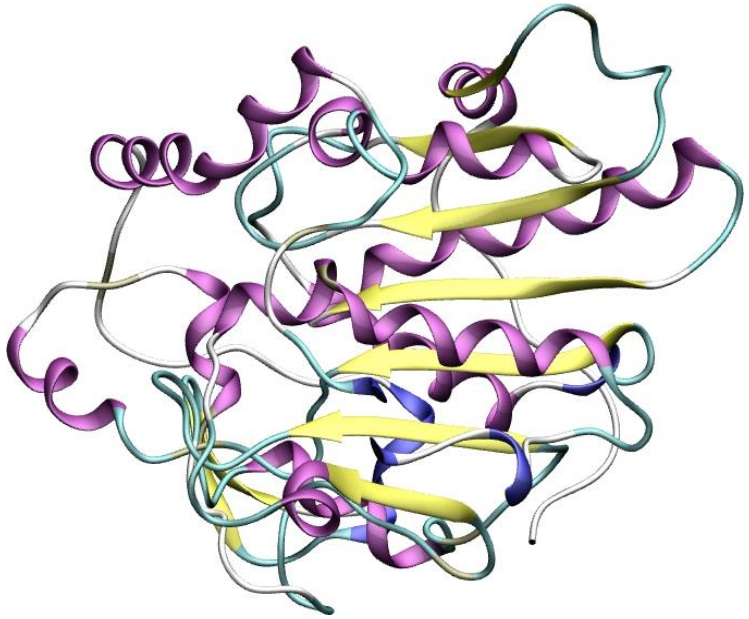
Biocatalysis in organic synthesis: Production of enantiomerically pure amino acids, drugs, aromas and building blocks



Amino acids: industrial impact

The use of enzymes and whole cell biocatalysts has proven particularly valuable in production of both **proteinogenic** and **nonproteinogenic** L-amino acids D-amino acids, and enantiomerically pure amino acid derivatives, which are of great interest as **building blocks** for active ingredients that are applied as **pharmaceuticals, cosmetics, and agricultural products**.

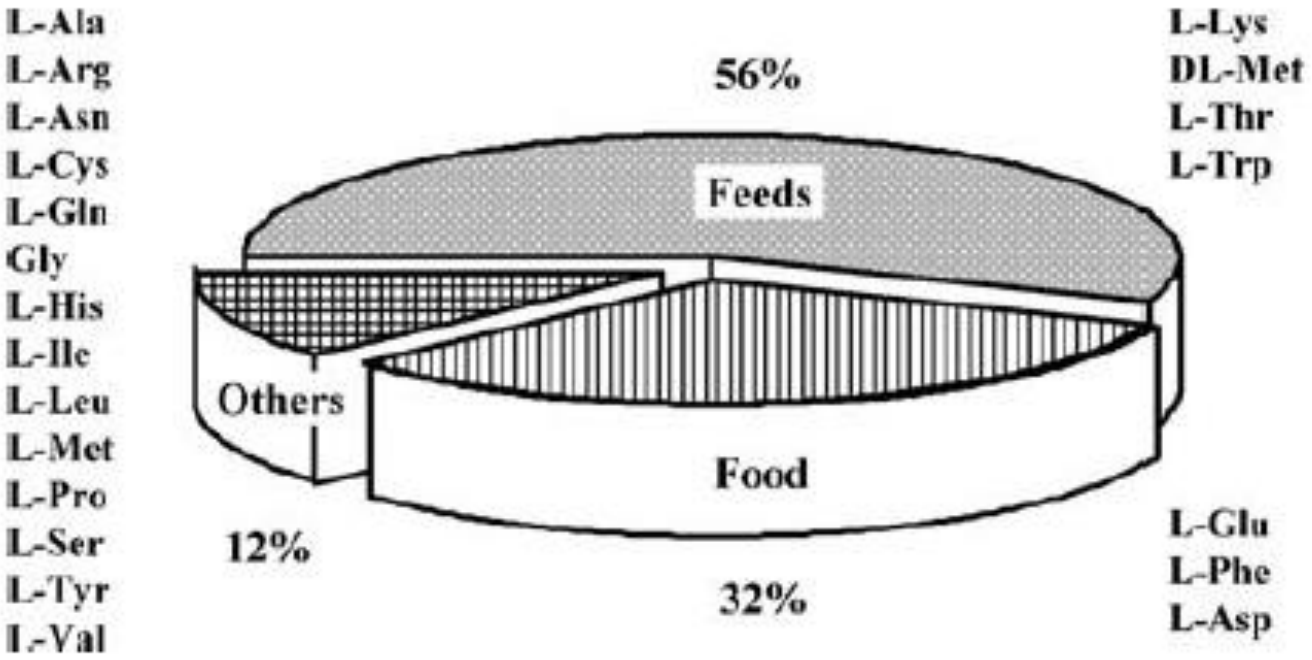


Fig. 1 Global market amino acids, 2004: US $4.5 billion

Amino acids: industrial impact

Of the 20 standard protein amino acids, the **9 essential amino acids** L-valine, L-leucine, L-isoleucine, L-lysine, L-threonine, L-methionine, L-histidine, L-phenylalanine, and L-tryptophan occupy a key position in that they are not synthesized in animals and humans but must be **ingested with feed or food**.

In terms of market volume, development over the last 20 years has been tremendously bullish in the so-called **feed amino acids** L-lysine, DL-methionine, L-threonine, and Ltryptophan, which constitute the largest share (56%) of the total amino acid market, estimated in 2004 at approximately US $4.5 billion.

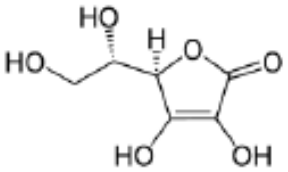
Also substantial is the share of the **food sector**, which is determined essentially by three amino acids: L**-glutamic acid** in the form of the flavorenhancer monosodium glutamate (MSG) and the amino acids L**-aspartic acid and** L**-phenylalanine**, both of which are starting materials for the peptide sweetener L-aspartyl Lphenylalanyl methyl ester (Aspartame), used, for example, in “lite” colas.

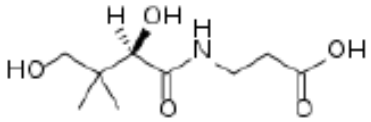
The amino acid market for synthesis applications is growing at an annual rate of 7% (US $1 billion in the year 2009), of which the share of amino acids for **peptide sweeteners** alone is expected to be more than US $400 million.

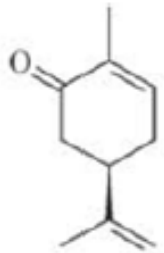
Amino acids: industrial impact

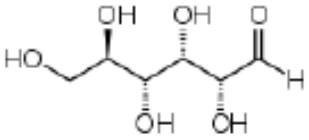
The remaining **proteinogenic** amino acids are required in the **pharmaceutical and cosmetics** industries and are also ideal raw materials for synthesis of chiral active ingredients, which in turn find application in such sectors as pharmaceuticals, cosmetics, and agriculture.

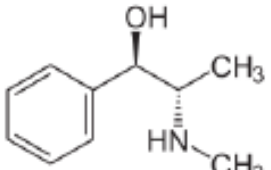
Chiral pool











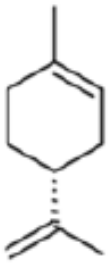
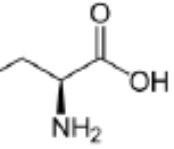
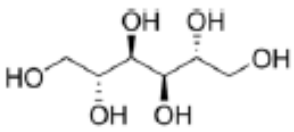


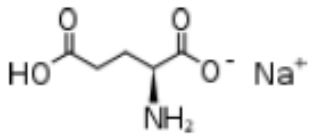
Table 2. Representative substances from the chiral pool[a]

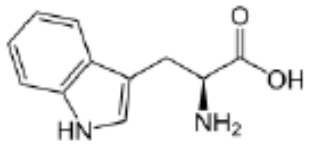
Compound	Approx. price (US dollars kg^{-1})
Ascorbic acid	13
(+)-Calcium pantothenate	16
(−)-Carvone	23
Anhydrous dextrose	1.2
Ephedrine hydrochloride	62
(+)-Limonene	3
→ L-Lysine	3.2
Mannitol	7.5
Monosodium glutamate	2
Norephedrine hydrochloride	24
Quinidine sulphate	130
Quinine sulphate	75
Sorbitol	1.7
→ L-Threonine	12–50, depending on grade
→ L-Tryptophan	68

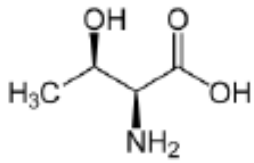
[a] Data from *Chemical Marketing Reporter*, Schnell Publishing, New York, 13 April (1990); reproduced by permission of the Editor.

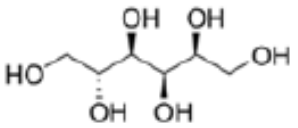


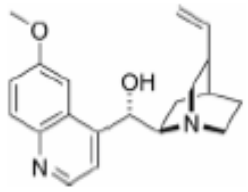


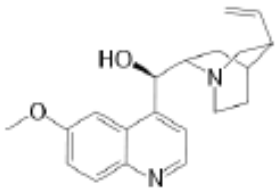


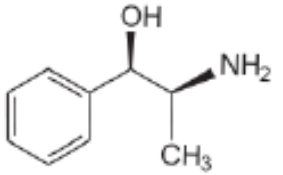












PRODUCTION OF AMINOACIDS

Extraction of amino acids from protein hydrolysate as a method of obtaining L-amino acids is now of only limited importance; although still relevant for production of **L-serine, L-proline, L-hydroxy-proline, and L-tyrosine**, for example, it is not suitable for large-scale production of amino acids.

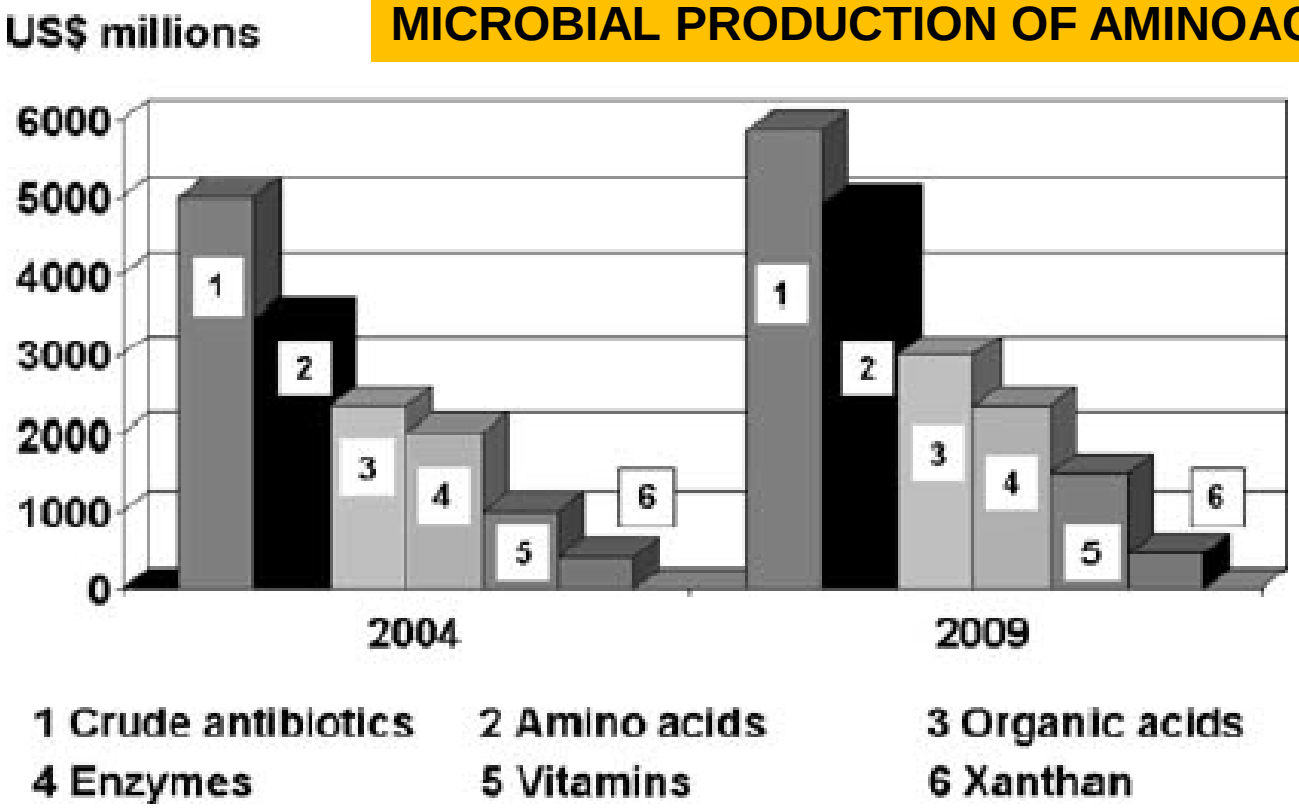
The extraction method for obtaining **L-glutamate** was superseded nearly 50 years ago by fermentation, following a sharp increase in demand for the flavor-enhancer MSG.

MICROBIAL PRODUCTION OF AMINOACIDS

The discovery of the soil bacterium, Corynebacterium glutamicum, which is capable of producing L-glutamic acid with high productivity from sugar, paved the way for the success of the fermentation technique in amino acid production (Kinoshita et al. 1957). It was advantageous that the wild strain could be used on an industrial scale under optimized fermentation conditions for **mass production of glutamate.**

Fermentation methods **are gaining importance for the preparation of enantiomerically pure compounds like amino acids, β-lactam antibiotics and vitamins. Many fermentations are complex multistep reactions, involving several different enzymes of living cell system.**

MICROBIAL PRODUCTION OF AMINOACIDS



In the world market for fermentation products (ethanol excluded),has been US $17.8 billion in 2009, the amino acids are the second most important category, after antibiotics, with fermentation products exhibiting the highest growth rates .

Biotechnological production of sodium glutamate

The fermentation process is in principle very simple: a fermentation tank is charged under sterile conditions with a culture medium containing a suitable carbon source, such as **sugar cane syrup**, as well as the required nitrogen, sulfur, and phosphorus sources, and some trace elements. A culture of the production strain prepared in a prefermenter is added to the fermentation tank and stirred under specified conditions (temperature, pH, aeration).
The L**-glutamic acid released by the microorganism** into the fermentation solution is then obtained by **crystallization** in the recovery section of the fermentation plant.

MSG (**1.5 million tons**) is currently produced each year by this method, making L-glutamic acid the number one amino acid in terms of production capacity and demand (Ajinomoto 2003).

Biotechnological production of lysine: *Corynebacterium glutamicum*

Lysine is a preferred additive to animal feeds for pig breeding (as the first limiting amino acid) and poultry (second limiting amino acid, after methionine).

