ALMA MATER STUDIORUM UNIVERSITY OF BOLOGNA

Dipartimento di Chimica “G.Ciamician”

# Biocatalysis: mechanism, synthesis, an applications. Part II

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Scuola di Dottorato in Scienze Chimiche

Bologna, 23 giugno 20

# Some selected milestones of industrially relevant biotransformation and biocatalytic processes

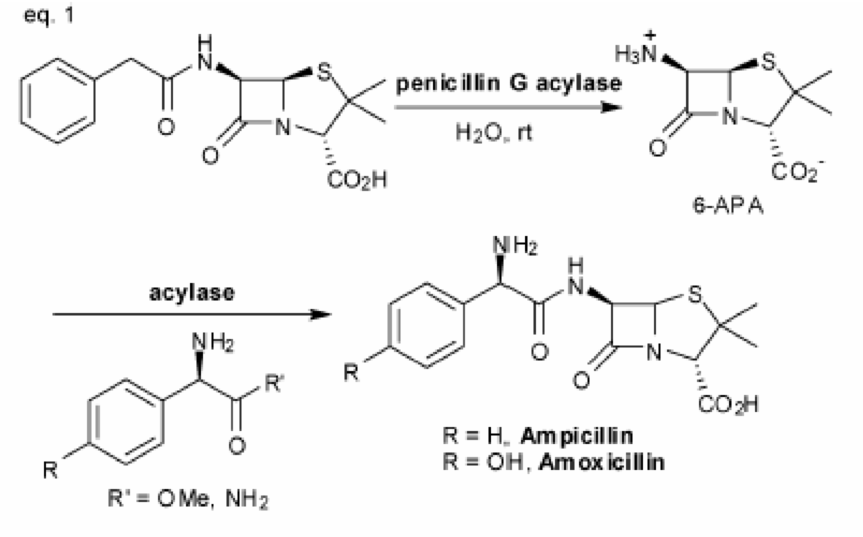
| year | process |
|---|---|
| 5000 BC | Vinegar production |
| 800 BC | Casein hydrolysis with chymosin for cheese production |
| 1670 | “Orlean” process for the industrial bio-oxidation of ethanol to acetic acid |
| 1680 | Antoni van Leeuwenhoek first to see microorganisms with his microscope. |
| 1897 | E. Buchner discovers yeast enzymes converting sugar into alcohol |
| 1934 | Regioselective biooxidation of sorbite to sorbose for the Reichstein Vitamin C synthesis |
| 1940 | Sucrose inversion using an invertase |
| 1950 | Bioconversion of steroids |
| 1970 | Hydrolysis of penicillin to 6-aminopenicillanic acid |
| 1973 | First successful genetic engineering experiments |
| 1974 | Glucose to fructose isomerisation with immobilized glucose isomerase |
| 1985 | Enzymatic process for the production of acrylamide |
| 1990 | Hydrolysis by protease (trypsin) of porcine insuline to human insuline |



*Organic Process Research & Development* **2006**, 10, 572-580

# Nomenclature

**Bioconversion:** Chemical conversion of a substance using biological methods (enzymes or whole cells, biocatalysis or biotransformation).




**Biotransformation:** Chemical conversion of a substance into a desired product with the aid of a living whole cell, containing the necessary enzyme(s).

**Biocatalysis:** Chemical conversion of a substance into a desired product with the aid of a free or immobilized enzyme.

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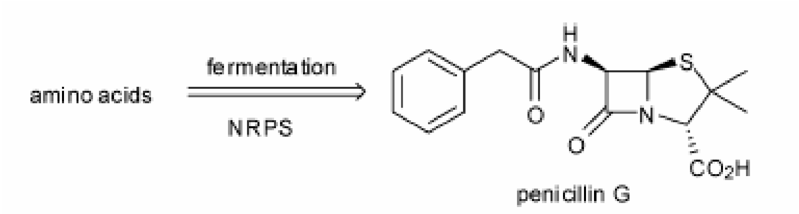
**Biosynthesis:** De novo production of an entire molecule by a living organism. Unlike biotransformation which acts on a starting substance or educt, biosynthesis is not dependent on educts or starting substances, but only on nutrients.

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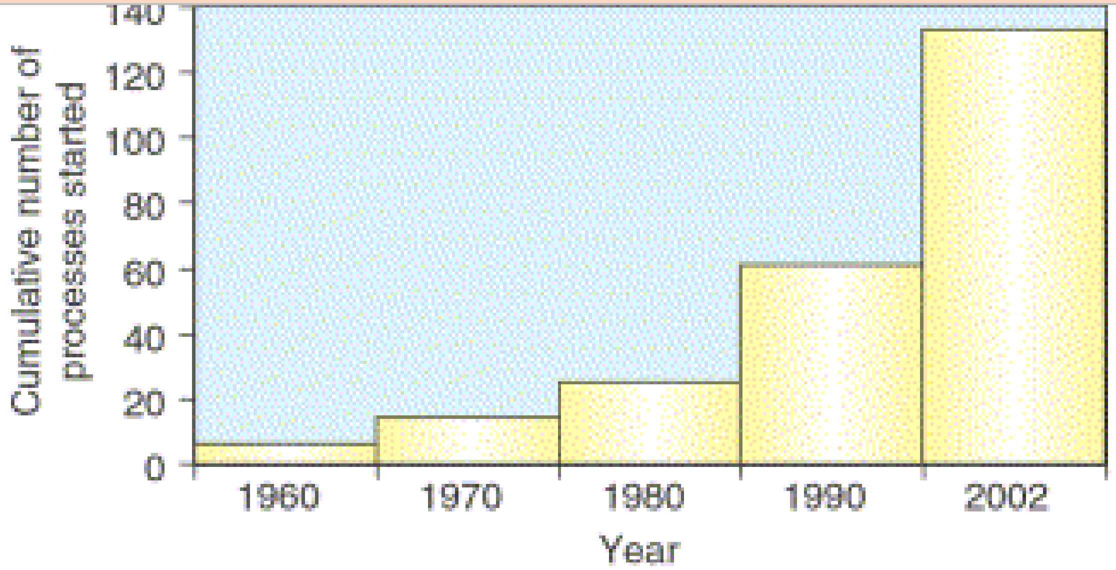
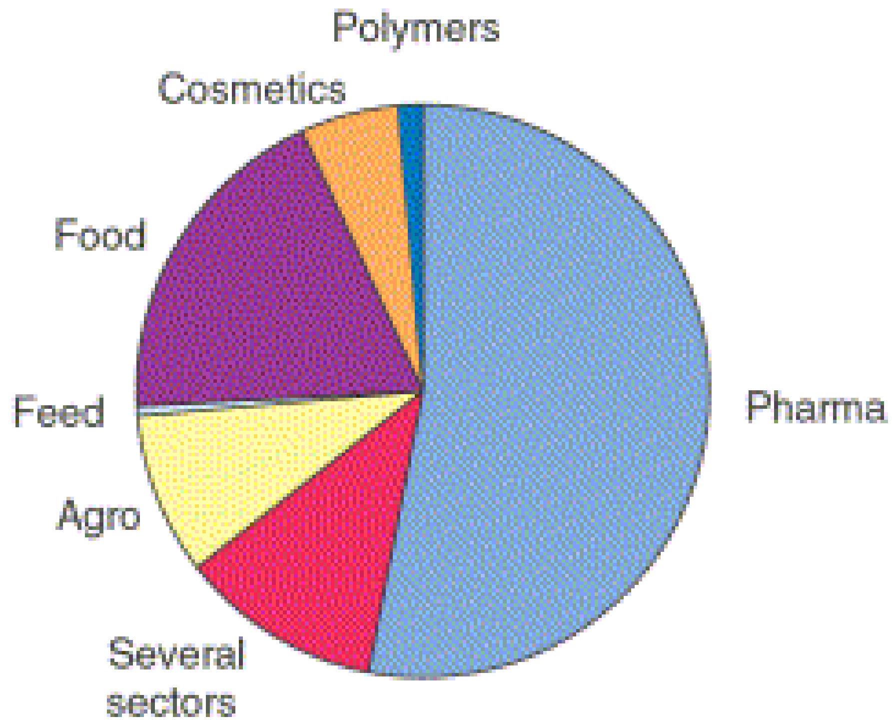
# Enzymes in Industry




**Cumulative number of biotransformation processes that have been started on an industrial scale**

**Industrial sectors in which the products of industrial biotransformations are used (based on 134 processes).**

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*Current Opinion in Biotechnology* **2002**, *13*, 548–556

# Enzymes in Industry

**Table 1**

**The application of enzyme technology in the chemical industry.**

| Industry sector* | Impact (estimate)†‡ Today | Near future | Distant future |
|---|---|---|---|
| Organics | | | |
| Food and feed additives | +++ | +++ | ++ |
| Fine chemicals | + | ++ | +++ |
| Drugs (antibiotics, intermediates) | ++ | ++ | ++ |
| Plastic materials and synthetics | + | ++ | ++ |
| Soaps, cleaners, personal care products (lipases, proteases) | + | ++ | ++ |
| Inorganics | – | + | + |
| Miscellaneous chemical products (adhesives, pulp, textile and oil processing, waste water treatment) | + | + | ++ |
| Agricultural chemicals (herbicides, intermediates) | + | + | ++ |

*As listed in Wittcoff and Reuben [36]. †Based on Tables 3, 4 and 5 and [10]. ‡+++, very high; ++ , high; + , moderate; – , low.

