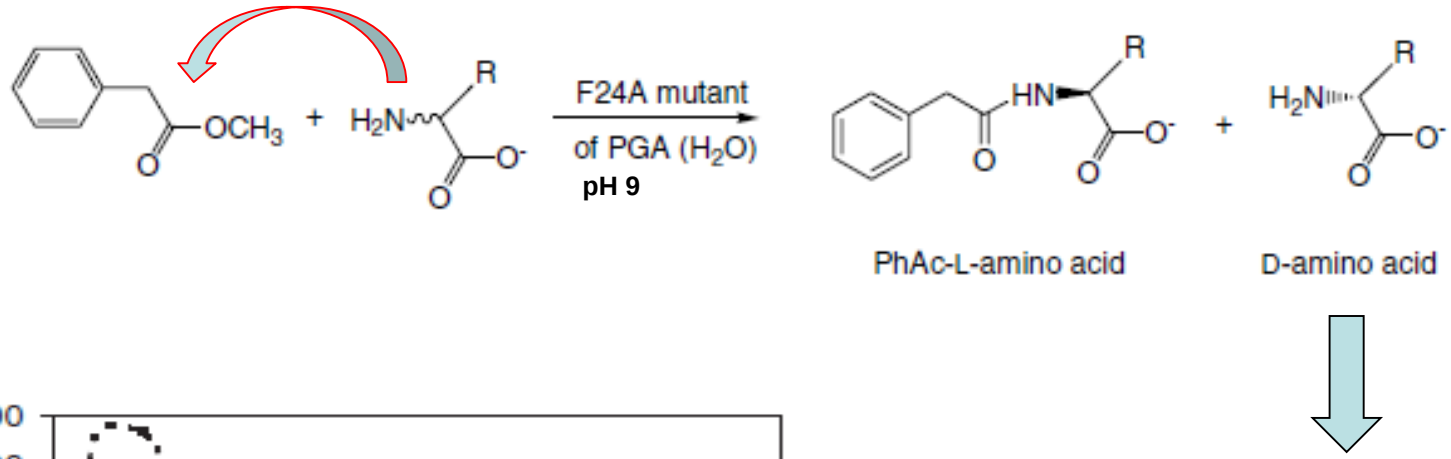


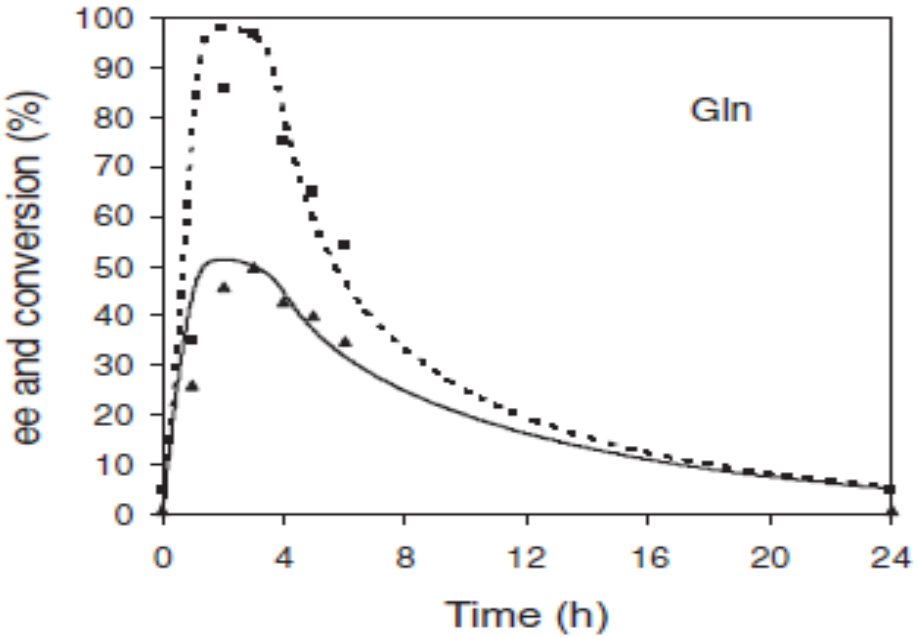
$$e.e.\% = \frac{c_R - c_S}{c_R + c_S} \times 100$$

NB!!! Changes throughout the reaction

Carboni c. et al, *Tetrahedron: Asymmetry* 17 (2006) 245–251

Resolution of aminoacid racemates *via* acylation: max 50% yield





It is easier to achieve higher ee% of the unreacted reagent by pushing the reaction beyond 50% : lower yield but higher ee%

Reaction must be stopped at the most favourable time

Expressing quantitatively enantioselectivity in biocatalysis

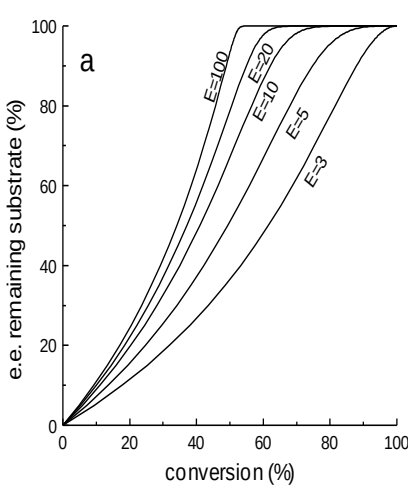
selectivity: $\dfrac{(k_{cat}/k_m)_R}{(k_{cat}/k_m)_S}$