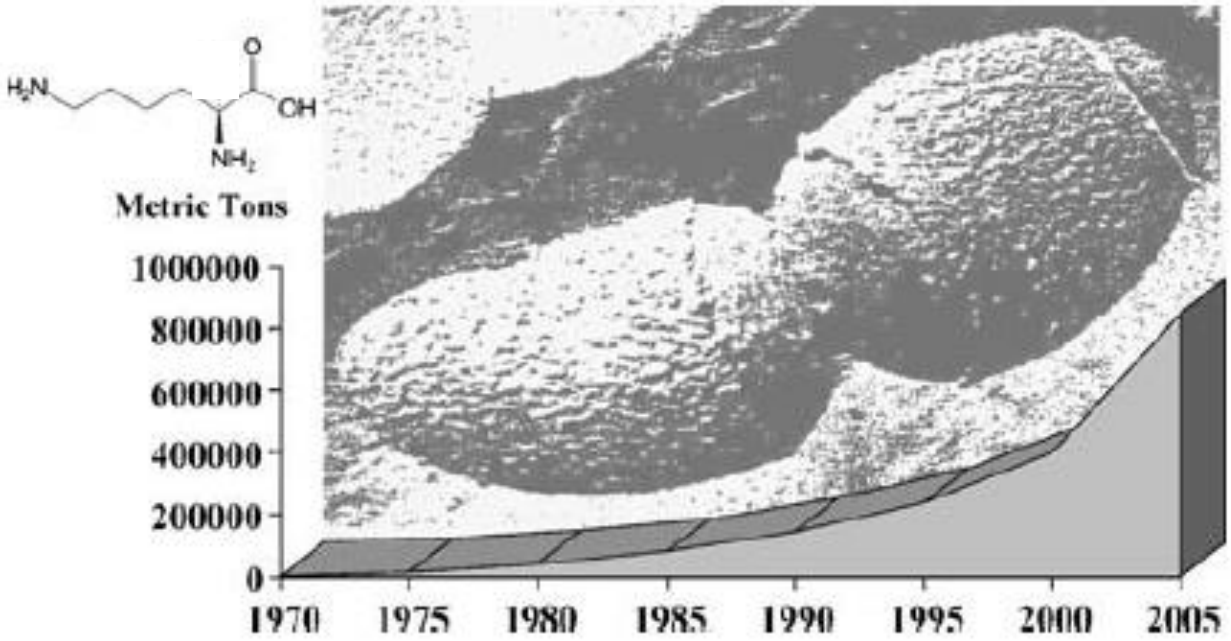


Fig. 3 Global market for L-lysine (1970–2005). The picture shows the lysine-producing mutant of *C. glutamicum*—after cell division

Biotechnological production of lysine

Production of lysine hydrochloride in 2005 is estimated at 850,000 tons.
The main producers of lysine are the companies Ajinomoto (Japan), ADM (USA), Cheil-Jedang (South Korea), and Global BioChem (China) as well as BASF and Degussa (Germany).

The strains used are exclusively high-performance mutants of ***C. glutamicum***, usually fermented by the fed-batch process, in which nutrients are added in a controlled manner in accordance with the requirements of the culture solution, allowing optimal yields and productivities.

Competitiveness is determined not only by the performance of the production strain, but can also be increased by a conveniently produced product form. Thus, in addition to the classic product form lysine **hydrochloride, other forms such as granulated lysine sulfate (Biolys) and** liquid lysine have also become established where the production is more economical and generates **less liquid and solid waste**.

Biotechnological production of lysine

Corynebacterium glutamicum

C-source + N-source + salts + trace elements

fermentation

cell separation → biomass

ion exchange → waste

evaporation → ammonia

cristallisation → mother liquor

drying

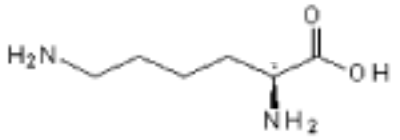
L-Lysine·HCl

fermentation

evaporation

spray drying

Biolys®



Selected amino acid producing strains

The amino acids **L-phenylalanine and L-cysteine**, both of which were previously produced mainly with the help of enzymes, can now be obtained more cost effectively by fermentation with ***E. coli*** strains and are thus available to a larger and growing market.

Almost all proteinogenic amino acids, with a few exceptions, can be produced industrially by specially developed mutants of ***C. glutamicum*** or *E. coli*.

Amino acid	Strain/mutant	Titer (g/l)	Estimated yield (g/100 g sucrose)
L-Lysine HCl	*C. glutamicum* B-6	100	40–50
L-Threonine	*E. coli* KY 10935	100	40–50
L-Tryptophan	*C. glutamicum* KY9218/pIK9960	58	20–25
L-Tryptophan	*E. coli*	45	20–25
L-Phenylalanine	*E. coli* MWPWJ304/pMW16	51	20–25
L-Arginine	*Brevibacterium flavum* AJ12429	36	30–40
L-Histidine	*C. glutamicum* F81/pCH99	23	15–20
L-Isoleucine	*E. coli* H-8461	30	20–30
L-Serine	*Methylobacterium* sp. MN43	65	30–35
L-Valine	*C. glutamicum* VR 3	99	30–40

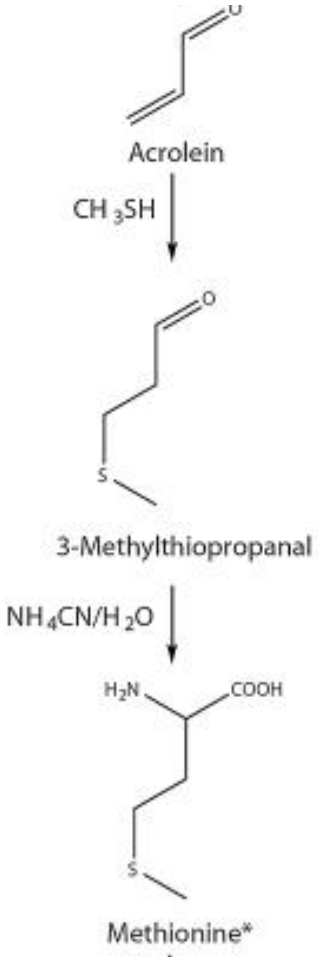
Large scale chemical production of D,L-Methionine

sulfur-containing amino acid methionine, is the first limiting amino acid in poultry, is of particular importance.

Researchers at Deutsche Gold- und Silber-Scheideanstalt (Degussa AG since 1980 of Evonik) studied the use of the synthetic amino acid methionine to treat the widespread nutritional edema, the result of chronic protein insufficiency suffered by soldiers returning home from the war. The first technically feasible synthesis of D,L-methionine at Degussa was achieved by Werner Schwarze, Hans Wagner and Hermann Schulz in 1946/47.

Large scale chemical production of D,L-Methionine

Despite the experience gained from lysine and threonine fermentation, attempts to develop a cost-effective production of L-methionine by the fermentation pathway have so far proved unsuccessful



The fact that the **D-form, not found in nature, is enzymatically converted into the nutritive L-form** in the animal organism by means of an oxidase and transaminase allows direct use of the synthetic racemic mixture.

Enzymatic production of enantiomerically pure aminoacids

- For other amino acids, there is no comparable enzyme system for conversion of the D-form, and there is no fermentation process with adequate yield.
- For these amino acids, it is necessary to produce the enantiomerically pure form using enzymatic procedures.
- The racemates are generally produced by chemical synthesis.

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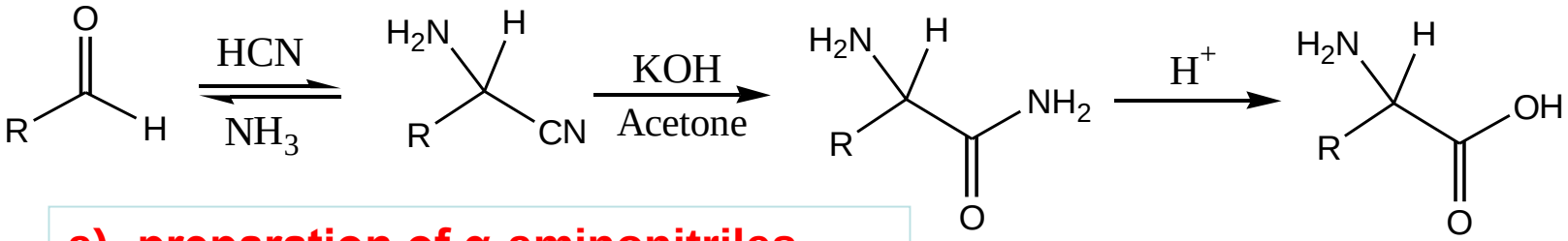
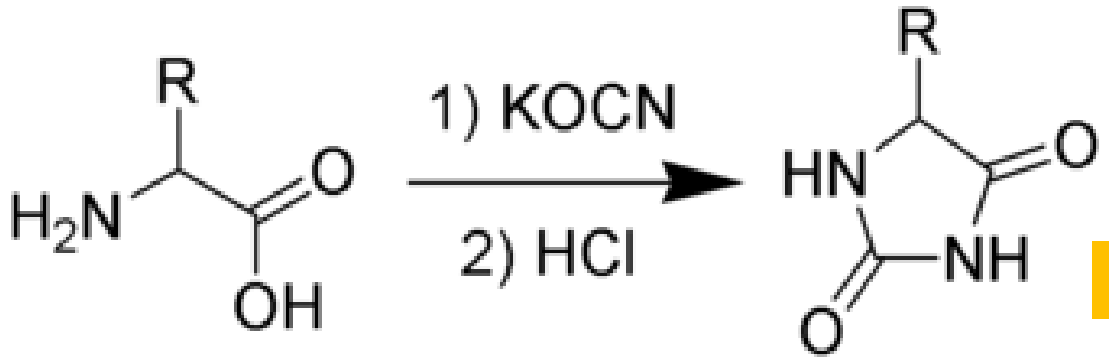
Synthesis of intermediates of D,L-amino acids:



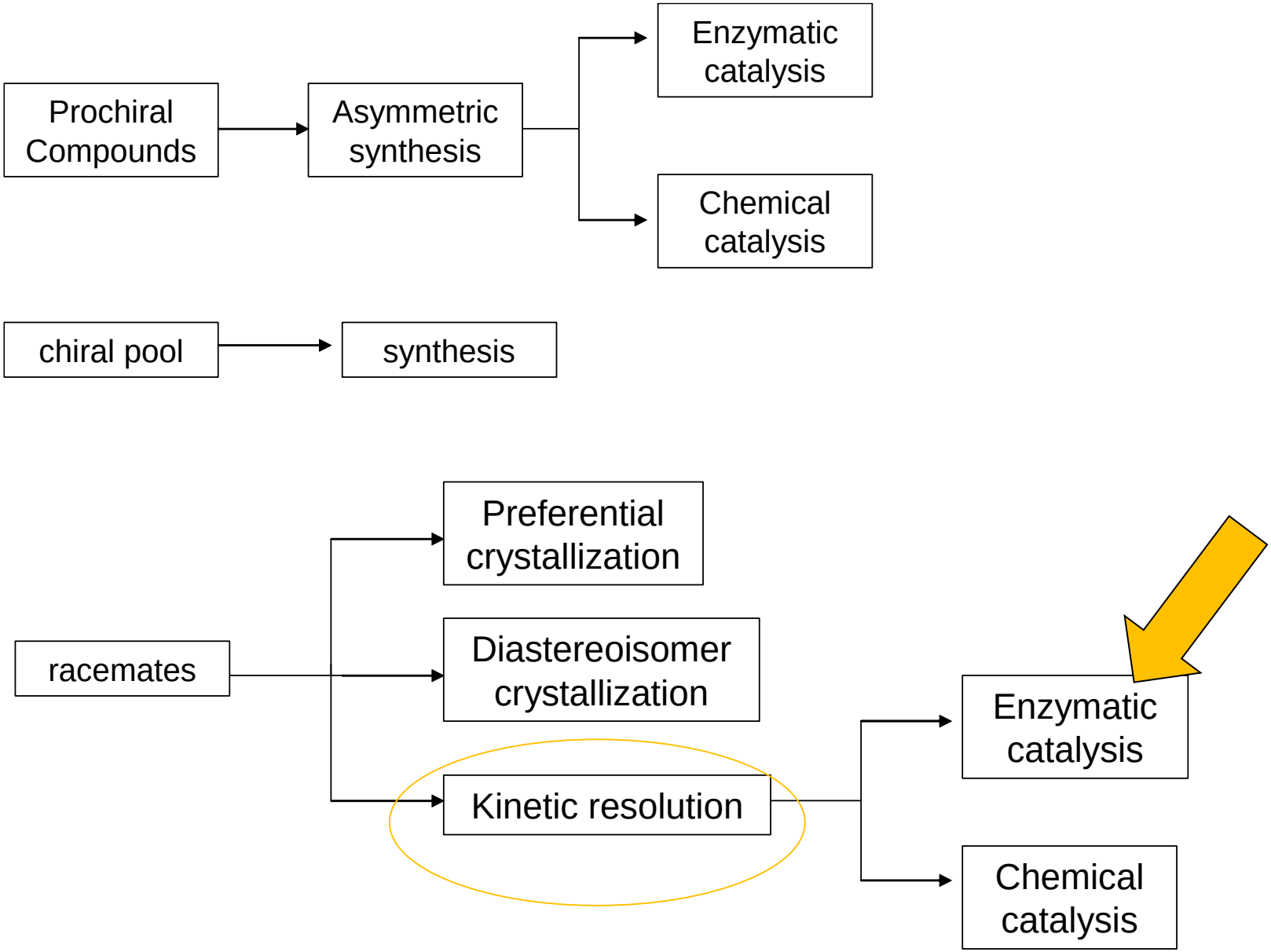
a) preparation of α-aminonitriles,
b) hydrolysis of the nitrile.

1. Strecker



2. Hydantoine

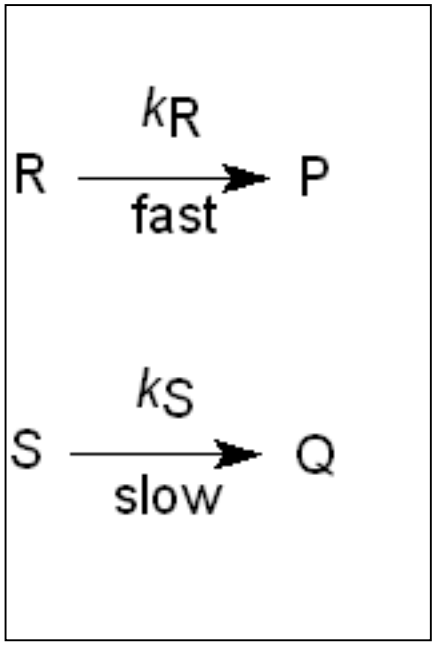
Synthetic methods for the production of pure enantiomers



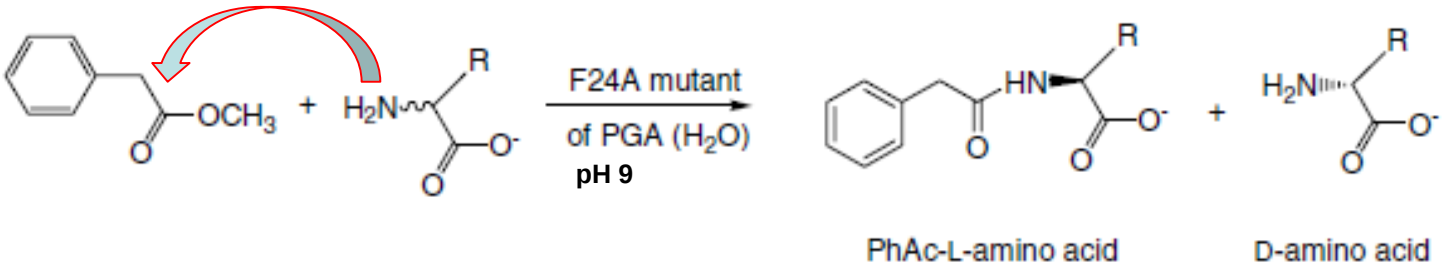
Kinetic resolution of racemates: typical application of hydrolases

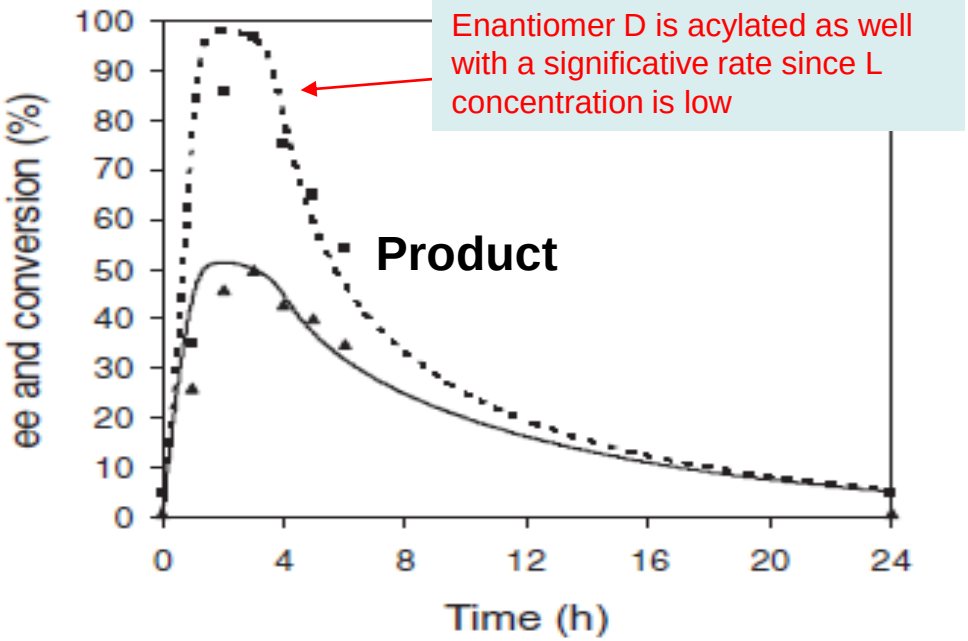
When an enzymatic catalytic reaction is followed in time, *ideally* only one enantiomer reacts and the reaction stops at 50% conversion.