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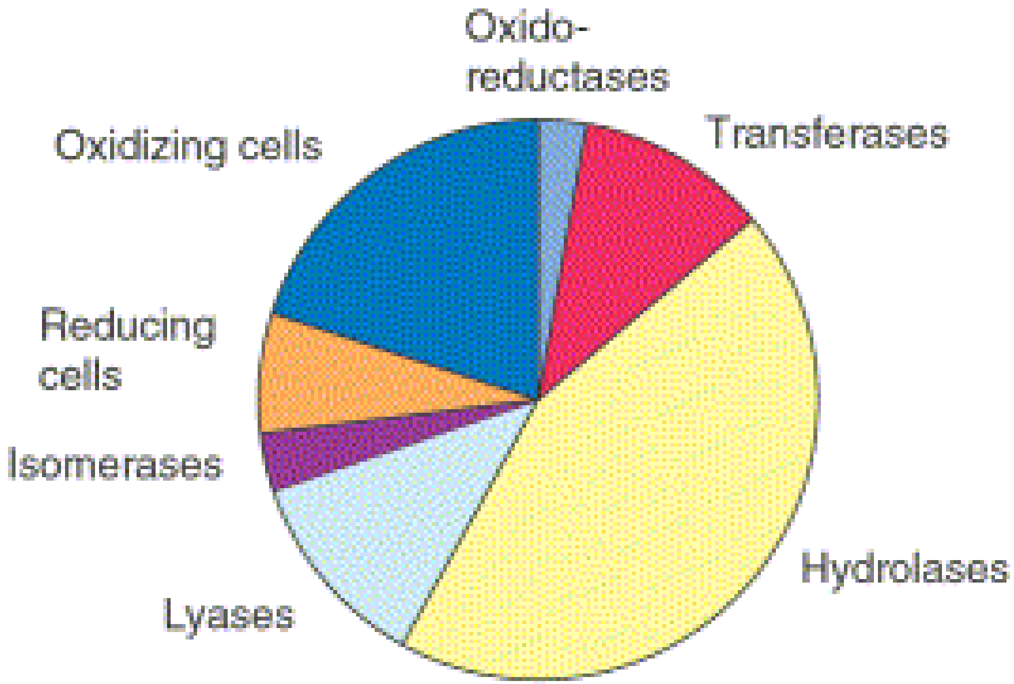
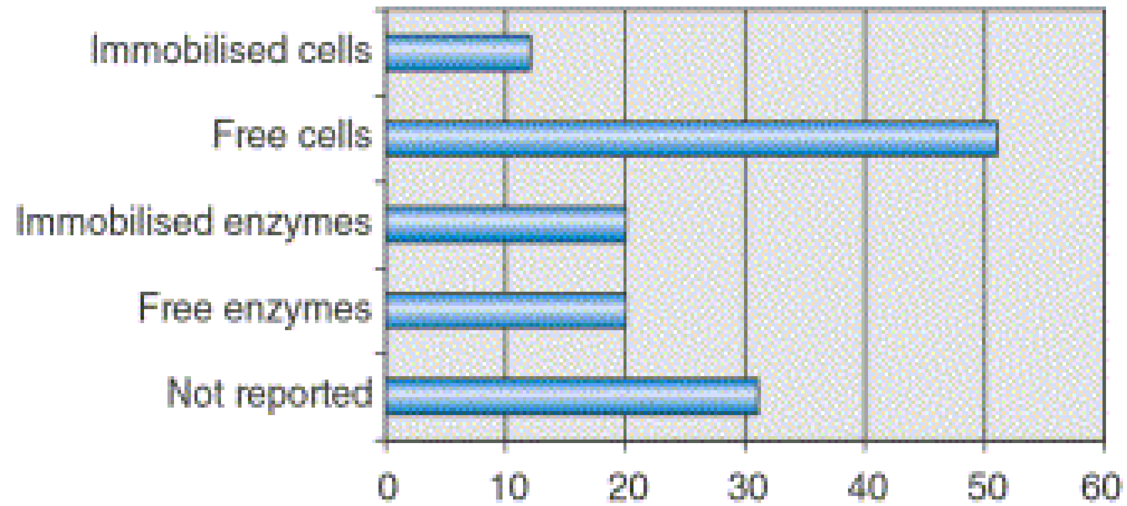
**The use of enzymes in the chemical industry in Europe** Schmid *et al.*
**Current Opinion in Biotechnology** 2002, **13**:359–366

# Enzymes in Industry




**Enzyme types used in industrial biotransformations (based on 134 processes)**

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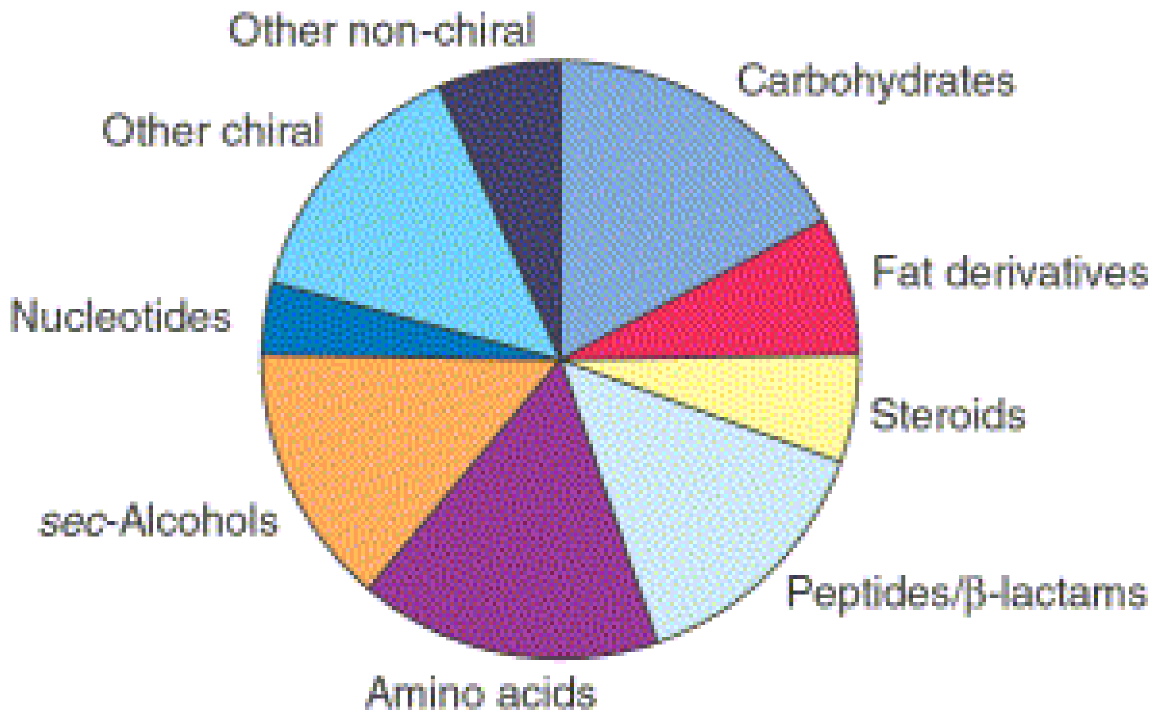
# Enzymes in Industry




**The type of compounds produced using biotransformation processes (based on 134 industrial processes).**

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**Box 2. Green credentials of biocatalysis**

Biocatalysis:

- operates in water (thus replacing organic solvents)
- has highly selective catalysis, including regio- and stereo-selectivity (thus reducing E-factors)
- operates in mild conditions, avoiding the need for protection (thus reducing E-factors)
- overcomes the use of some hazardous materials (resulting in improved LCA)
- uses renewable resources (resulting in improved LCA)
- can be modified, that is, the biocatalyst properties can be altered to suit the process (thus improving the ease of processing)
- is rarely endo- or exo-thermic (thus reducing energy requirements)
- provides a high yield as a result of selectivity and mild conditions (thus improving the efficiency of processing)
- is catalytic rather than stoichiometric (thus improving the ease of processing)



TRENDS IN BIOTECHNOLOGY **2008,** 26, 321-327

# Enzymes in Industry

**Pros:**

Selectivity
Environmentally friendly

**Cons:**

Substrate specificity
Substrate unsolubility in aqueous solvents
Cofactor dependency
Expensive

**Improvements:**

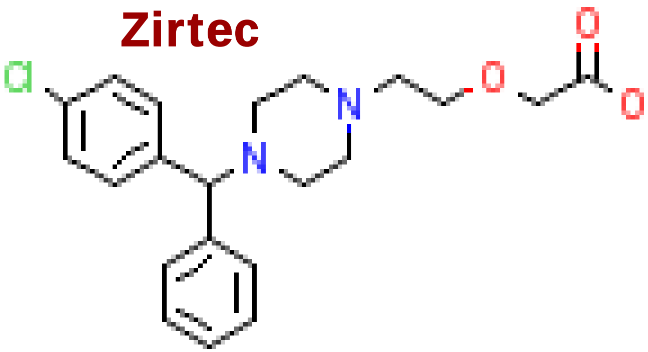
Engineered enzymes
Use in organic solvents
Cofactor recycle
Immobilization
Whole cells processes



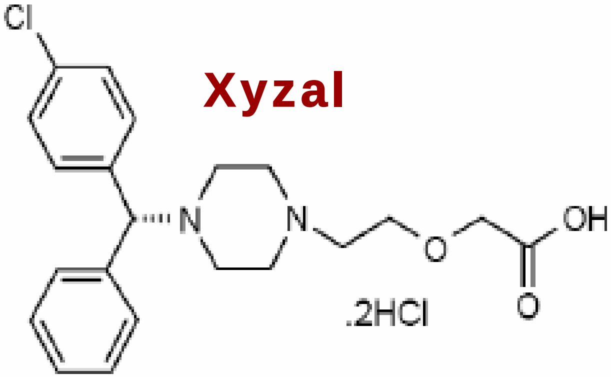
A. Straathof, *Current Opinion in Biotechnology*, 2002, 548

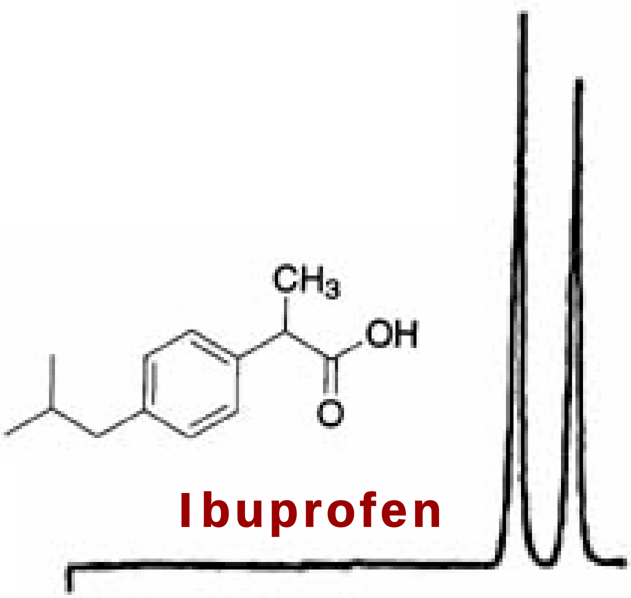
# Chiral Drugs

Zirtec

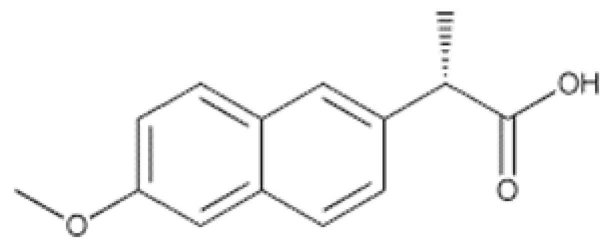



Xyzal




Ibuprofen




Naproxen

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# Chiral Drugs

The active enantiomer of a drug with the desired biological effect is called the **eutomer**

The other enantiomer is called the **distomer.**

**Eudismic ratio** is defined as the ratio of the biological activity of the eutomer to that of the distomer.

- the distomer may be inactive but....
- the distomer may possess harmful side effects e.g. thalidomide
- the distomer may be converted into the eutomer by the body e.g. ibuprofen.