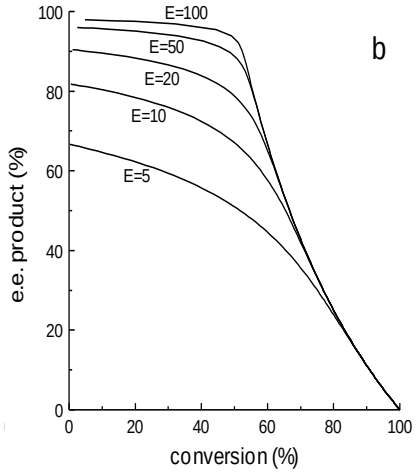
$$e.e.\% = \frac{c_R - c_S}{c_R + c_S} \times 100$$

Ratio of specificity constants

"E" : Enantiomeric Ratio

ee% changes during the reaction and is not informative





Calculating “E ” experimentally

$$E = \frac{\ln(A/A_0)}{\ln(B/B_0)} = \frac{V_A/K_A}{V_B/K_B}$$

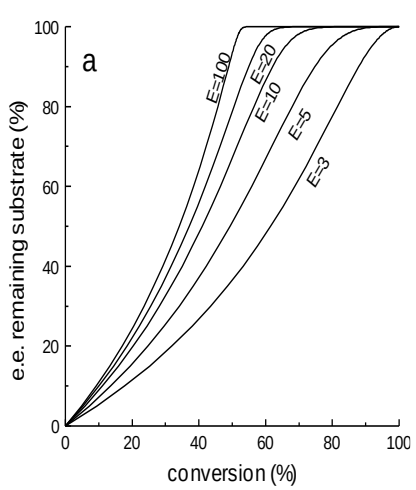
$$E = \frac{\ln[(1-c)(1-ee(L))]}{\ln[(1-c)(1+ee(L))]} = \frac{\ln[1-c(1+ee(P))]}{\ln[1-c(1-ee(P))]}$$

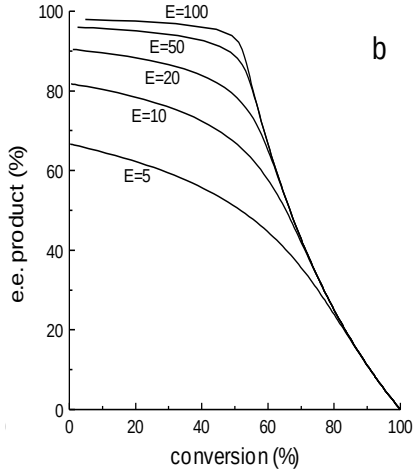
L: left, Remained unreacted
P: product

Experimental quantitative work:

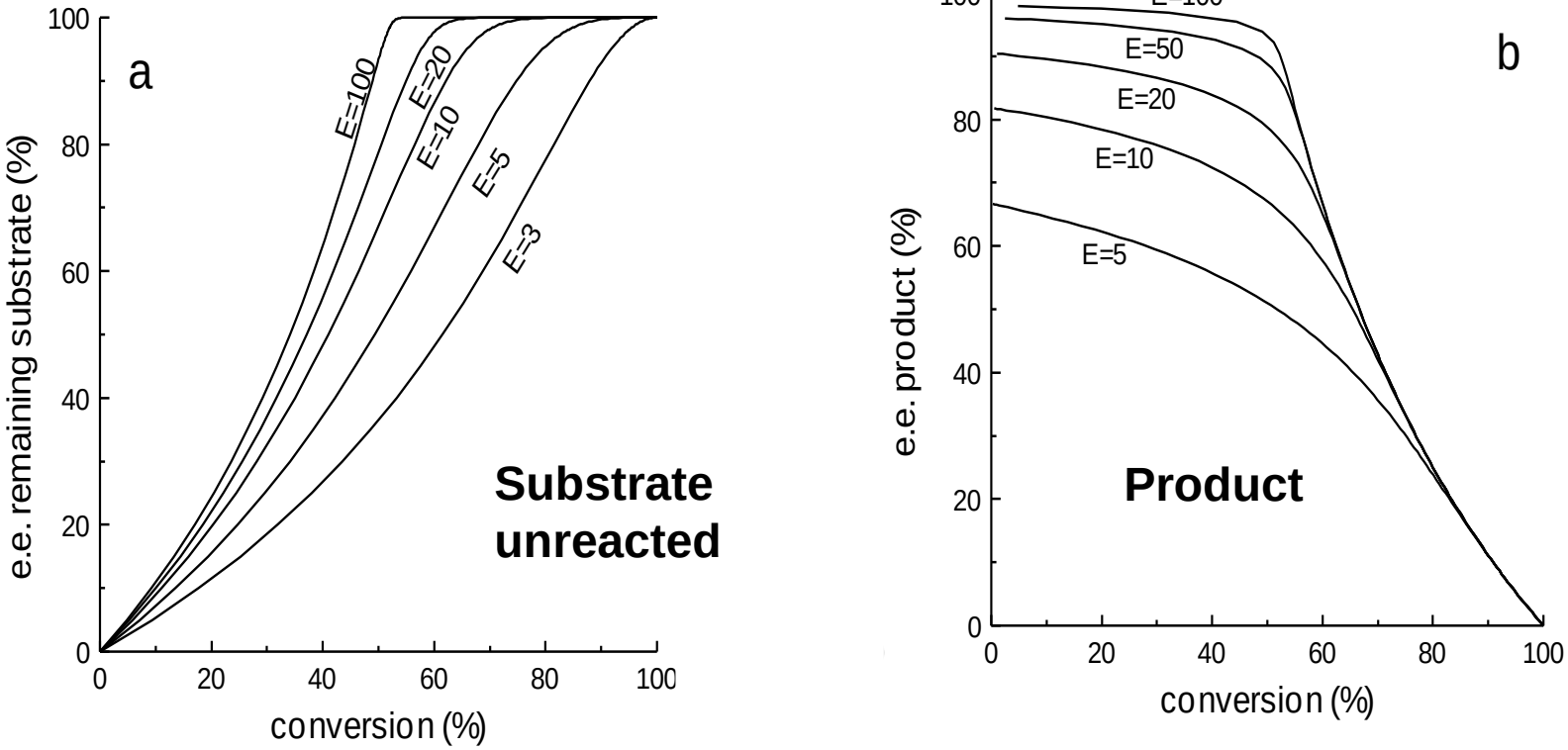
“*c*” (concentration by means of HPLC or ^{1}H NMR)