- **Resolutions have a maximum theoretical yield of 50%**
- **Unwanted enantiomer is wasted or at best recycled**



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



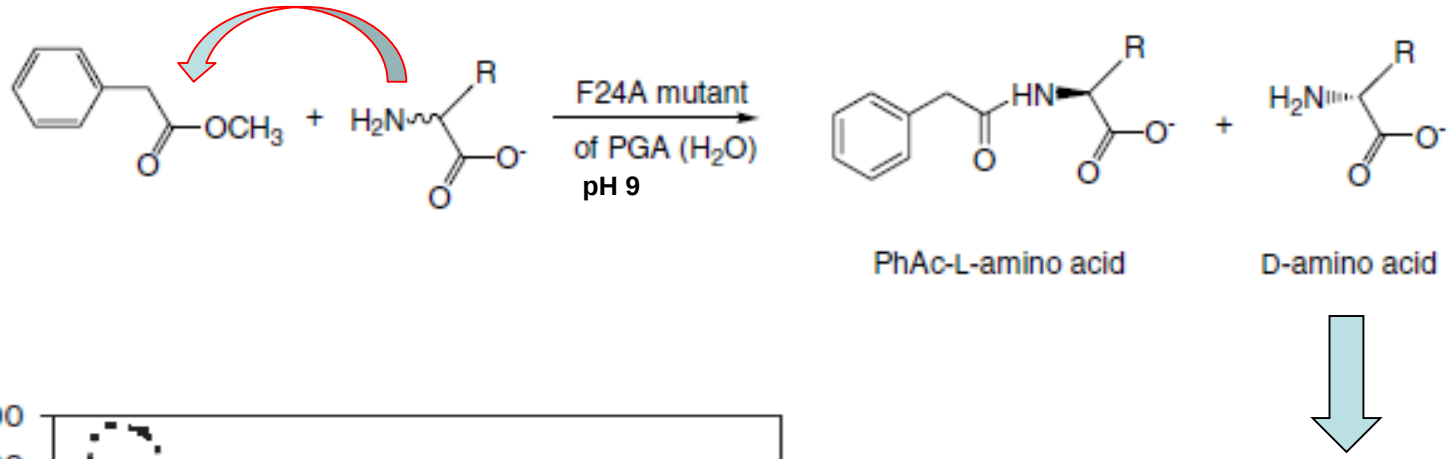


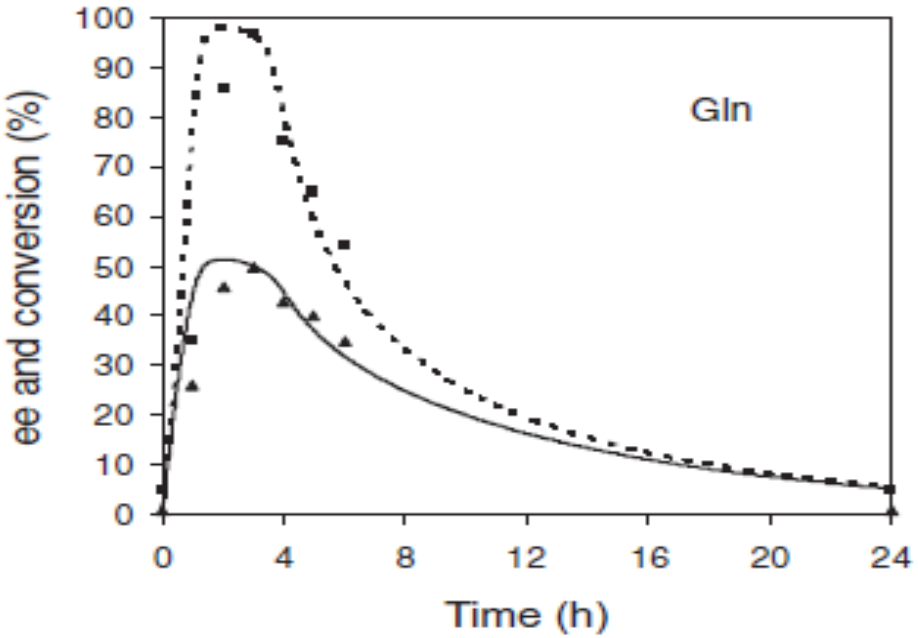
$$e.e.\% = \frac{c_R - c_S}{c_R + c_S} \times 100$$

NB!!! Changes throughout the reaction

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

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

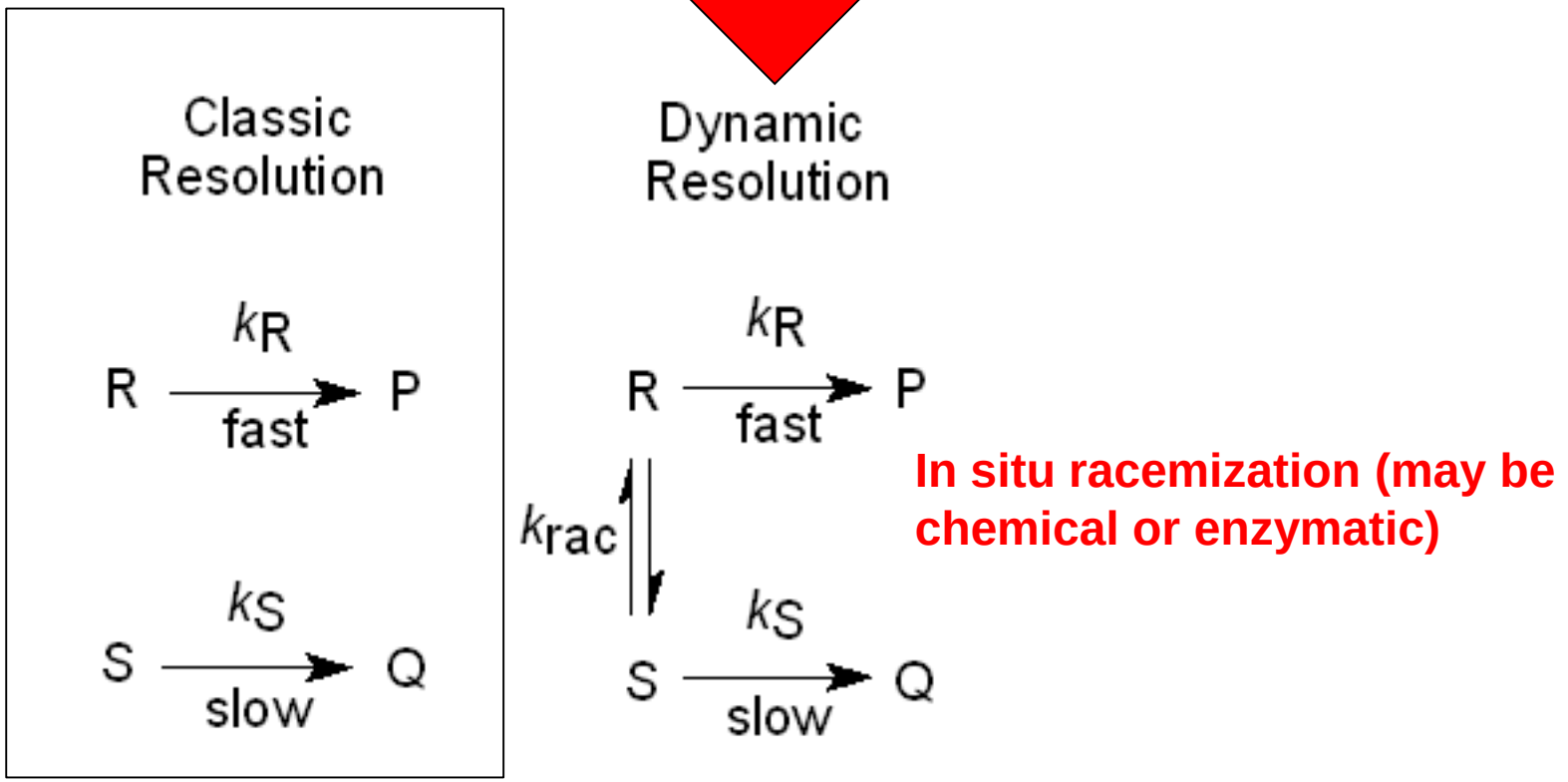




It is easier to achieve higher ee% of the unreacted reagent by pushing the reaction beyond 50% : lower yield but higher ee%

Reaction must be stopped at the most favourable time

How to overcome the maximum 50% yield?
Dynamic Kinetic Resolution

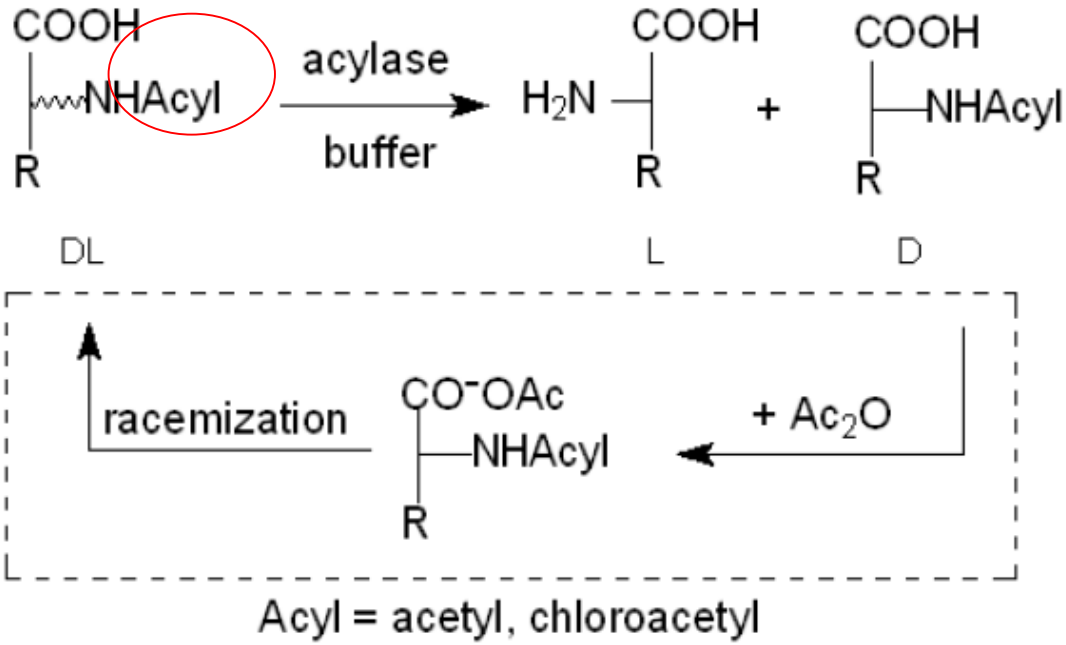


In situ racemization (may be chemical or enzymatic)

R, S = substrate enantiomers

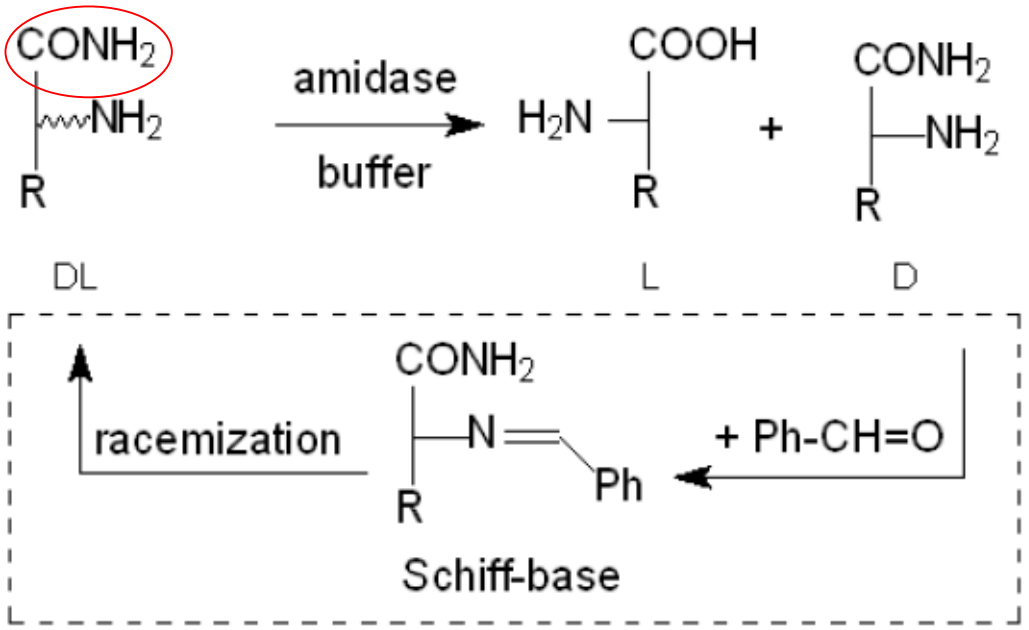
P, Q = product enantiomers

DKR: Hydrolysis of N-acylated amminoacids



- *rac* - *N* -acyl amino acids as substrates.
- use of acylases from porcine kidney or from *Aspergillus* or *Penicillium* sp.
- resolution of *N* -acetyl tryptophan and -phenylalanine on an industrial scale using immobilized enzymes in column reactors.
- the non-reacting D-enantiomer may be recycled *via* **racemization** of the corresponding **mixed anhydride** intermediate in a separate step.