Individual stereoisomers of a chiral drug should be tested as well as the racemate before a drug is marketed. This is because the presence of the distomer in a racemic drug can have important consequences on the biological activity**.**

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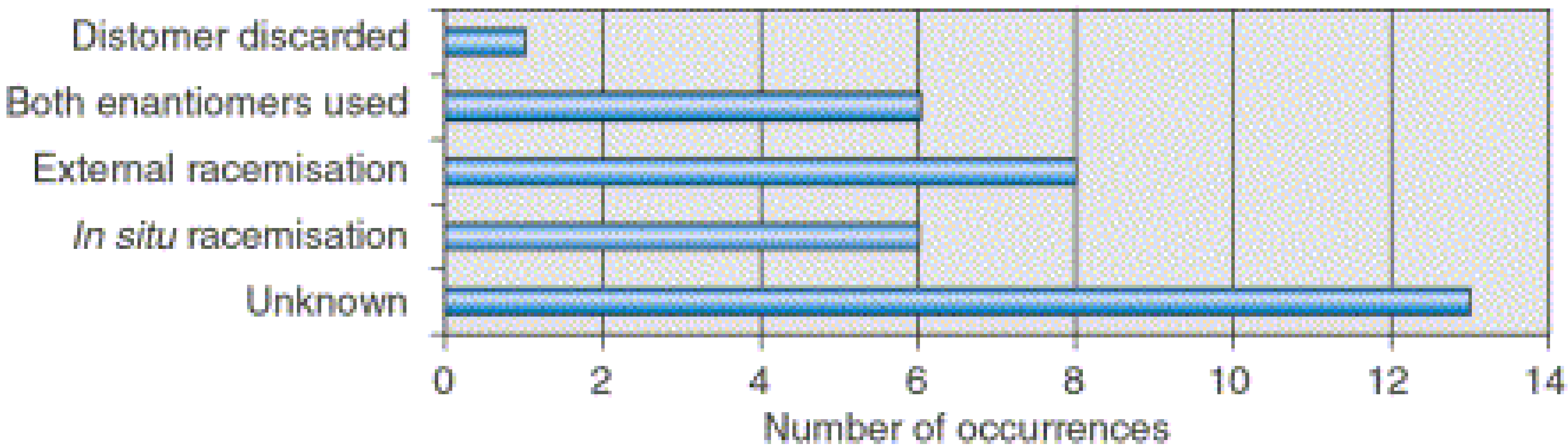
# Chiral Drugs




Current Opinion in Biotechnology

# Synthesis of pure enantiomers

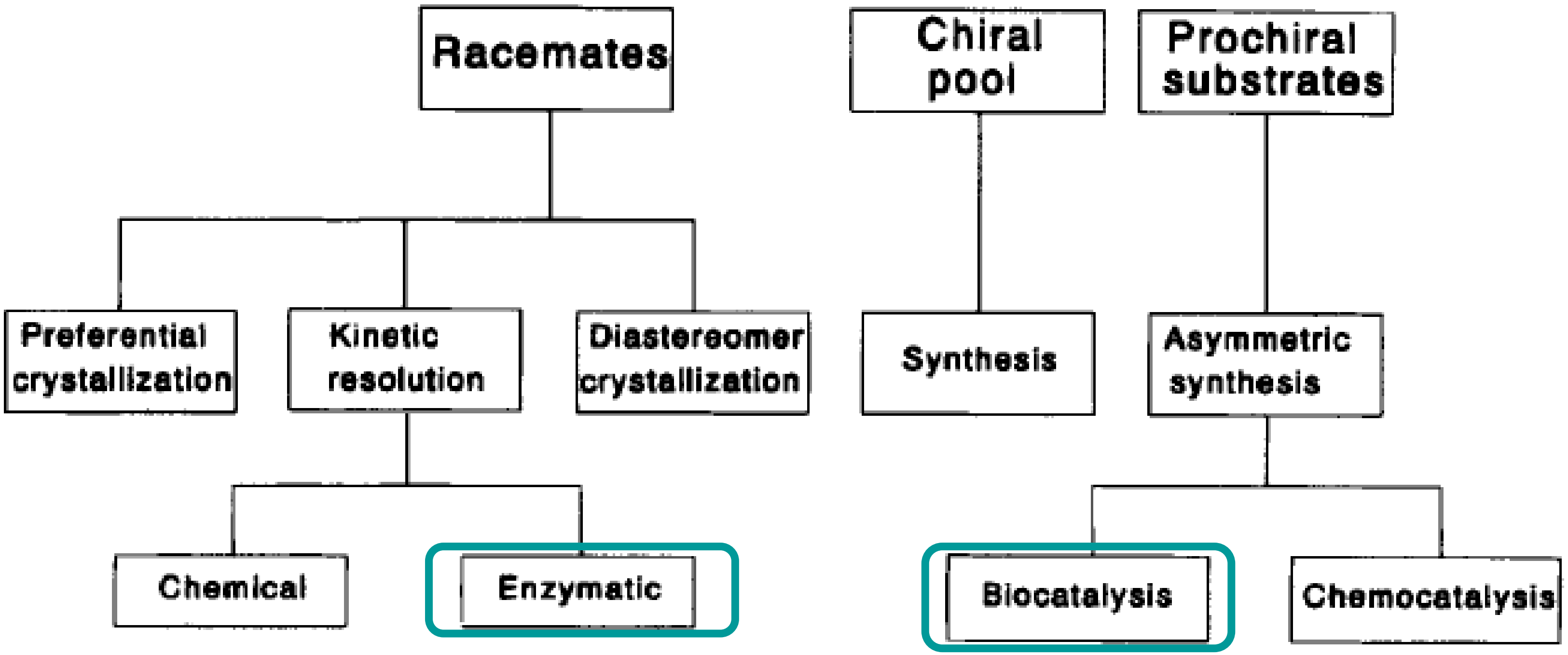



**Fig. 1.** Synthetic routes to pure enantiomers.

*J. Chem. Tech. Biotechnol.* 1996, 67, 1–14

# Biocatalysis in stereoselective synthesis

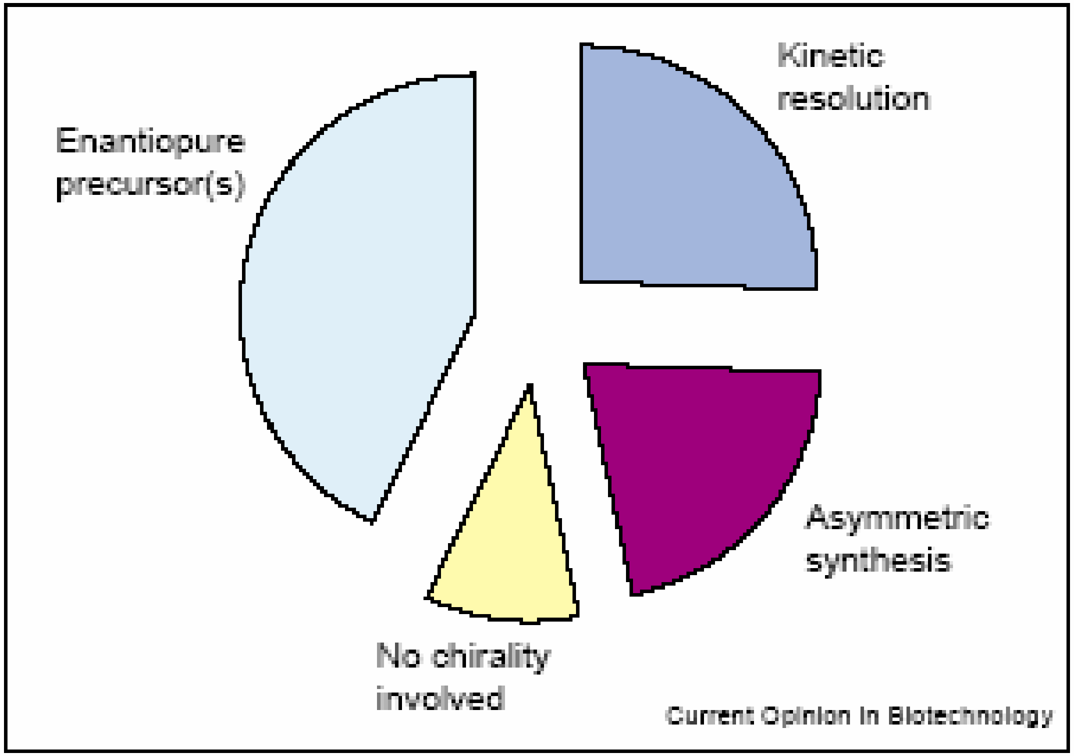
***Resolutions:***

**kinetic resolution (KR)**

**dynamic kinetic resolution (DKR)**

***Desymmetrization***

***Asymmetric Synthesis***




Source of chirality for the products of industrial biotransformations (based on 134 processes).

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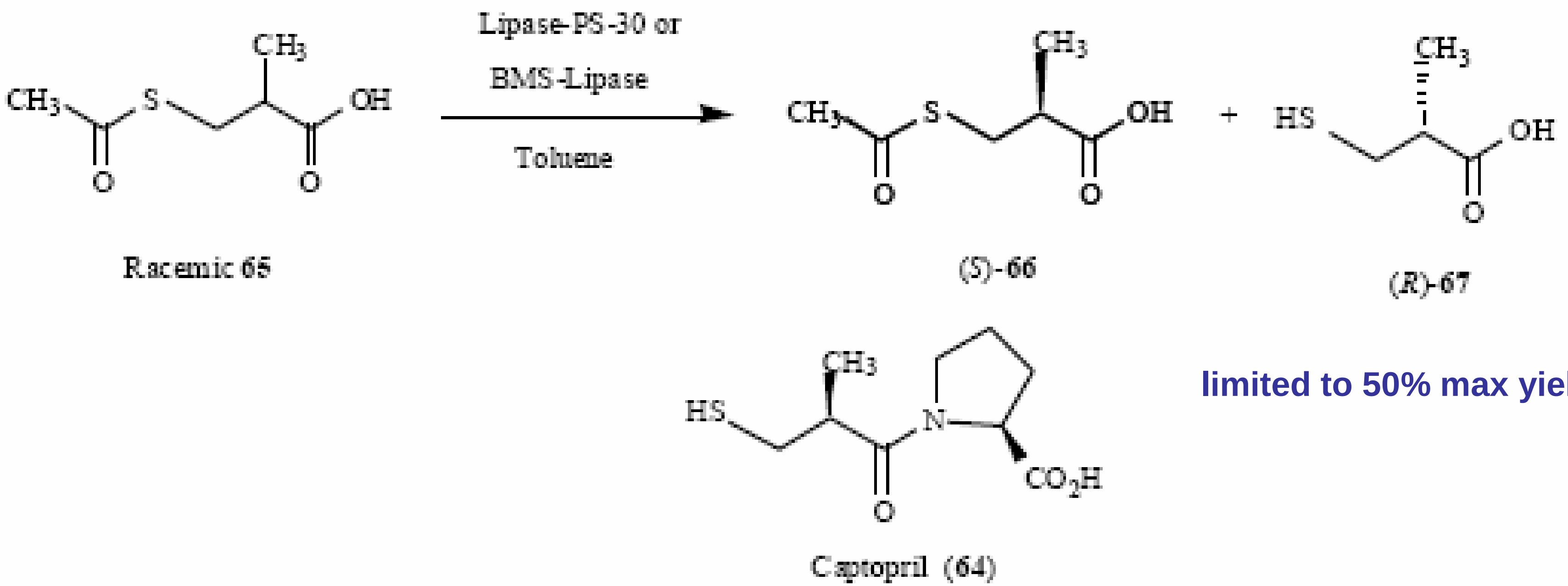
# Enzymes and kinetic resolution