e.e. % (chral GC or HPLC)





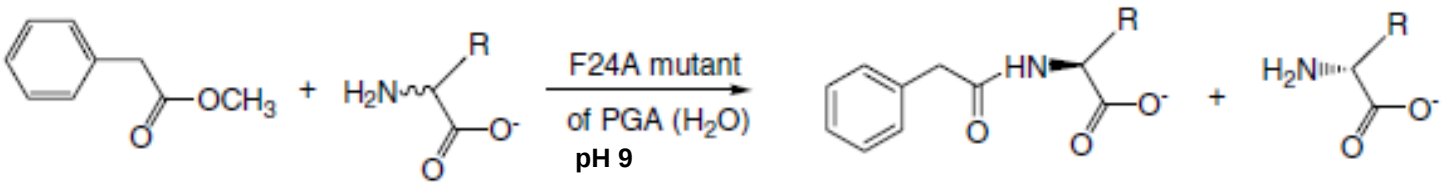
“E value” for practical applications

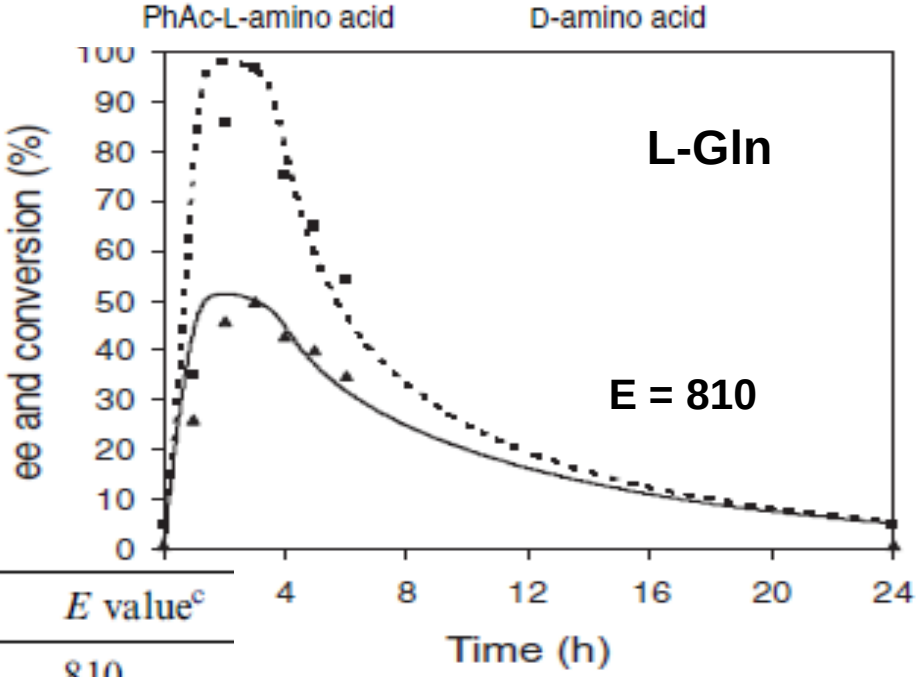


for the left unreacted substrate: E >20 (yield≈ 40%; e.e.% >99).

Product enantiomerically pure: E > 100

Example: amino acid resolution





ee_D max (%)	ee_L[a] (%)	Conversion[a,b] (%)	*E* value[c]
97	>99	49.5	810