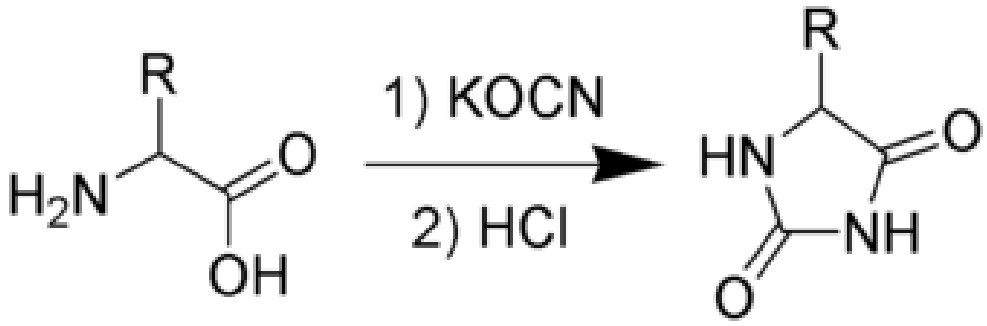
DKR: Hydrolysis of amides of aminoacids



- use of L-selective amidases from *Pseudomonas* , *Aspergillus* or *Rhodococcus* sp.,
- hydrolyze **L-amino acid amides** from a racemate.

The possibility to **recycle** the unreacted D-configured amide *via* its corresponding Schiff-base with benzaldehyde in a separate step makes this procedure economical.

Synthetic intermediates of D,L-amino acids: hydantoines



hydantoines

A promising route to enantiomerically pure amino acids, both L- and D-enantiomers, is based on conversion of hydantoins via hydantoinases and, additionally, carbamoylase.

5-substitued hydantoins

1. **hydantoinase**
2. **carbamoylase,**
3. **hydantoin racemases**

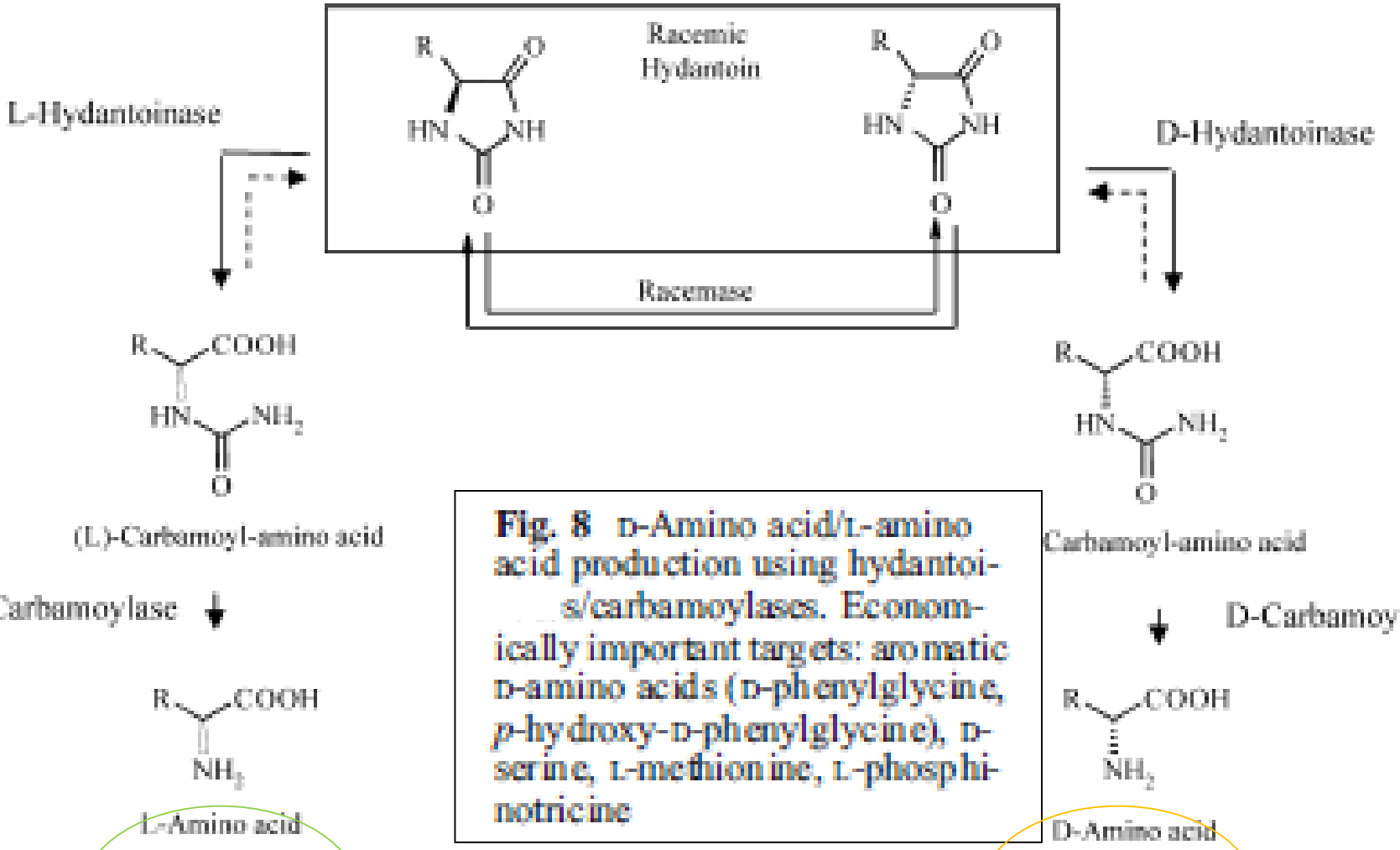


Fig. 8 D-Amino acid/L-amino acid production using hydantoi-s/carbamoylases. Economically important targets: aromatic D-amino acids (D-phenylglycine, *p*-hydroxy-D-phenylglycine), D-serine, L-methionine, L-phosphinotricine

Dynamic Kinetic Resolution (DKR)

Racemization using enzymes

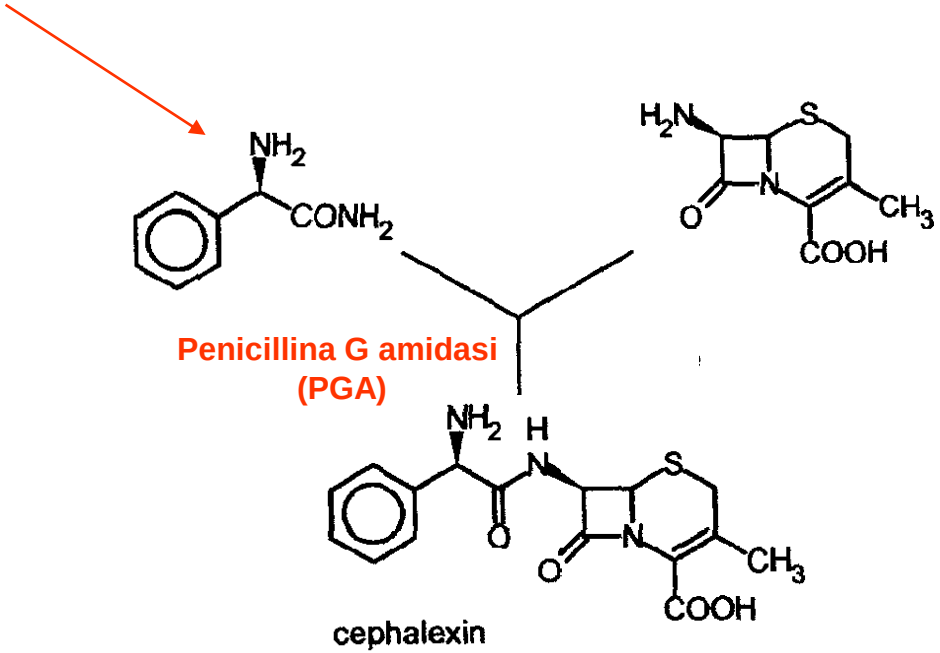
The use of an **enzyme,** rather than a transition metal catalyst, represents an attractive option for combined DKR reactions in view of the likely mild conditions associated with enzyme-catalyzed racemization processes.

Racemases belong to the group of enzymes EC 5.1.X.X and contain notable members such as mandelate racemase and various **amino acid racemases**.

Method of the 5-substitued hydantoins for side chain antibiotics production

D-Phenylglycin
D-p-hydroxyphenyglycine

Side chains of beta lactams antibiotics (ampicillin, cephalexin amoxycillin)



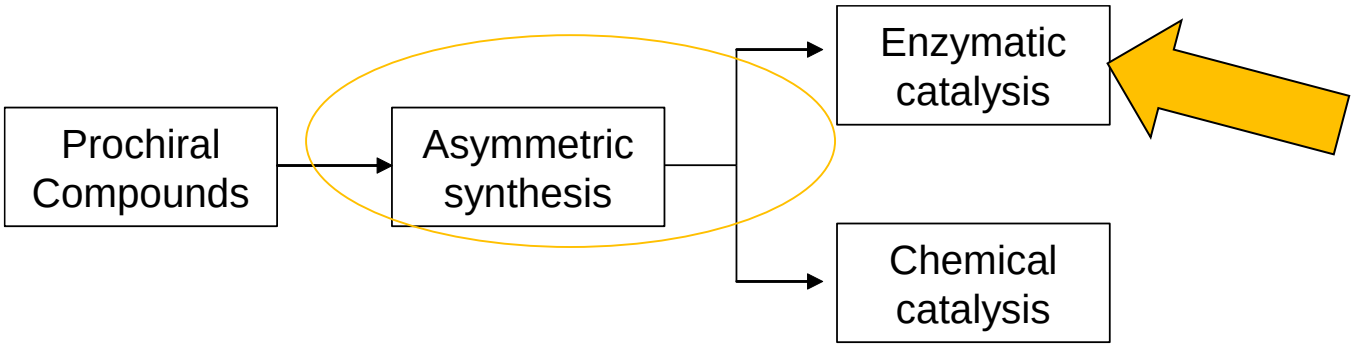
Recordati (IT)

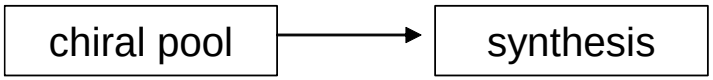
Method of the 5-substitued hydantoins

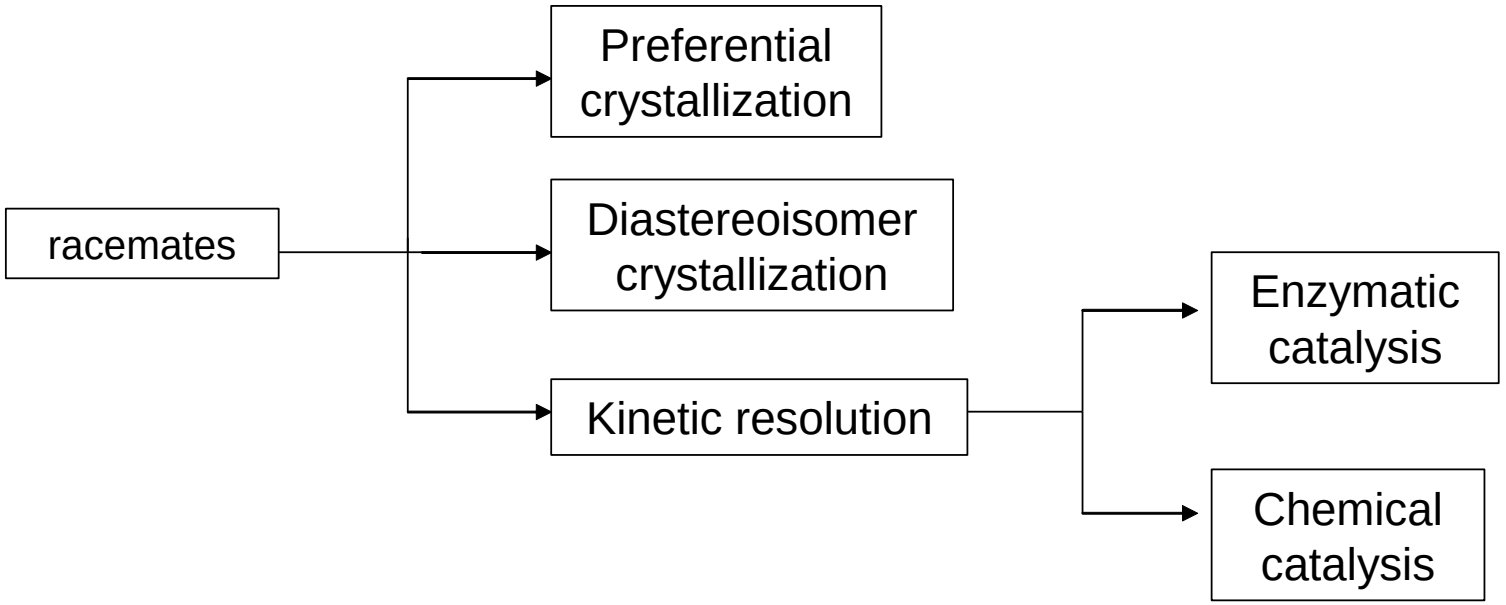
D-serine
L-methionine

Degussa (D):
whole **cells coexpressing** **L-carbamoylase + hydantoin racemase** + **hydantoinase**

Enantiomerically pure aminoacids *via* enzymatic asymmetric synthesis







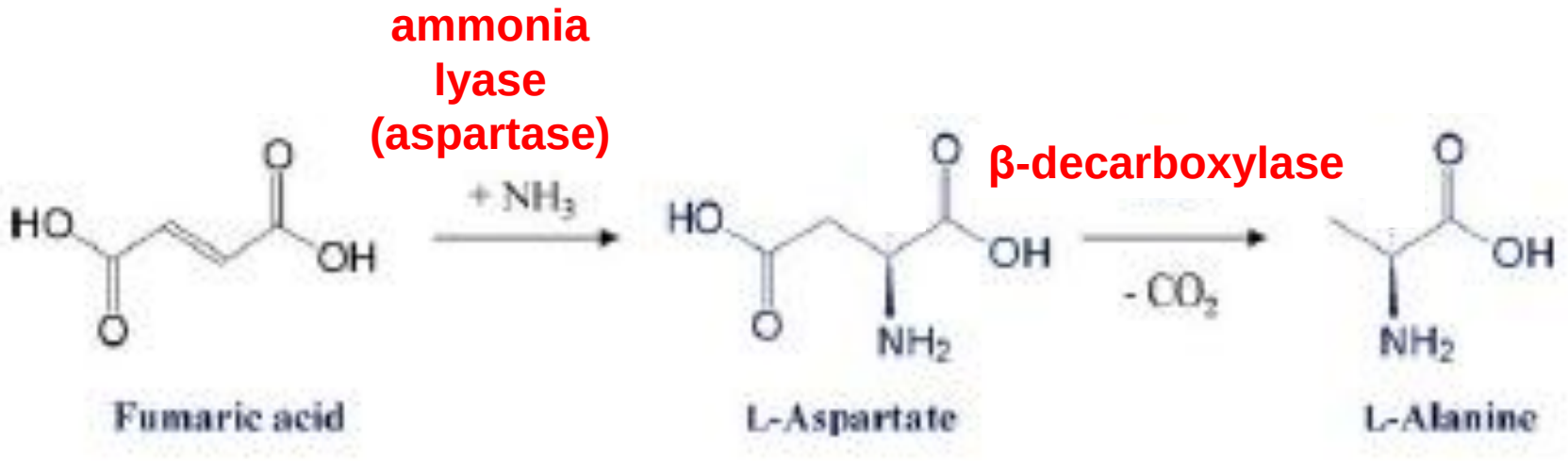
L-Aspartic acid: Asymmetric enzymatic synthesis

Achiral substrate + chiral (enantioselective) biocatalyst

1. addition of ammonia to fumaric acid catalyzed by **ammonia lyase** from *E.coli* , also called aspartase