limited to 50% max yield

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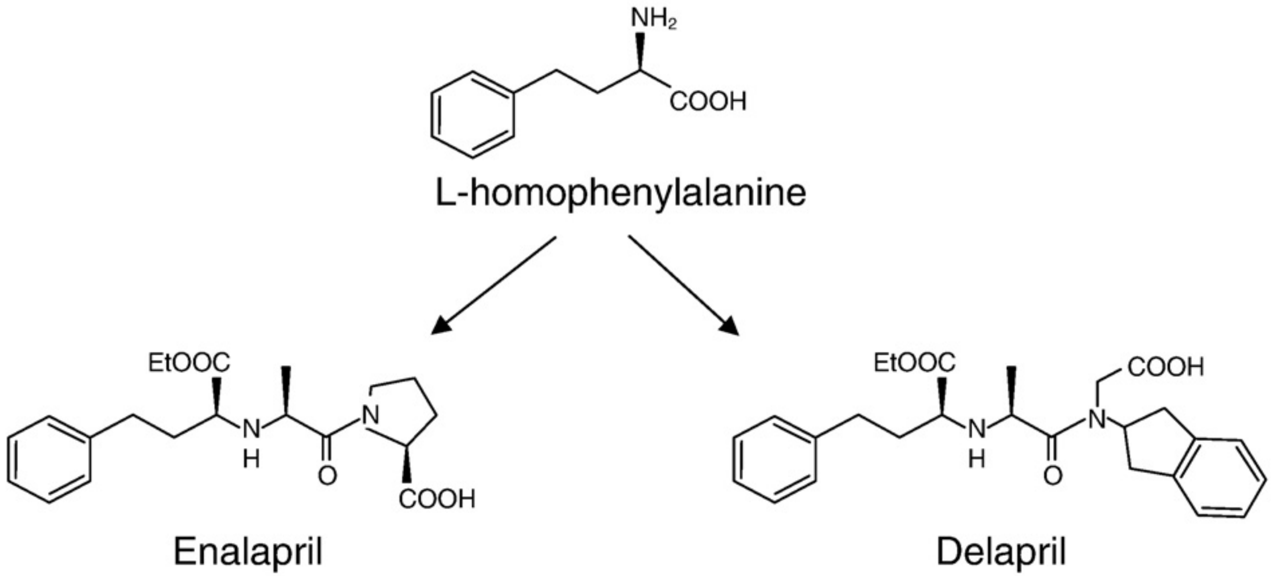
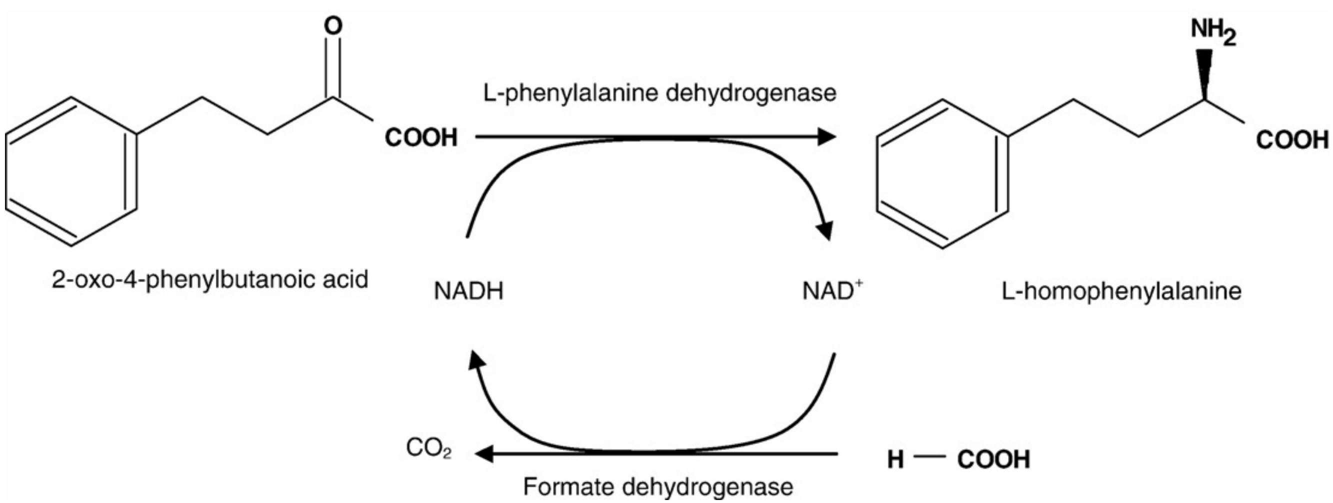
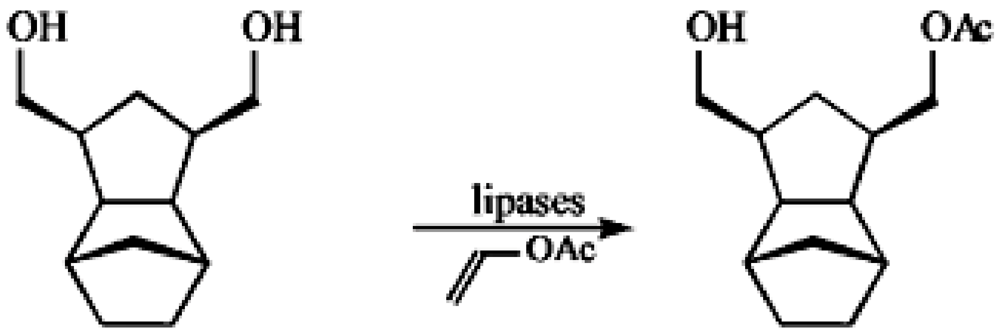
# Enzymes and “Prils” synthesis




**100% max yield**

# Enzymes and desymmetrization




3

(+)-4

**Scheme 2.** Lipase-catalysed acetylation of *meso-exo*-3,5-dihydroxymethylenetricyclo[5.2.1.0$^{2,6}$]decane, (**3**).

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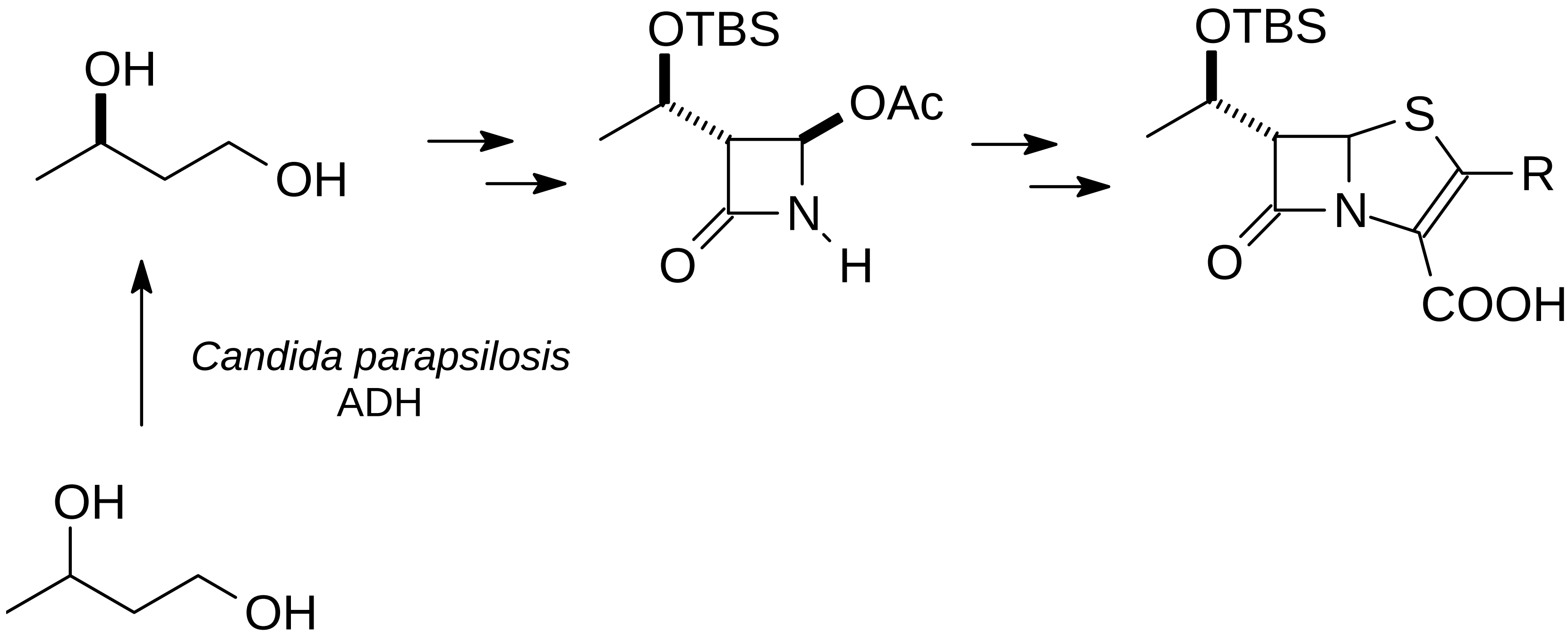
# Enzymes in synthesis of antibiotics

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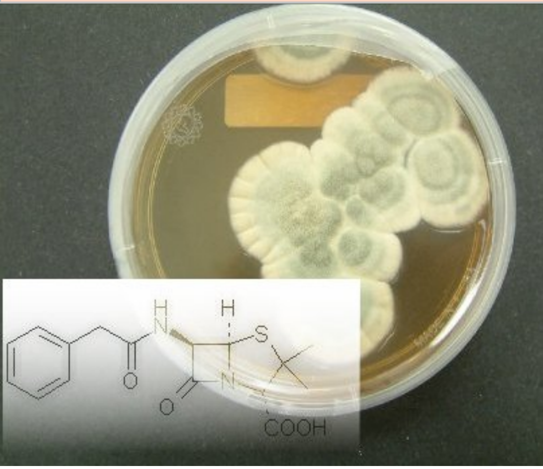
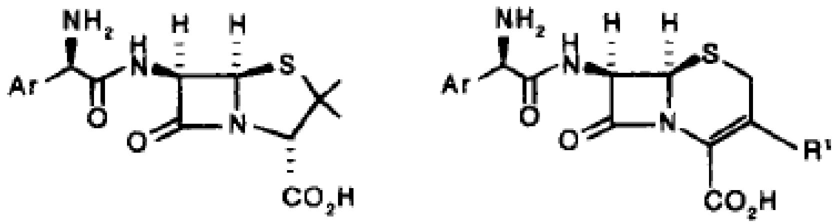
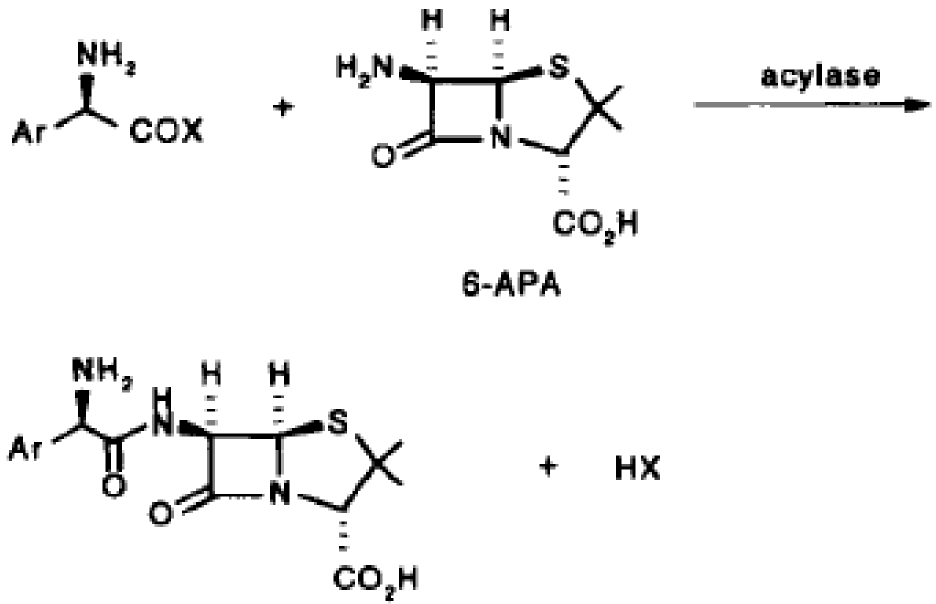
# Enzymes in synthesis of antibiotics




| | | |
|---|---|---|
| Ar = $C_6H_5$ | Ampicillin | Cefalexin |
| Ar = p-$HOC_6H_4$ | Amoxycillin | Cefadroxyl |




X = RO , $NH_2$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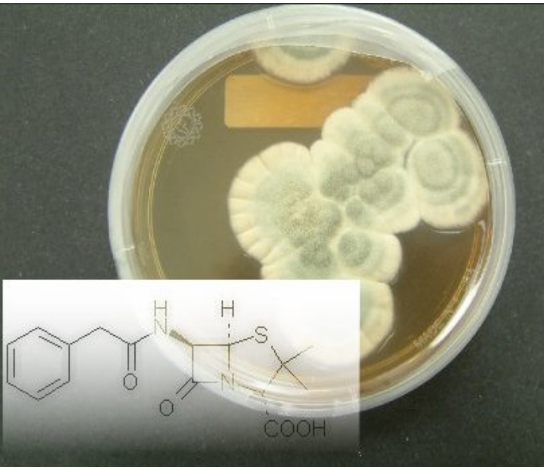
# Enzymes in synthesis of antibiotics



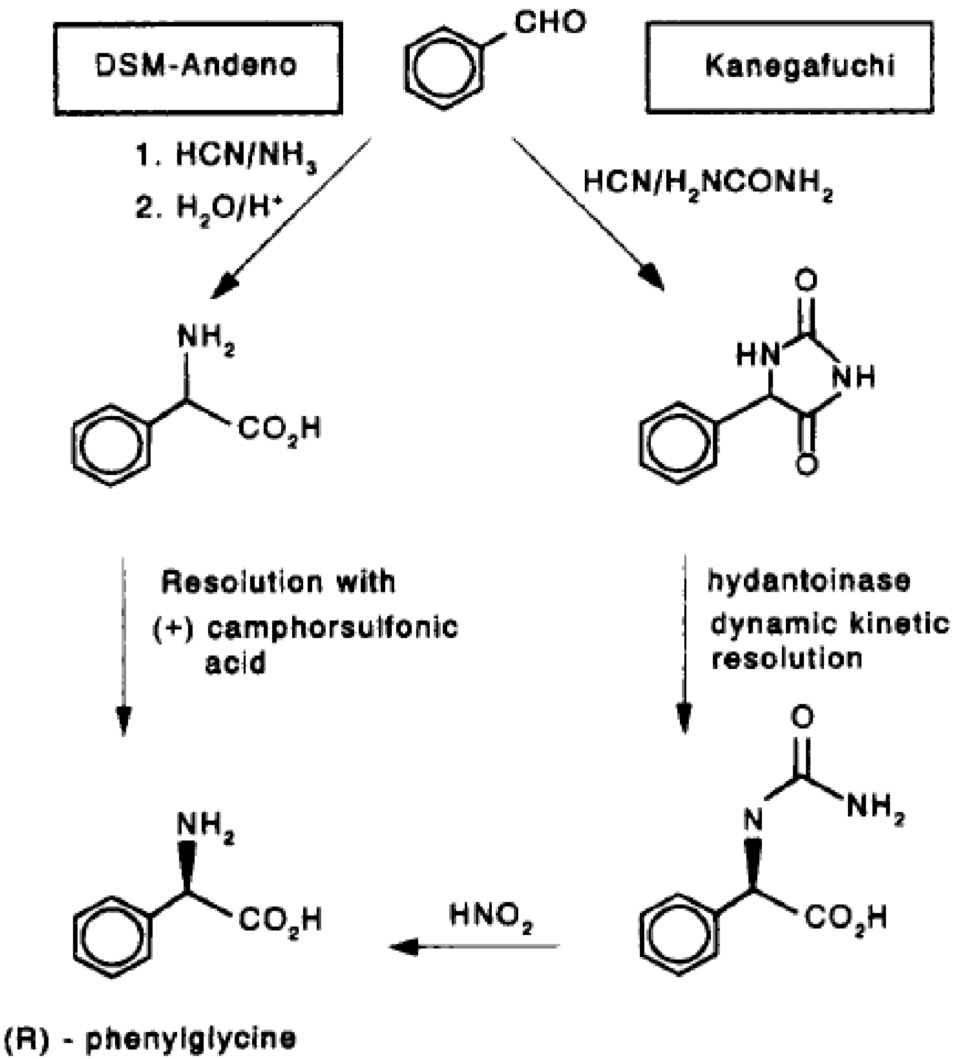




Fig. 7. Two routes to (*R*)-phenylglycine.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# Enzymes in synthesis of antibiotics