2. L-aspartate (which is required in large quantities for the sweetener Aspartame).

3. aspartate **β-decarboxylase** from *Pseudomona dacunhae* trasforms aspartic acid into **L-alanine**



- **Lyases catalyze the addition or removal of a chemical group without passing through hydrolysis, oxidation, transfer**

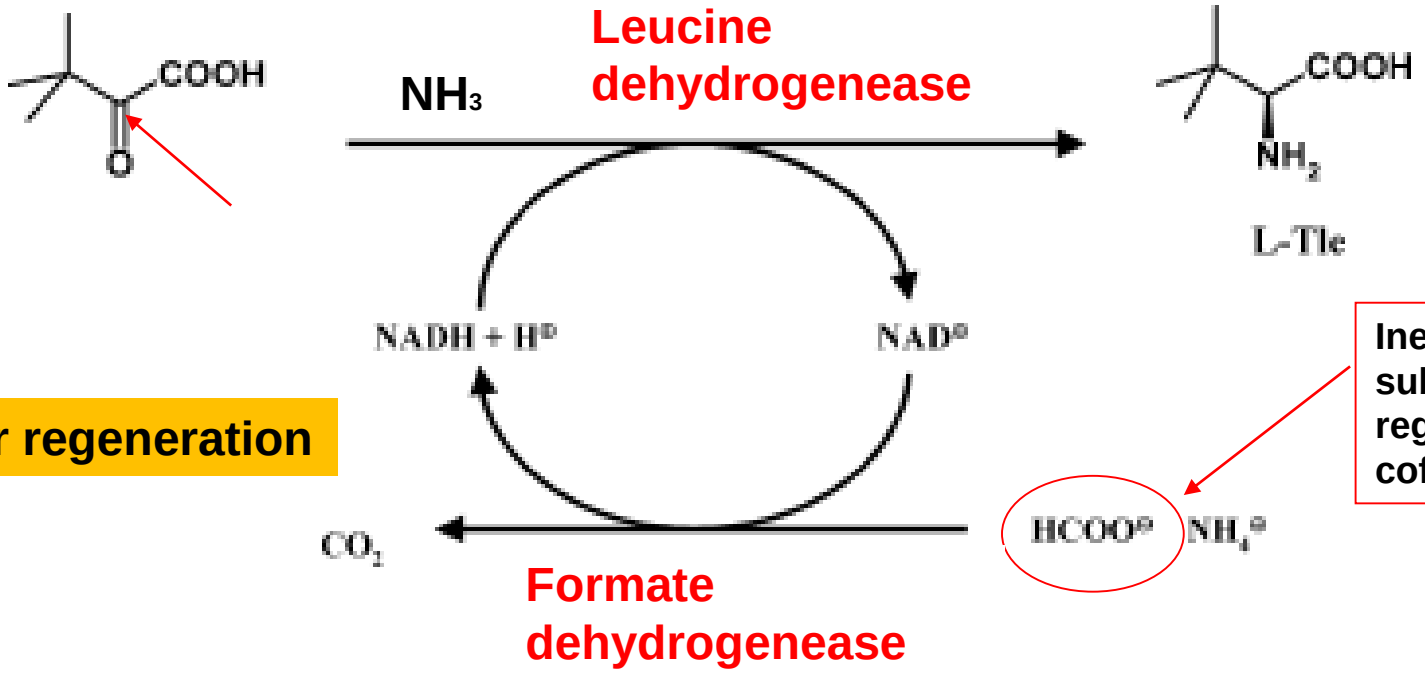
Lyases can act on

- **C - C bonds (decarboxylases; aldolases)**
- **C – O (hydratases or dehydratases)**
- **C – N**
- **C – S**
- **C – X**

Asymmetric enzymatic synthesis

Synthesis of L-*tert*-leucine via reductive amination of trimethyl pyruvate

***Prochiral compound*:**



Cofactor regeneration

Use of recombinant *E. coli* coexpressing **Leucine dehydrogenase** and NAD+-dependent **Formate dehydrogenase**.

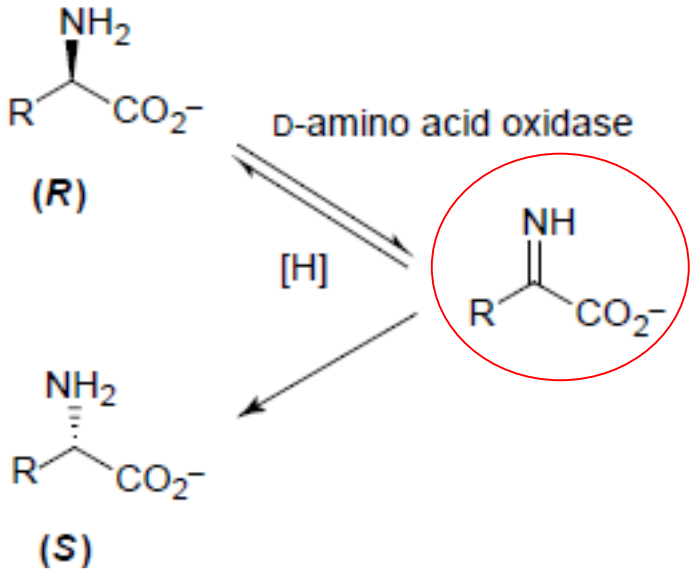
Deracemisation

Deracemisation reactions tend to **involve redox processes**, for example the **interconversion of amino acids via the corresponding imine**

1. combines a highly **enantioselective amino acid or amine oxidase**

2. with a ***nonselective chemical reducing agent***

R,S
Only R oxidized to achiral imine
Achiral amine reduced to both R and S



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.

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

1 Aripripazolo: Chinolinone, è una molecola che viene utilizzata come antipsicotico atipico.L'aripiprazolo è utilizzato soprattutto nel trattamento della schizofrenia, del disturbo bipolare e del disturbo depressivo maggiore

2 L'esomeprazolo è un inibitore di pompa protonica (IPP), una molecola utilizzata per la terapia di patologie gastrointestinali acido-correlate, quali l'ulcera e la malattia da reflusso gastroesofageo (GERD), oltre che per la prevenzione di possibili lesioni gastriche derivanti dall'assunzione di farmaci FANS.

3 La duloxetina è il principio attivo di un antidepressivo appartenente alla classe degli inibitori della ricaptazione della serotonina e noradrenalina (SNRI).

4 Adalimumab, noto anche come D2E7, è un anticorpo monoclonale umano IgG1 specifico per il fattore di necrosi tumorale alfa (Tumor Necrosis Factor alpha, TNF-alfa), approvato per il trattamento di artrite reumatoide, artrite idiopatica giovanile poliarticolare, artrite psoriasica, psoriasi, morbo di Crohn, spondilite anchilosante, colite ulcerosa.

5 La *rosuvastatina* (Crestor, Provisacor) è un farmaco usato per abbassare il colesterolo nei pazienti con livelli elevati. Appartiene alla classe delle statine.

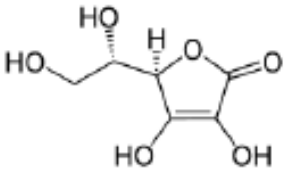
CHIRALITY AND PHARMACEUTICALS

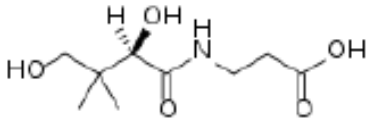
Recent rulings of the Food and Drug Administration (FDA) in the United States reflect the current situation in "chiral drugs": pharmaceutical industries will have to provide rigorous justification to obtain the FDA's approval of racemates.

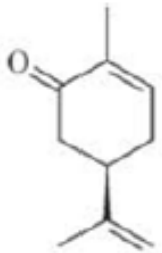
The chiral pool refers to relatively inexpensive enantiomerically pure products (approximate prices of 2-100 US $/Kg), which are readily available from nature, for example by plant extraction or fermentation (amino acids, alkaloids, lactic acid, e.g.), in the range of 10^2 to 10^5 tonnes per annum.

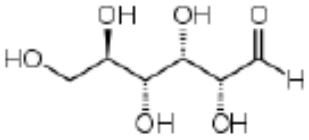
- Carboidrati C6, C4
- Terpeni: limonene, carvone, carene, pineni, derivati della canfora
- Alcaloidi
- Etc.

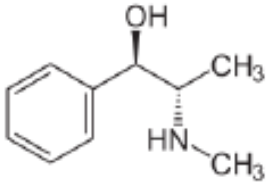
Chiral pool











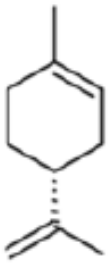
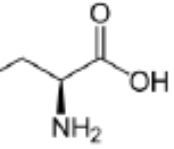
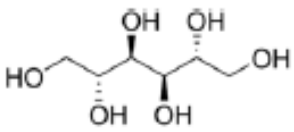


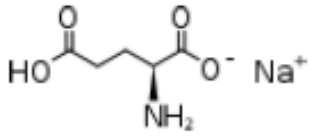
Table 2. Representative substances from the chiral pool[a]

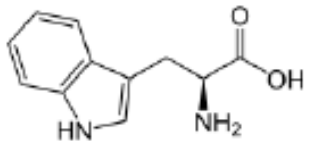
Compound	Approx. price (US dollars kg^{-1})
Ascorbic acid	13
(+)-Calcium pantothenate	16
(−)-Carvone	23
Anhydrous dextrose	1.2
Ephedrine hydrochloride	62
(+)-Limonene	3
L-Lysine	3.2
Mannitol	7.5
Monosodium glutamate	2
Norephedrine hydrochloride	24
Quinidine sulphate	130
Quinine sulphate	75
Sorbitol	1.7
L-Threonine	12–50, depending on grade
L-Tryptophan	68

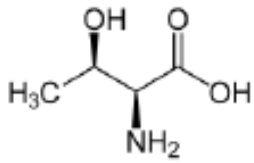
[a] Data from *Chemical Marketing Reporter*, Schnell Publishing, New York, 13 April (1990); reproduced by permission of the Editor.

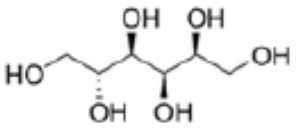


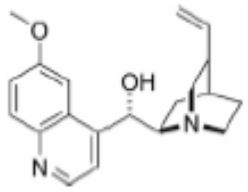


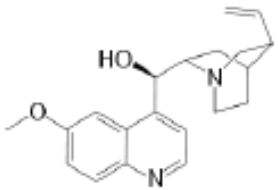


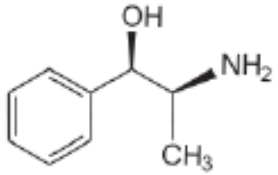


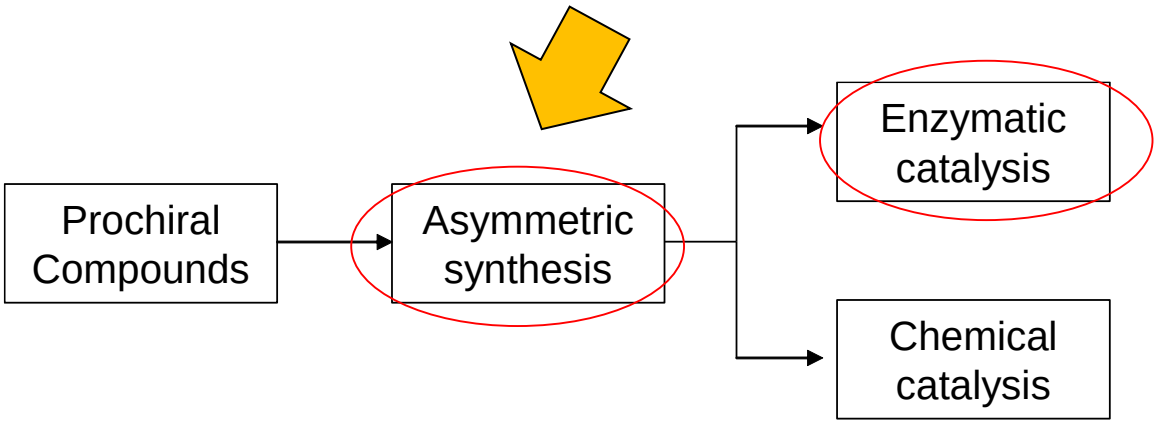


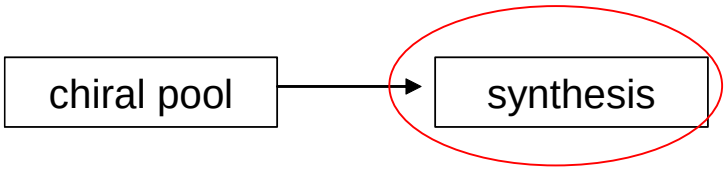




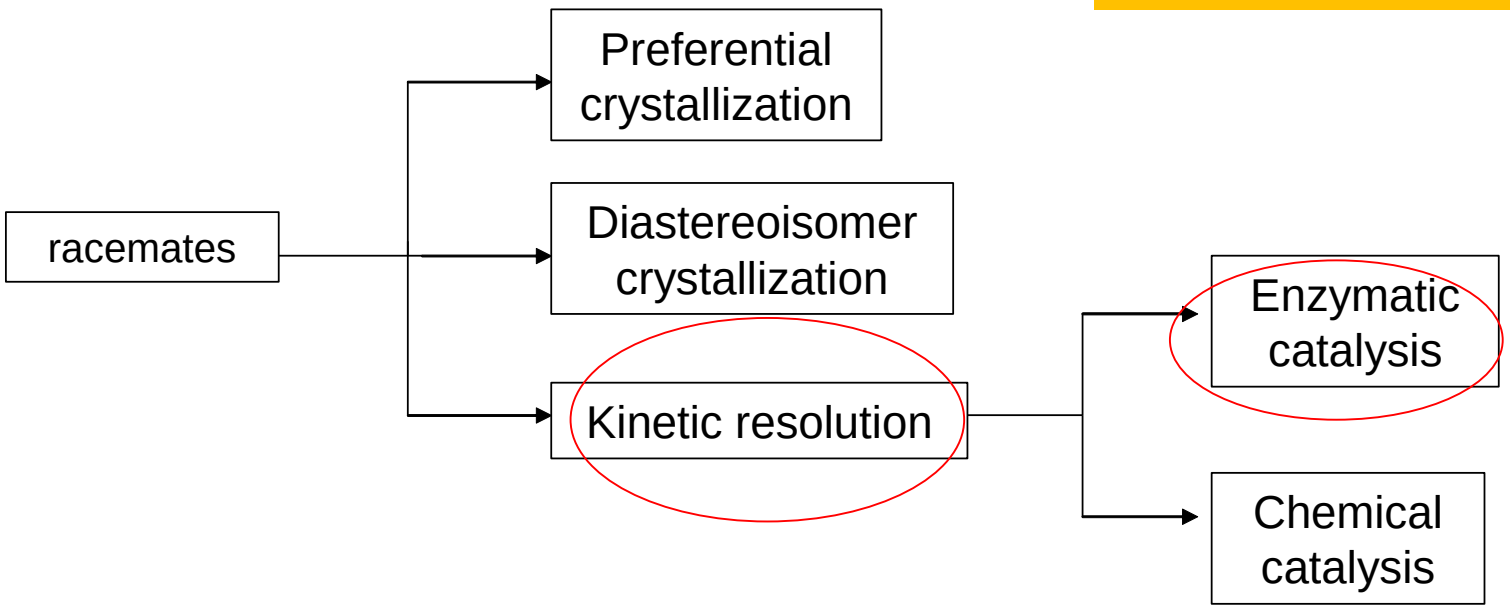








Synthetic methods for production of pure enantiomers



Asymmetric synthesis for the production of statin intermediates

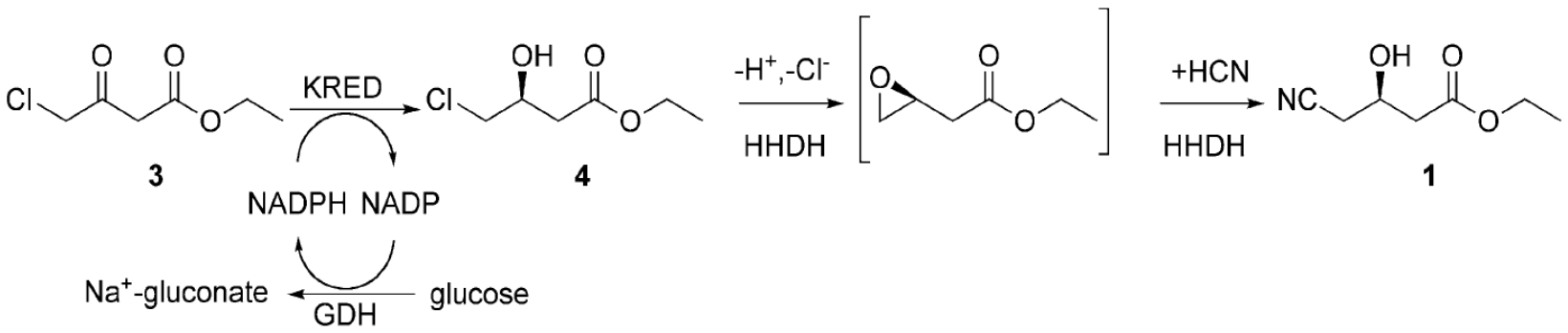
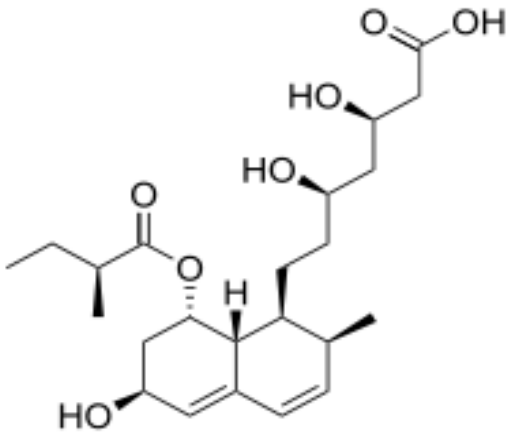


Fig. 4 Two-step, three-enzyme process for hydroxynitrile **1**.

1. **Ketoreductase**
2. **Glucose dehydrogenase for cofactor recycling**



pravastatina

3. halohydrin dehalogenase (HHDH) catalyses the replacement of the chloro substituent with cyano by reaction with HCN at neutral pH and ambient temperature

Asymmetric synthesis for the production of statin intermediates

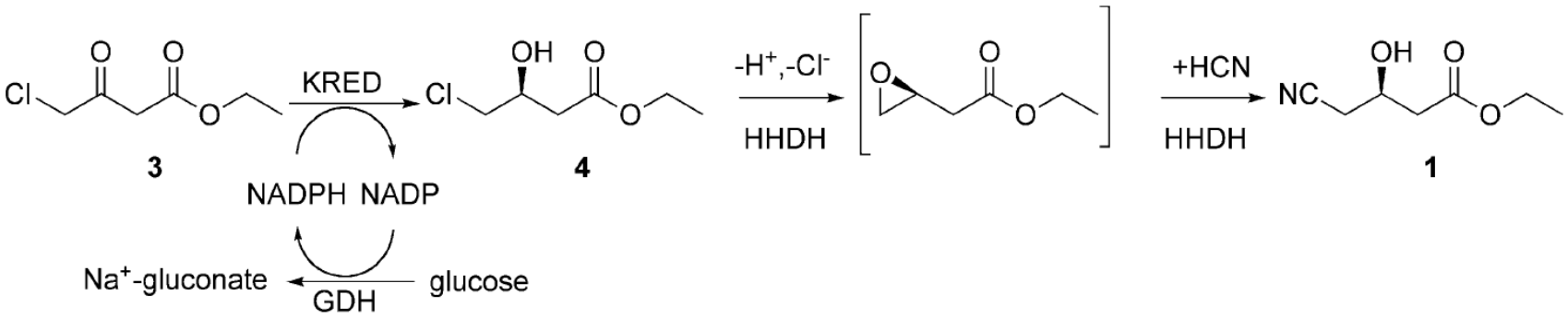


Fig. 4 Two-step, three-enzyme process for hydroxynitrile **1**.

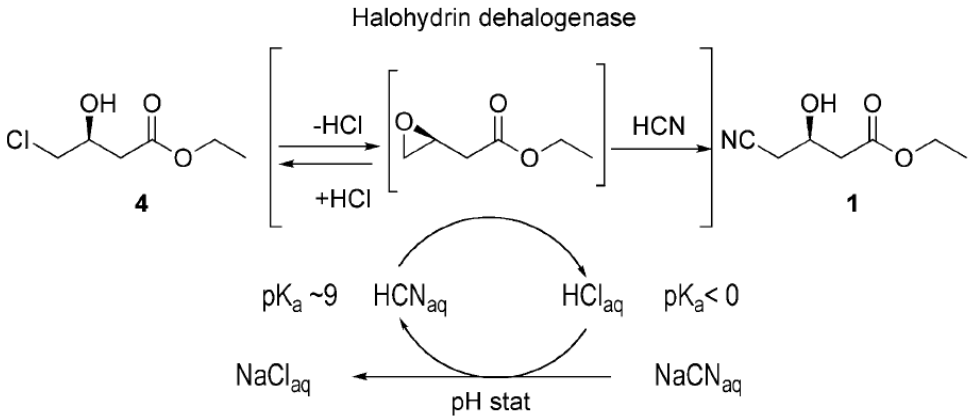


Fig. 6 HHDH-catalyzed cyanation of **4**.

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

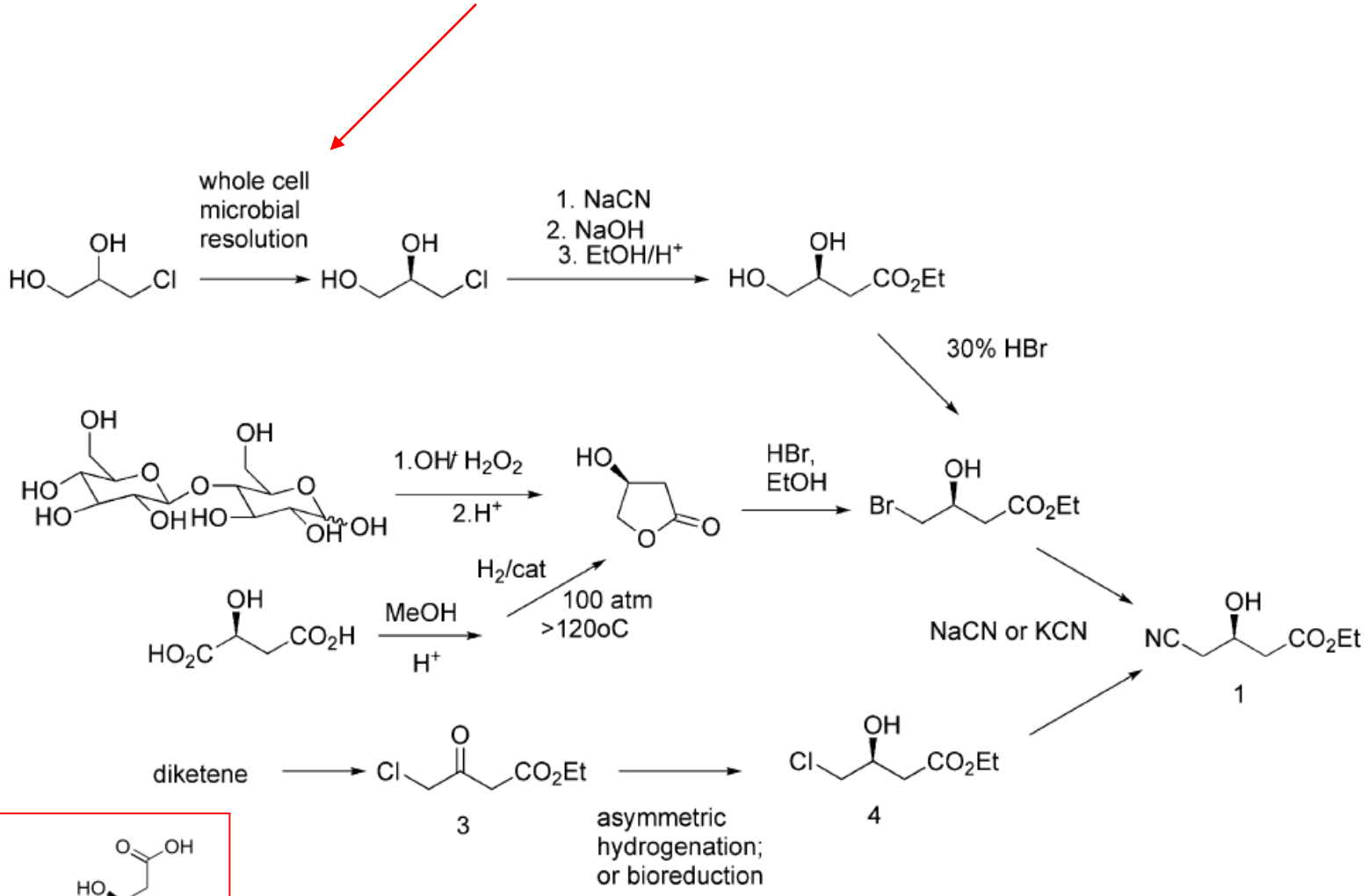
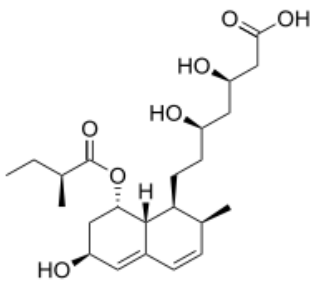


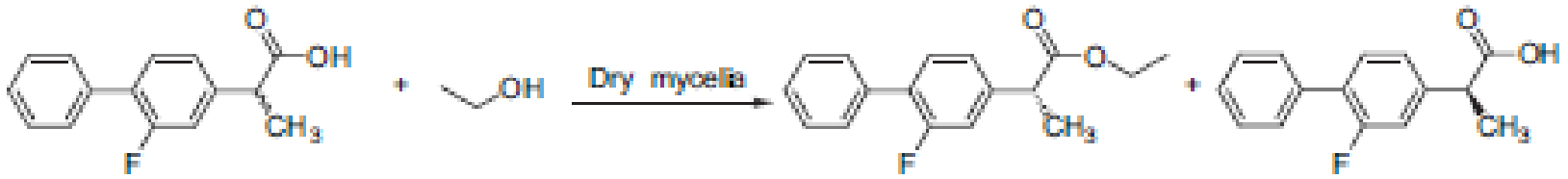
Fig. 3 Previous routes to the hydroxynitrile intermediate **1**.



pravastatina

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

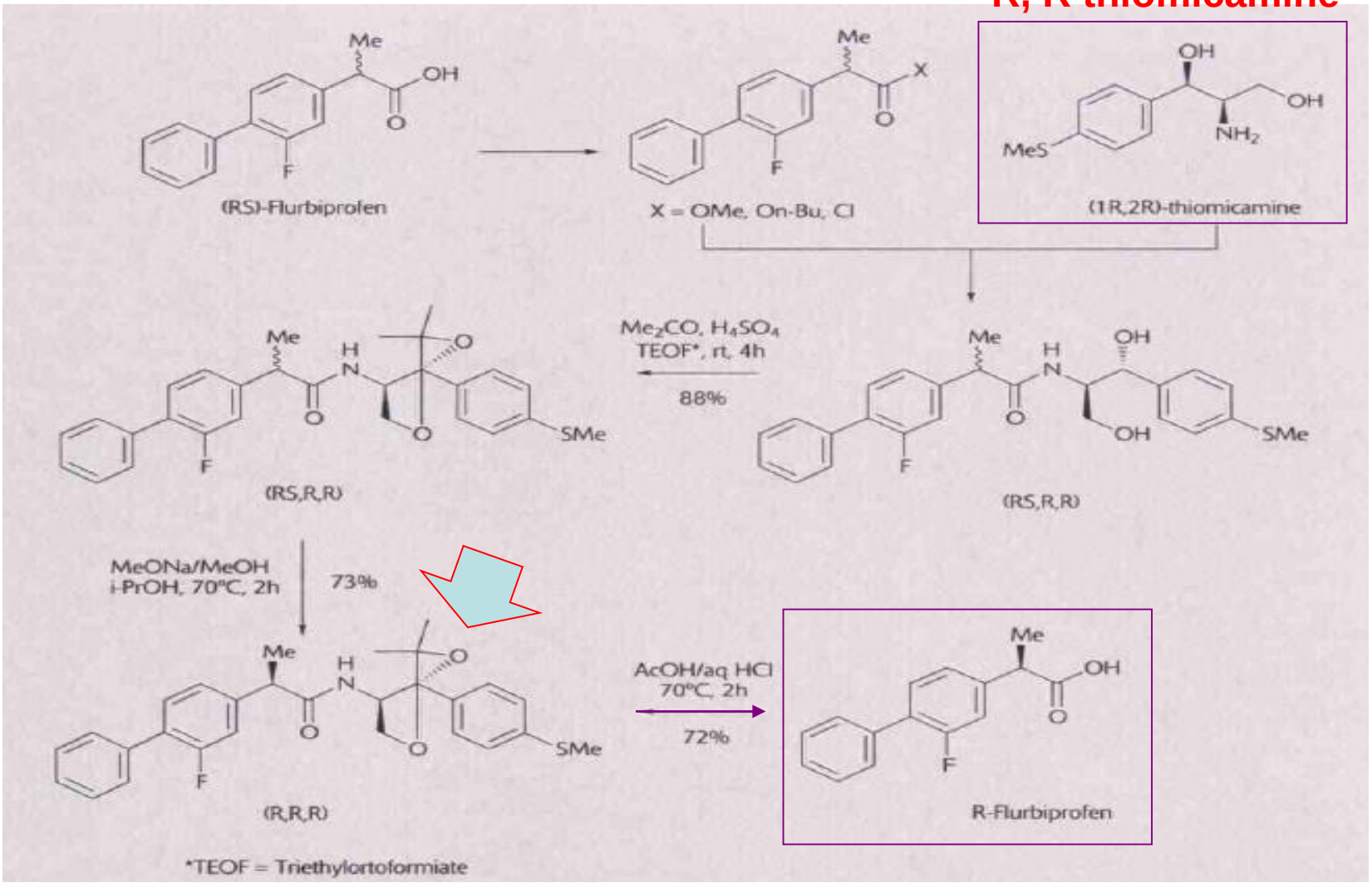
Dry mycelia of *Aspergillus oryzae* in toluene