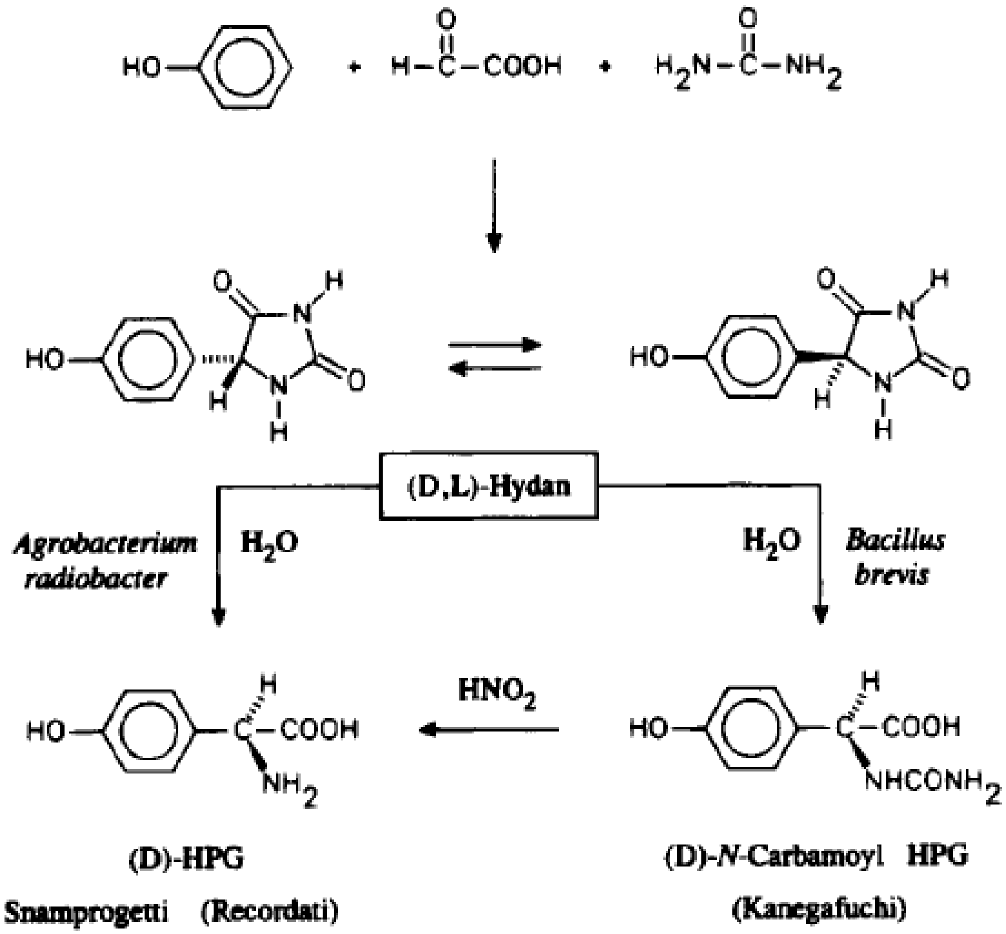


**Fig. 8.** The hydantoin route to (*R*)-*p*-hydroxyphenylglycine.

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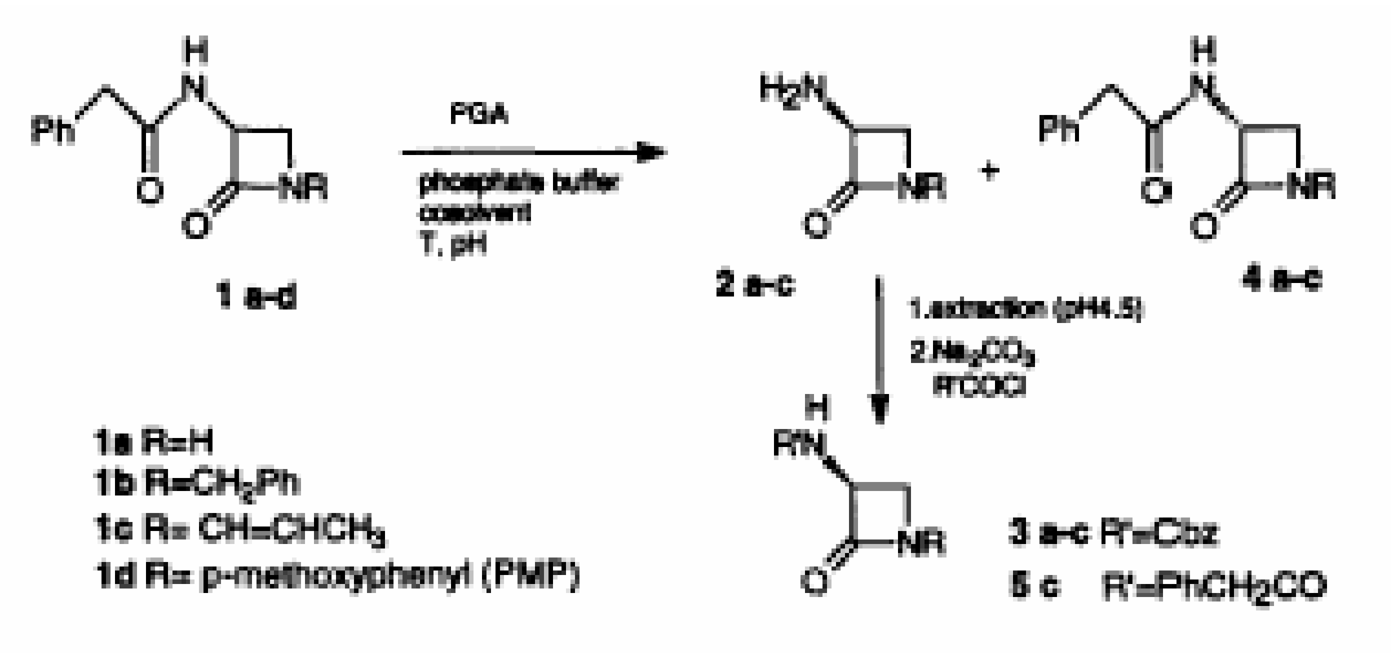
# Enzymes in synthesis of antibiotics



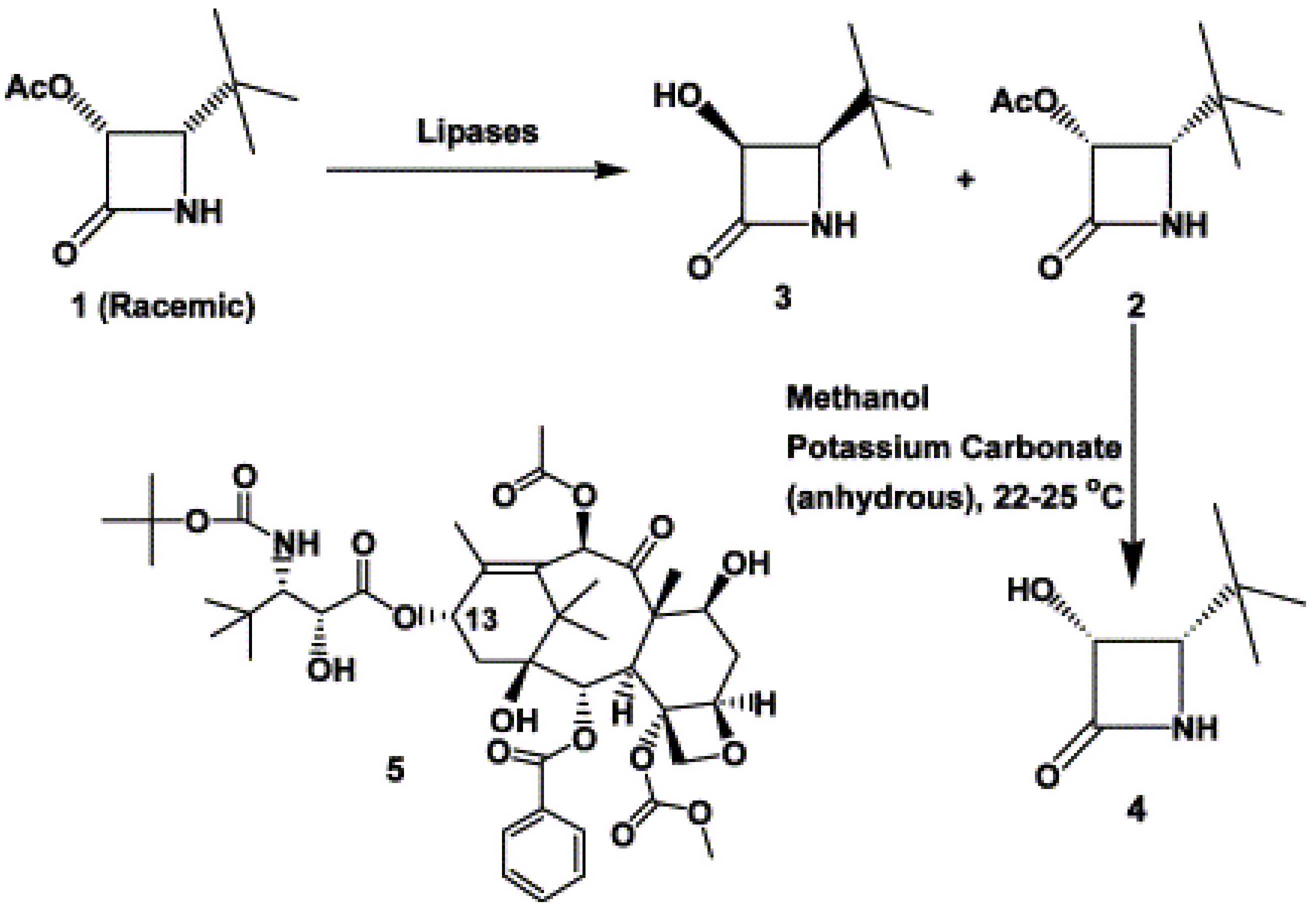

Scheme 1.

**Penicillin G acylase mediated synthesis of the enantiopure (S)-3-amino-azetidin-2-one**

G. Cainelli. D.Giacomini, P.Galletti, M.DaCol

*Tetrahedron: Asymmetry,* Vol. 8, No. 19, pp. 3231-3235, 1997

# Enzymes in synthesis of anticancer drugs

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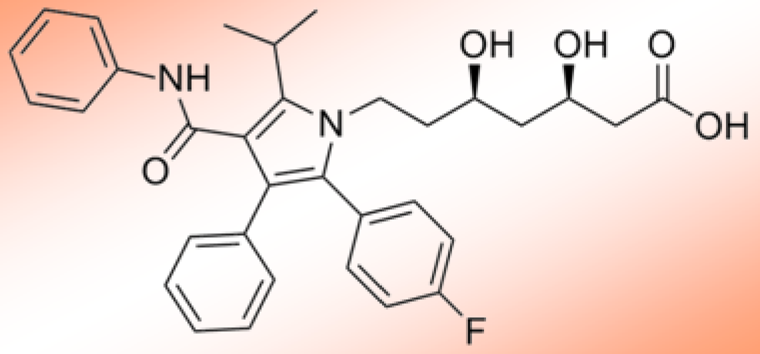
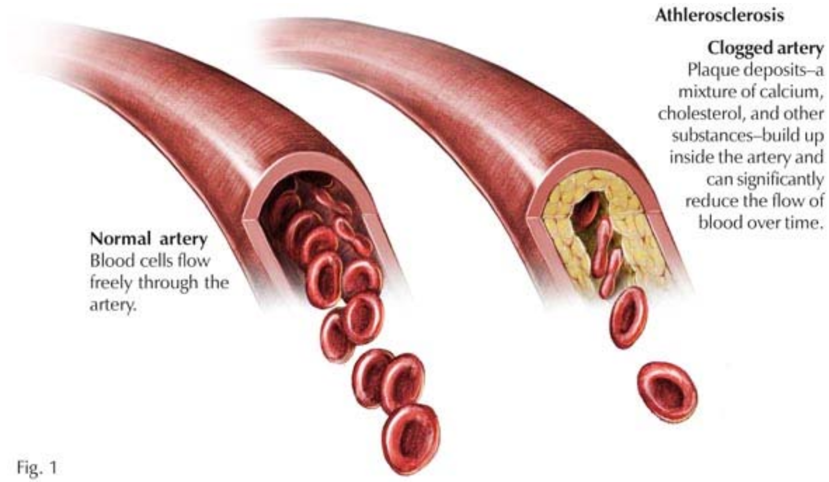
# Biocatalysis in Atorvastatin synthesis