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.**

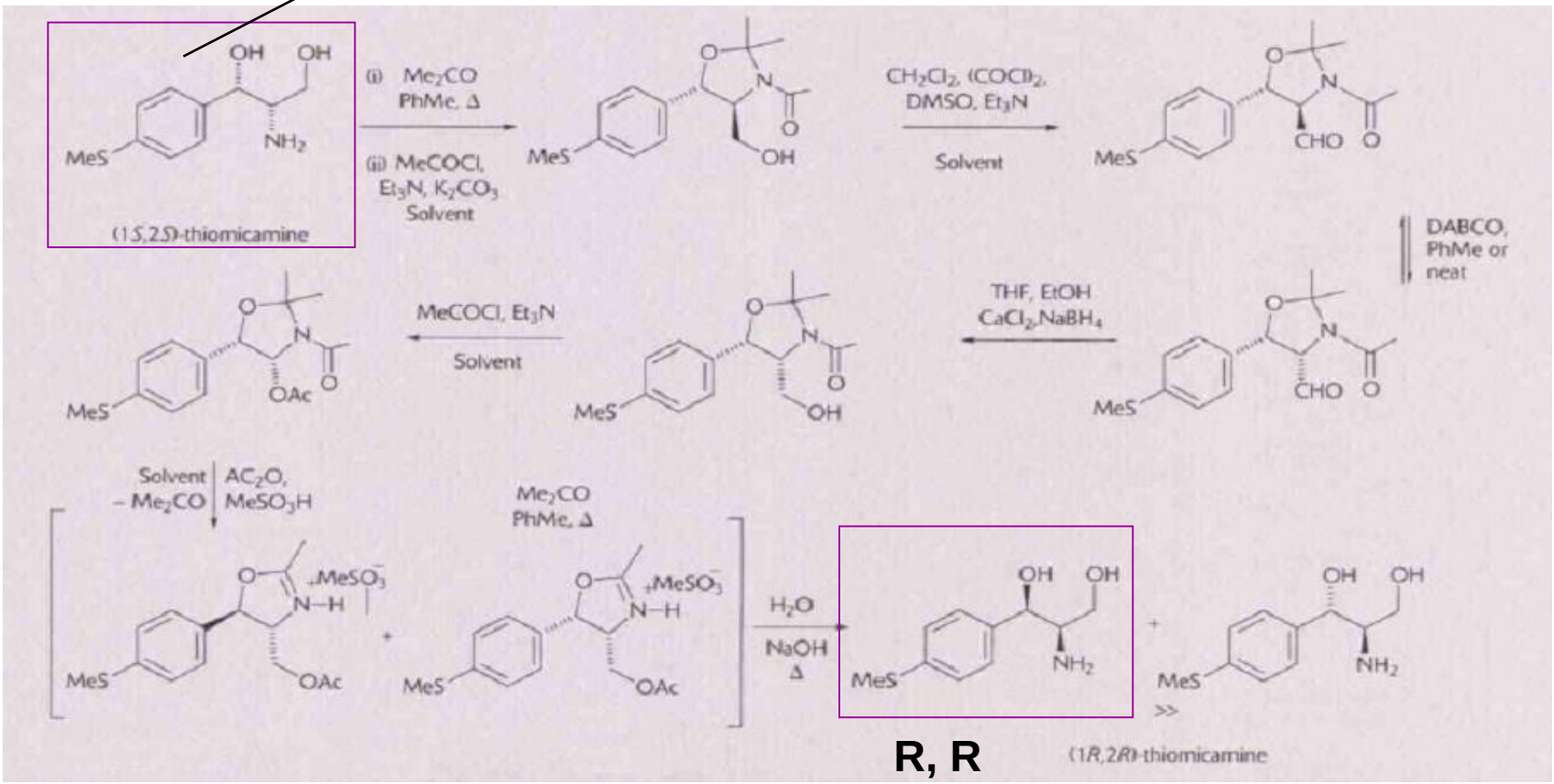
To date it has been the most commonly used industrial technique.

Chemical industrial resolution Zambon Group S.p.A

R, R thiomicamine



S, S: Intermediate in the production of tiamfenicolo



R, R

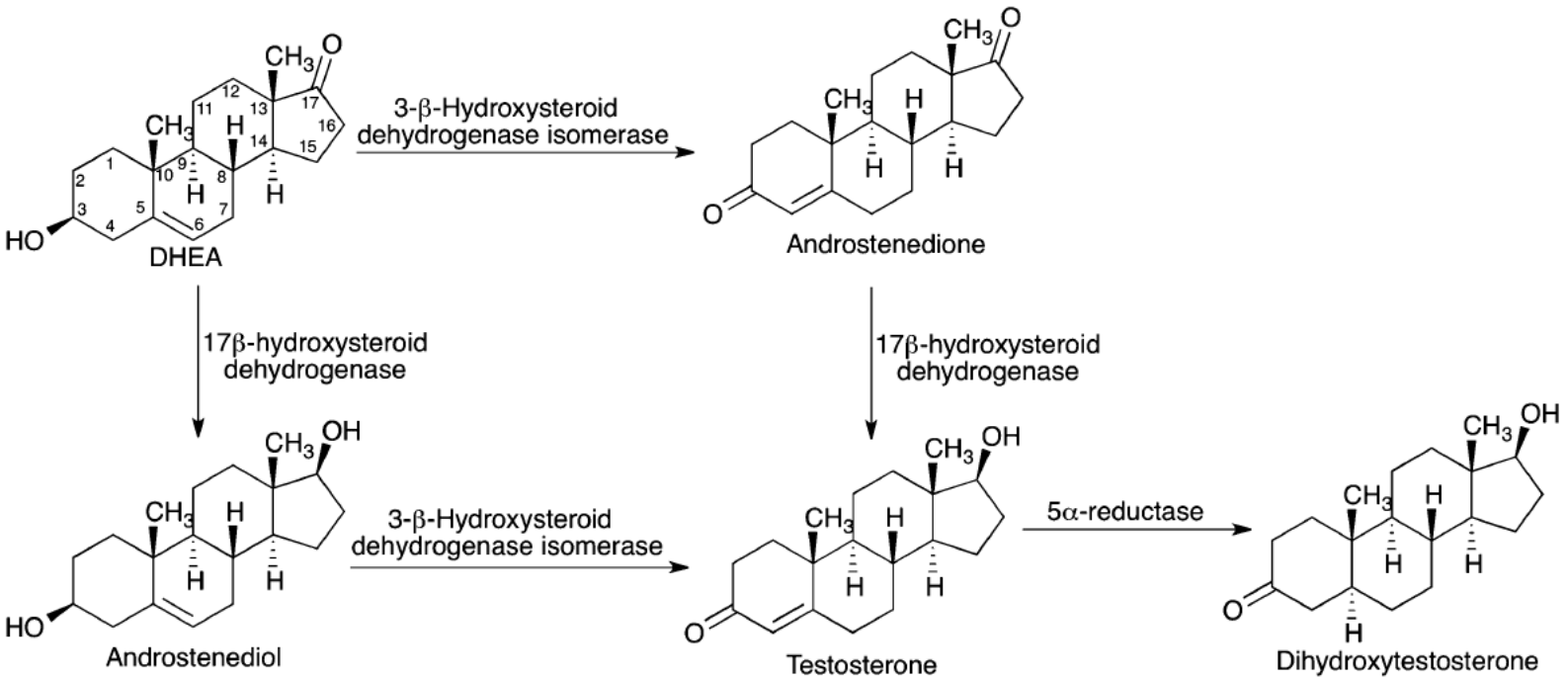
ZAMBON GROUP SpA

TIAMFENICOLO GLICINATO ACETILCISTEINATO

€ 43,38

Steroids synthesis

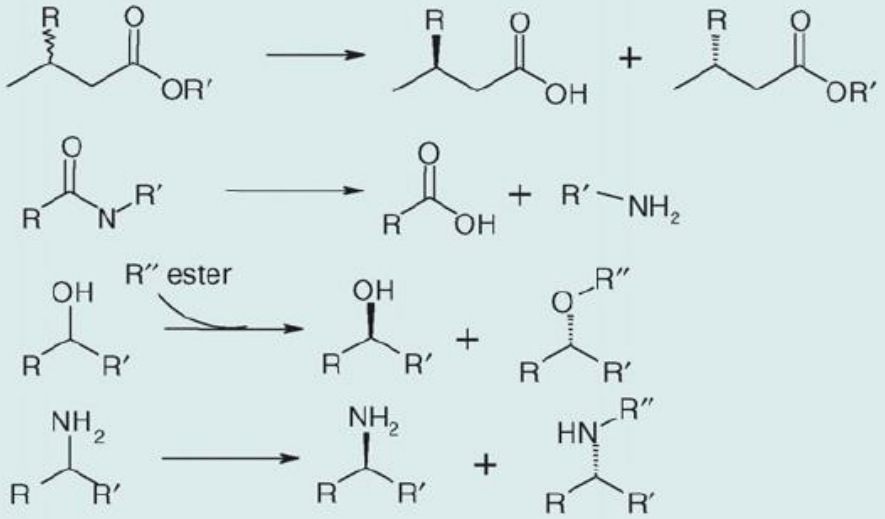
DHEA (deidroepiandrosterone), natural steroid (max in humans 15-20 anni)
Precursor of estrogens and androgens
produced from natural vegetal sources (tropical plant)



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

Established chemistries

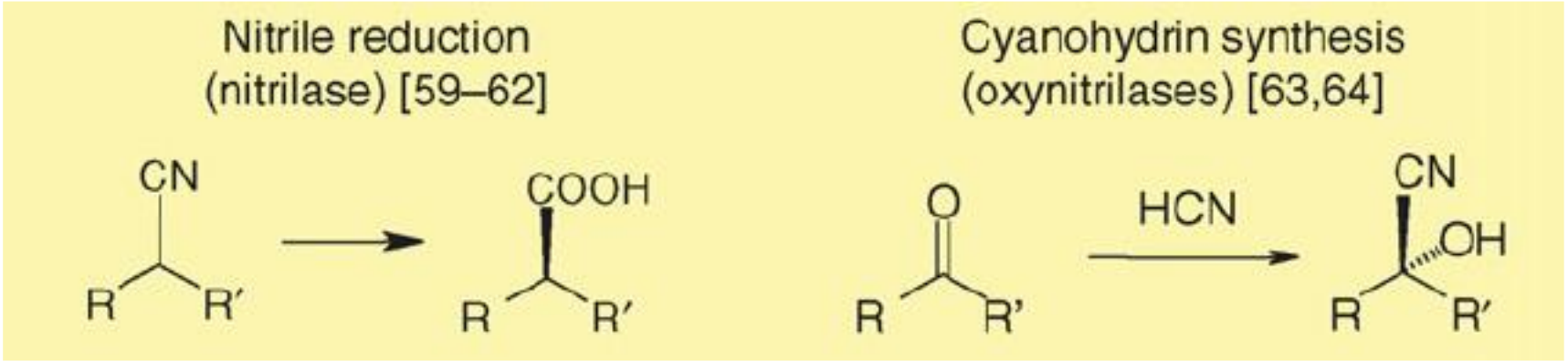
Resolution (lipase/protease) [20,21,34,56]



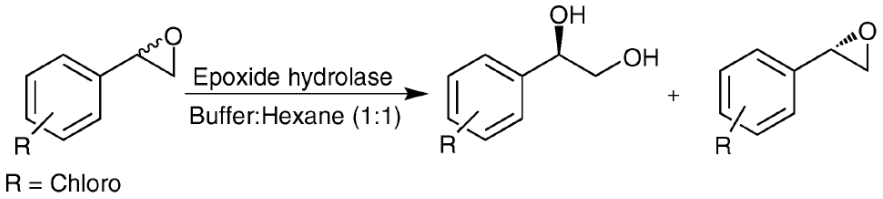
Hydrolysis (lipase/protease) [56,57]

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

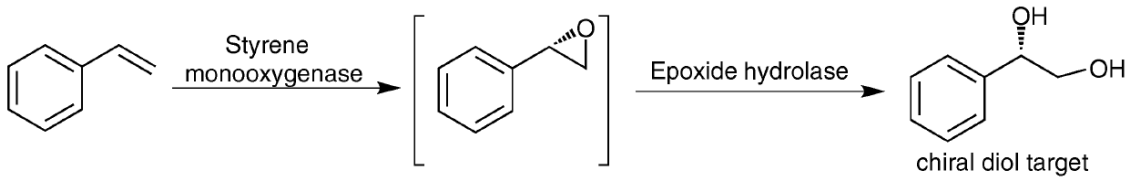
Playing with nitrile group



Playing with epoxides

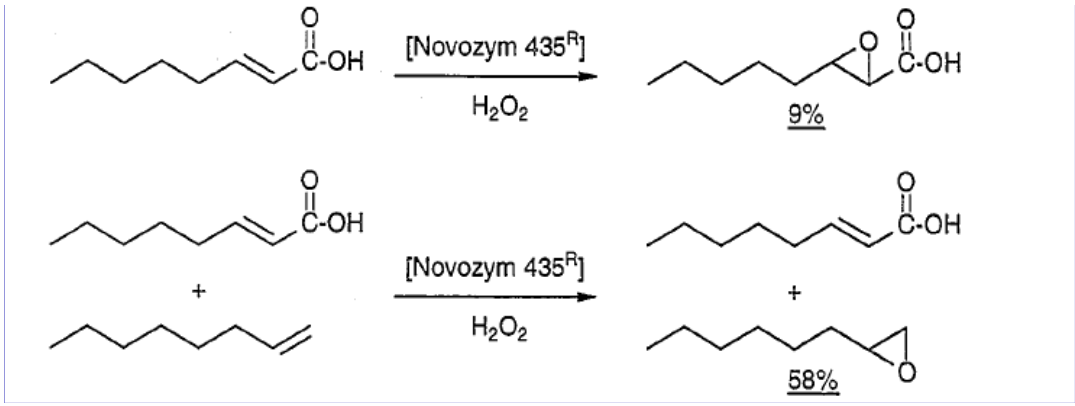


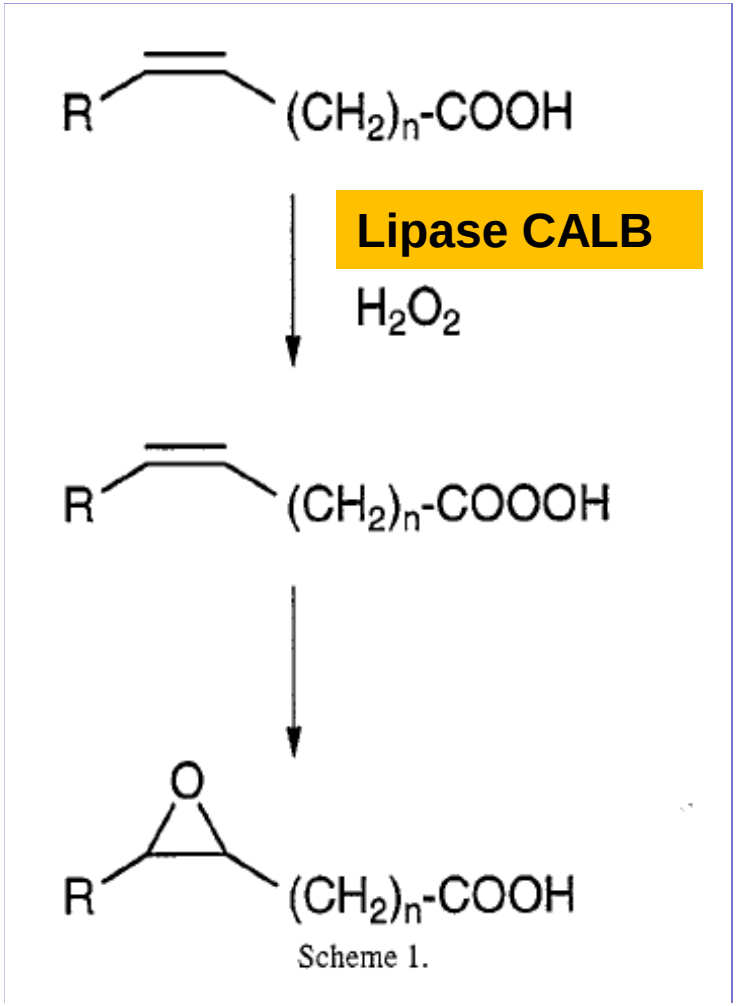
Scheme 6 Biocatalytic kinetic resolution of racemic 2-, 3-, and 4-chlorostyrene oxides using epoxide hydrolase.