**inhibitors of HMC-CoA**

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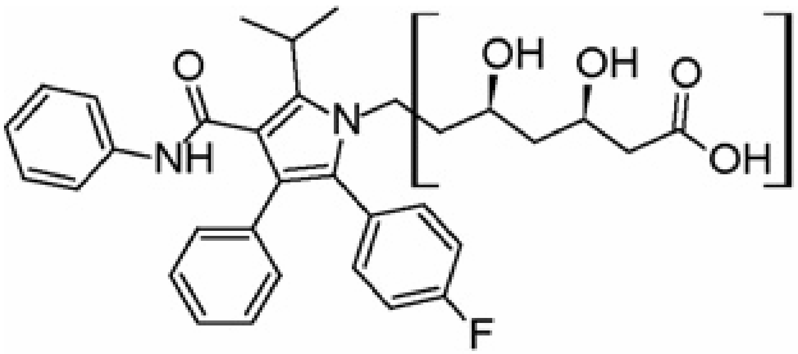
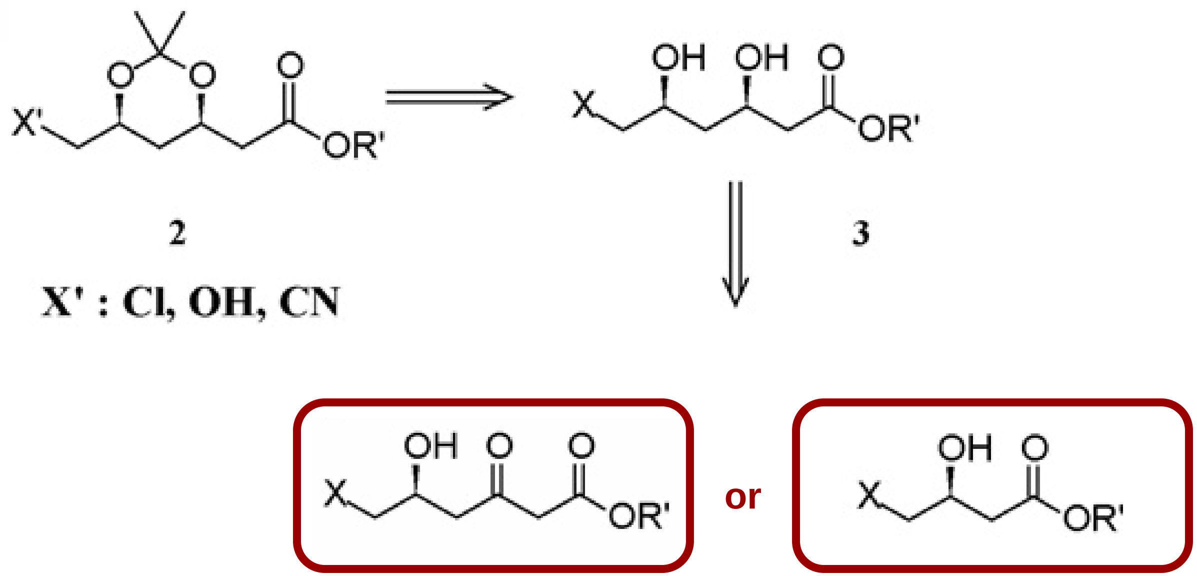
# Biocatalysis in Atorvastatin synthesis



**1**

Atorvastatin

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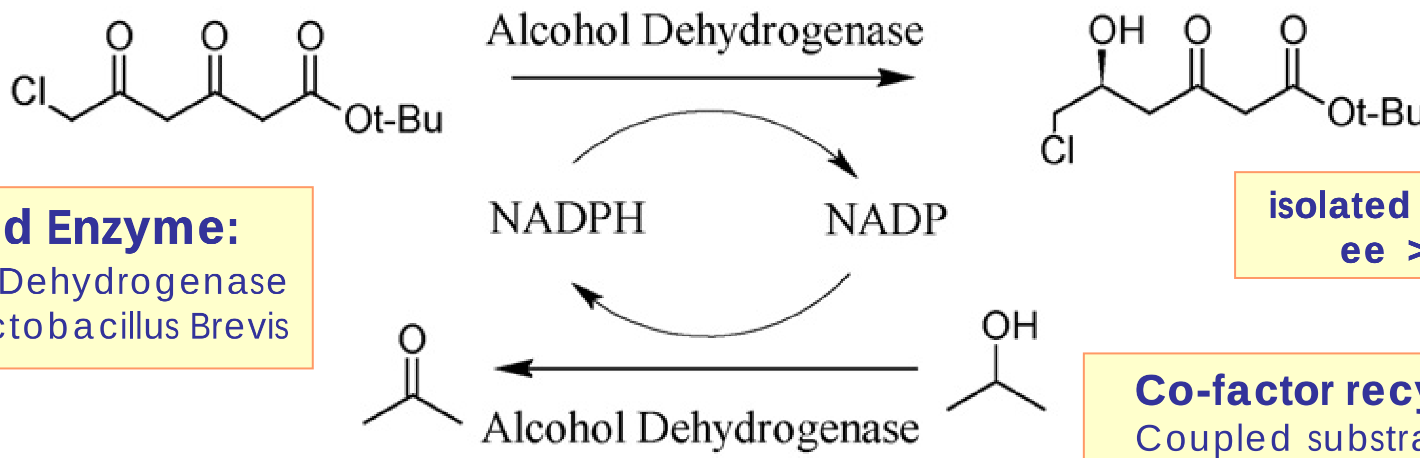
J.M. Patel *J. Mol. Cat.B: Enzymatic* 2009, 61, 123–128

# Dehydrogenase approach

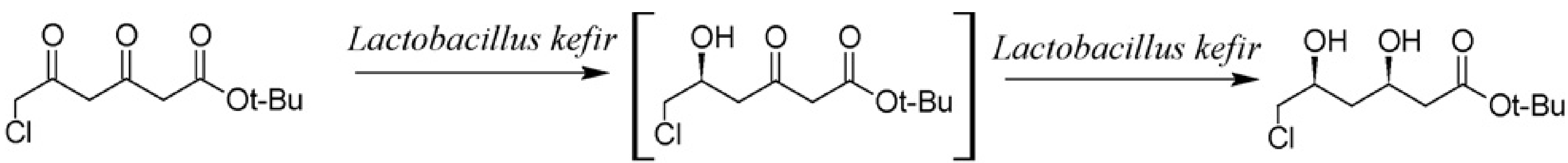



**Isolated Enzyme:**
Alcohol Dehydrogenase from Lactobacillus Brevis

**isolated yield 75%**
**ee > 99.5 %**

**Co-factor recycling:**
Coupled substrate process with one single enzyme (ADH) and isopropanol as co-substrate

**Whole cell:**
Alcohol Dehydrogenase from Lactobacillus Kefir

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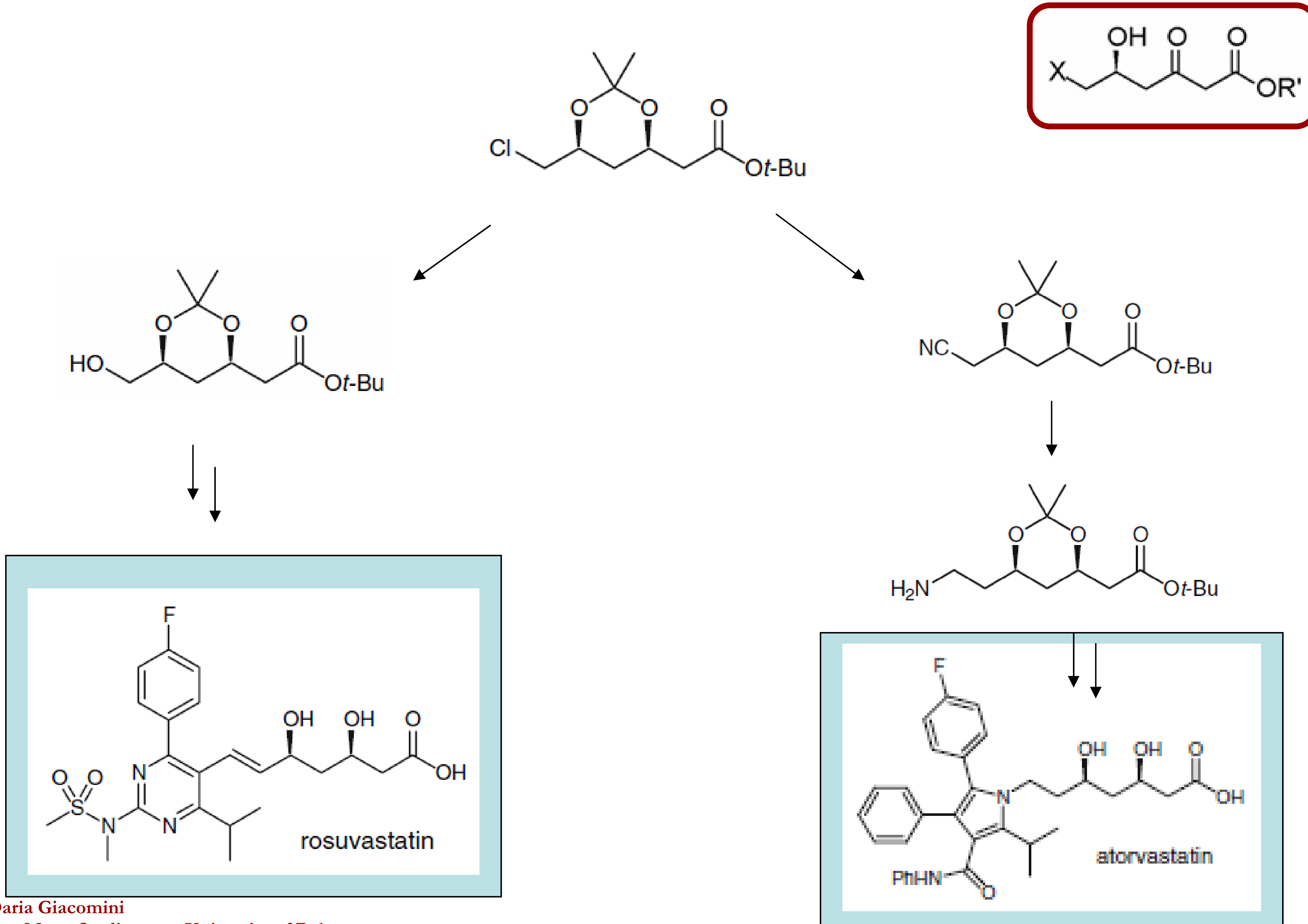
# Dehydrogenase approach