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

Chemo enzymatic epoxydation of unsaturated fatty acids

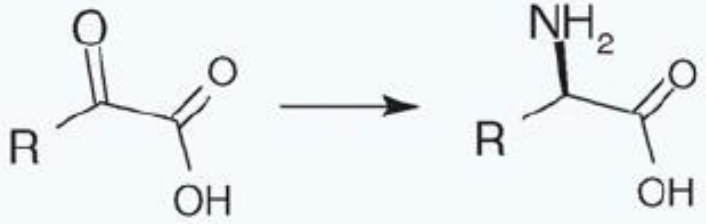




Scheme 1.

Emerging chemistries

Transamination (transaminase) [53,65,66]

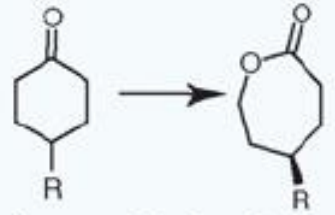


Enoate reduction (enoate reductase) [67–69]

Hydroxylation (cytochrome P450) [70,71]

Dihdroxylation (cytochrome P450) [72,73]

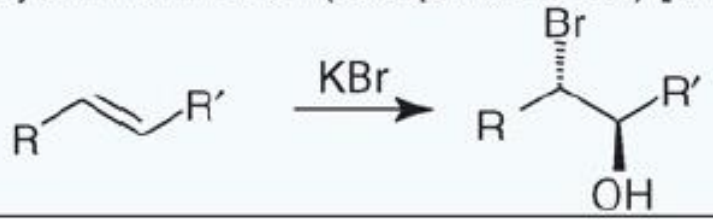
Baeyer-Villiger (monooxygenase) [74]



Hydrolysis (epoxide hydrolase) [75–77]

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

Halohydrin formation (haloperoxidase) [81–82]



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Biocatalysis for pharmaceutical intermediates: the future is now

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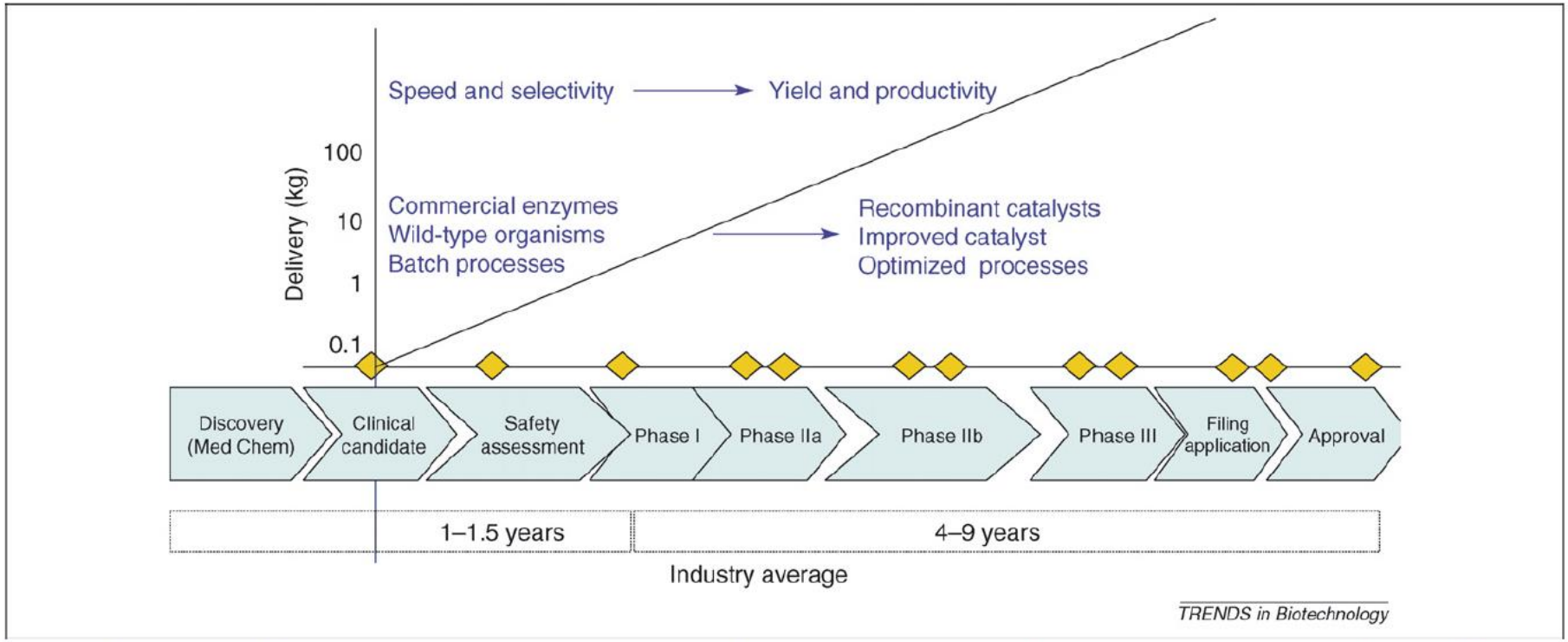


Figure 2. Development timeline for pharmaceutical products and processes. Initial development requires rapid synthesis of compounds to provide material for safety assessment. Later stage enables development time for optimized processes using recombinant catalysts. Abbreviation: Med Chem, medicinal chemistry.

Expressing quantitatively enantioselectivity in biocatalysis

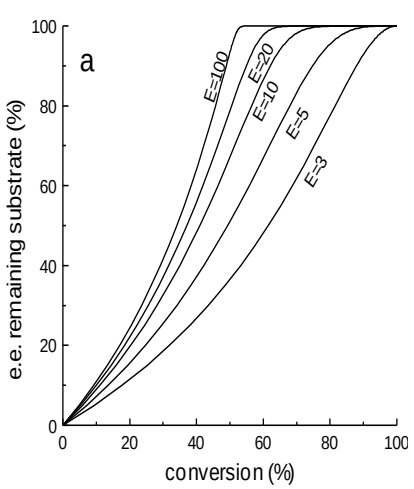
selectivity: $\dfrac{(k_{cat}/k_m)_R}{(k_{cat}/k_m)_S}$

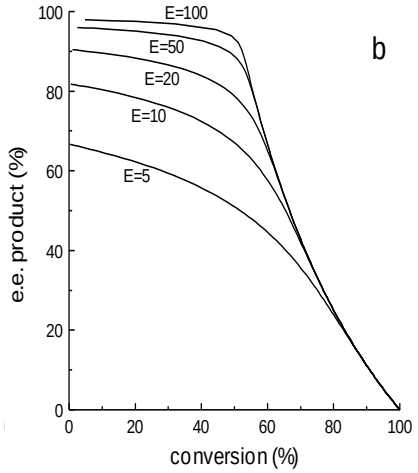
$$e.e.\% = \frac{c_R - c_S}{c_R + c_S} \times 100$$

Ratio of specificity constants

"E" : Enantiomeric Ratio

ee% changes during the reaction and is not informative





Calculating “E ” experimentally

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

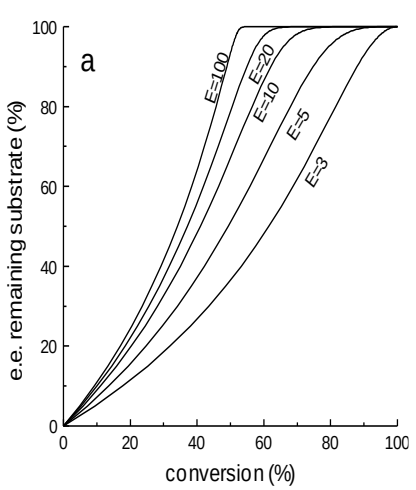
$$E = \frac{\ln[(1-c)(1-ee(L))]}{\ln[(1-c)(1+ee(L))]} = \frac{\ln[1-c(1+ee(P))]}{\ln[1-c(1-ee(P))]}$$

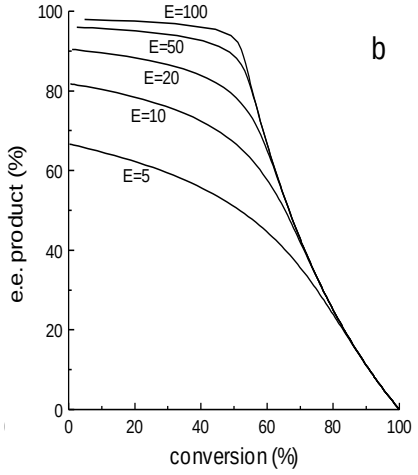
L: left, Remained unreacted
P: product

Experimental quantitative work:

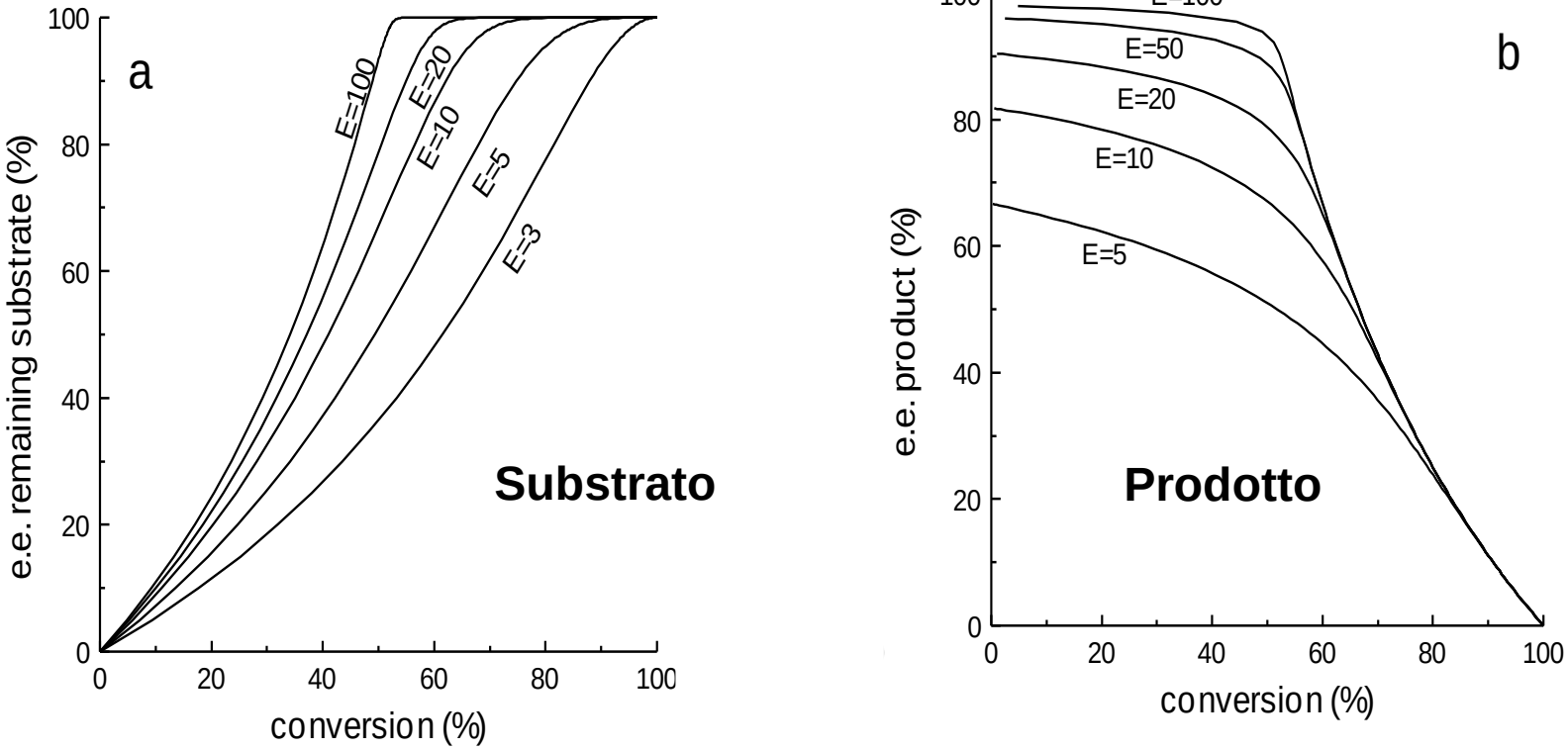
“*c*” (concentration by menas of HPLC or ^{1}H NMR)

e.e. % (chral GC or HPLC)





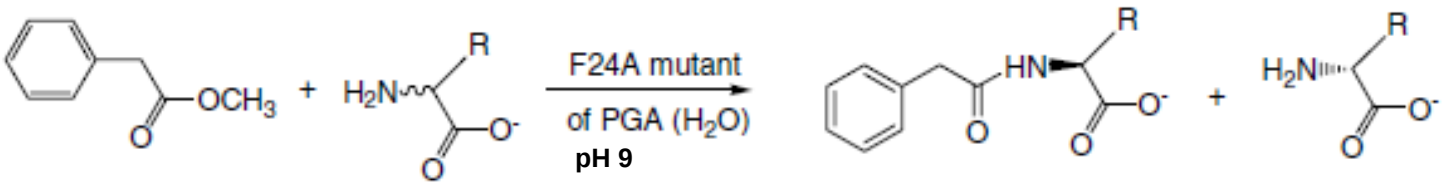
“E value” for practical applications

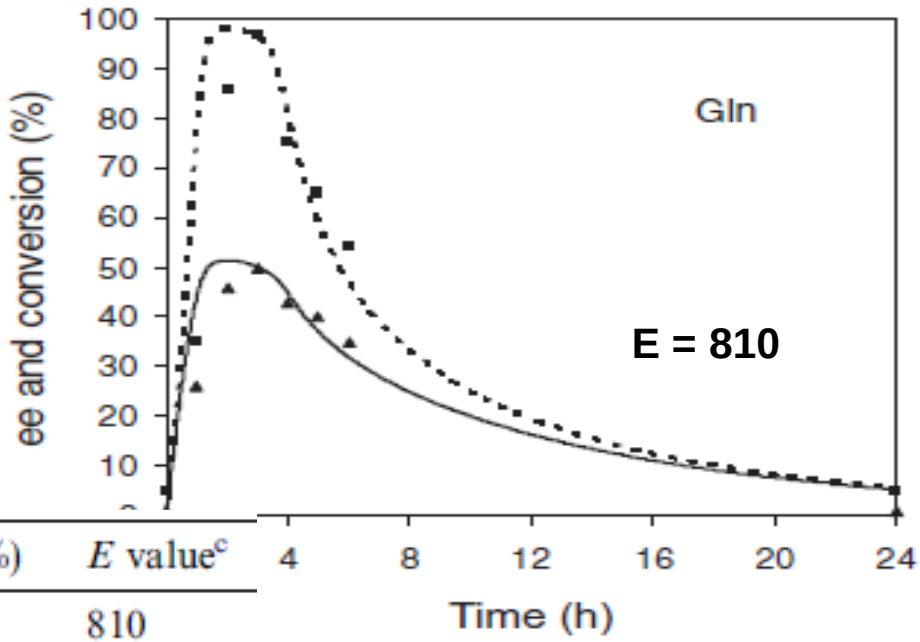


for the left unreacted substrate: E >20 (yield≈ 40%; e.e.% >99).

Product enantiomerically pure: E > 100

Esempio: risoluzione di amminoacidi





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