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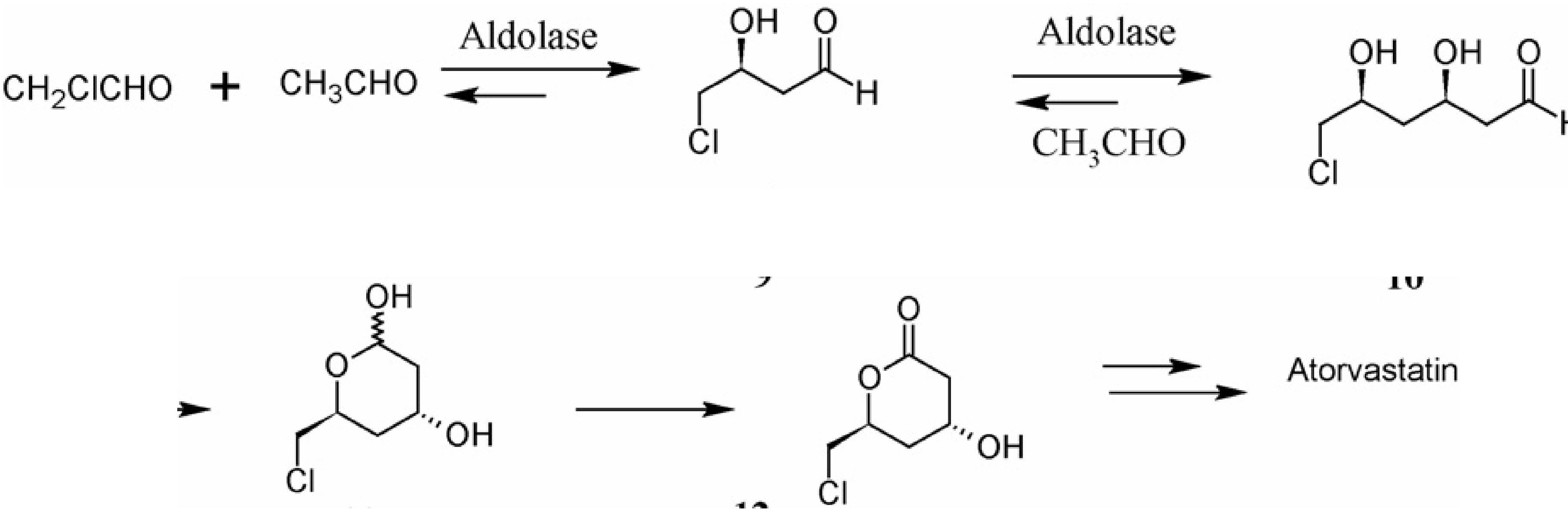
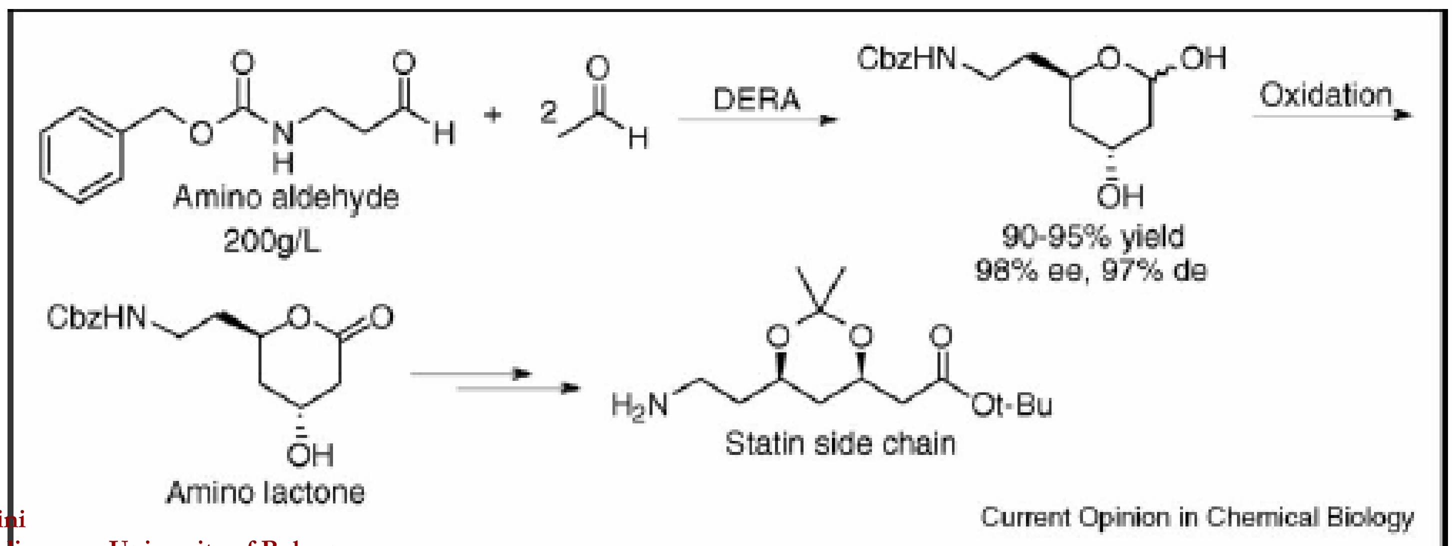
# Aldolase approach

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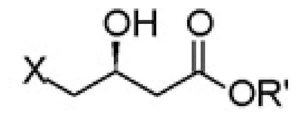
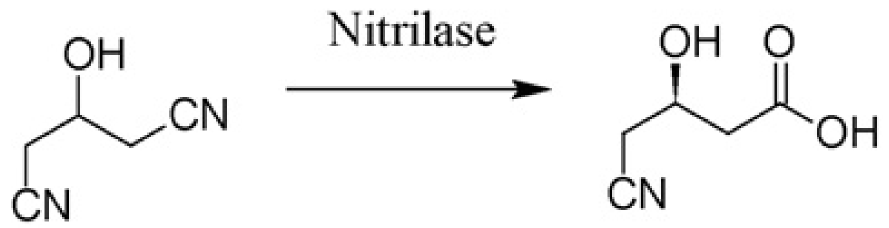
# Nitrilase approach




**Desymmetrization step**

wild type

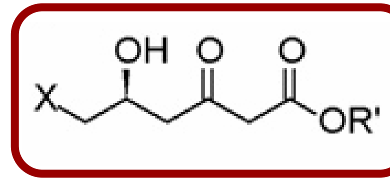
**0.2 M substrate: Y = 98% ee = 95%**

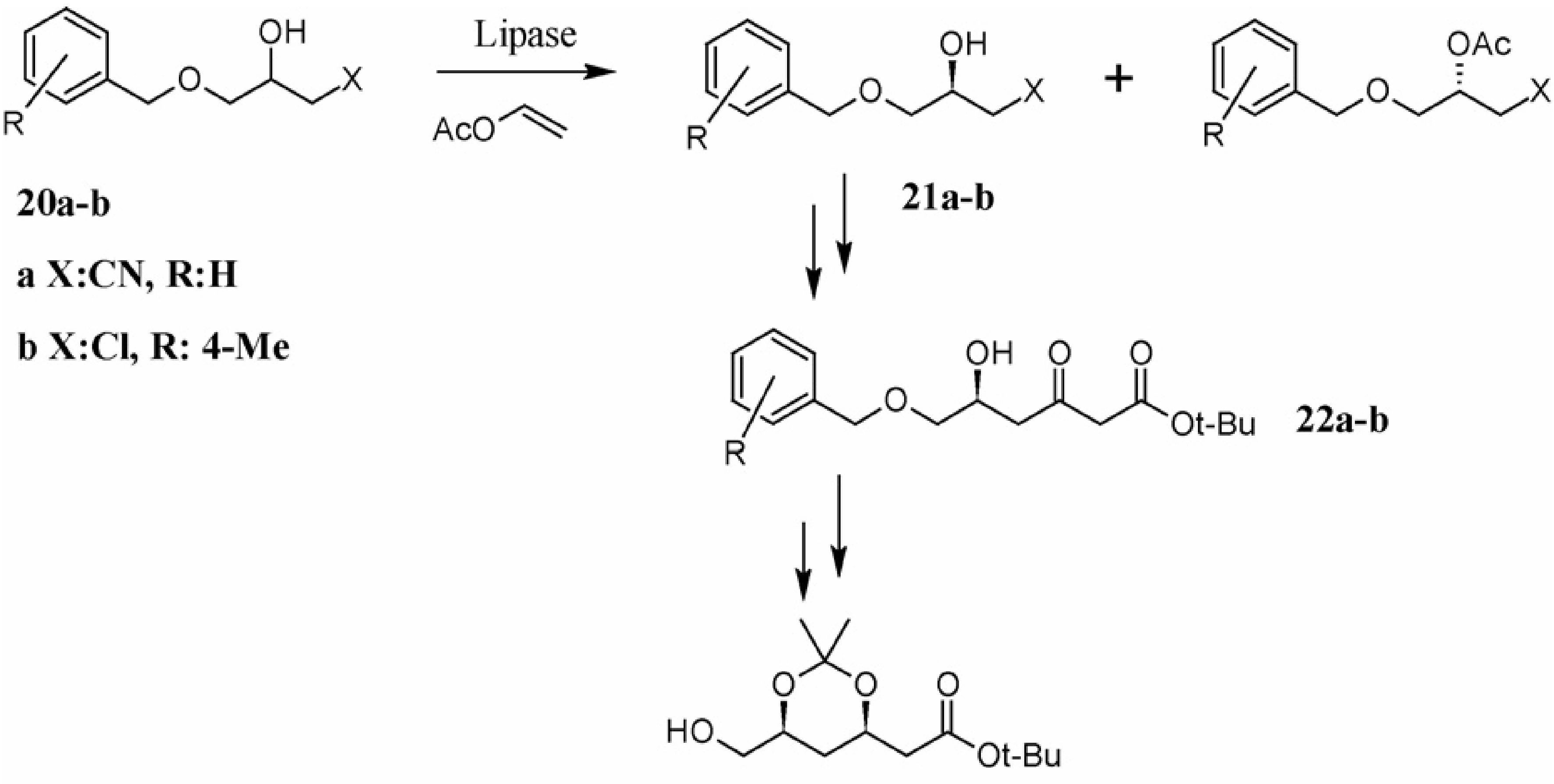
**3 M substrate: Y = 89% ee = 87%**

mutant

**3 M substrate Y = 96% ee = 98.5%**

# Lipase approach




**20a-b**

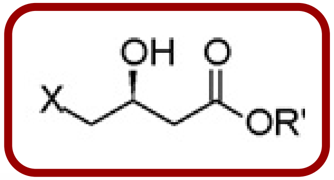
**a X:CN, R:H**

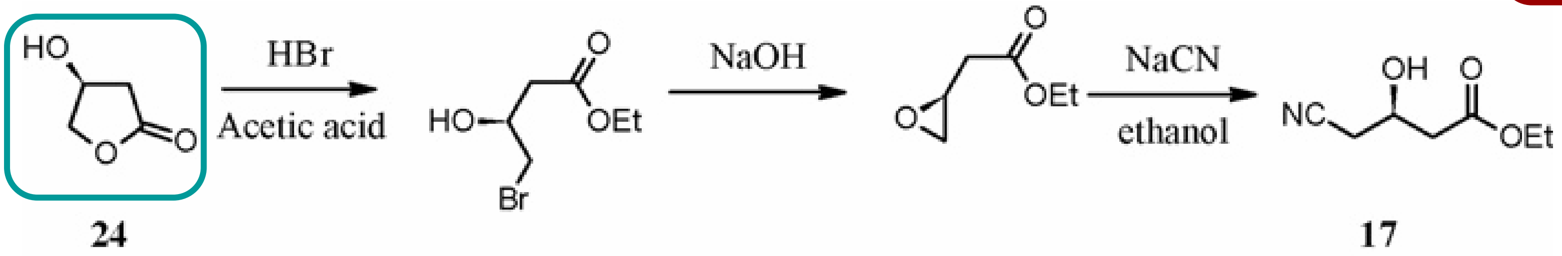
**b X:Cl, R: 4-Me**

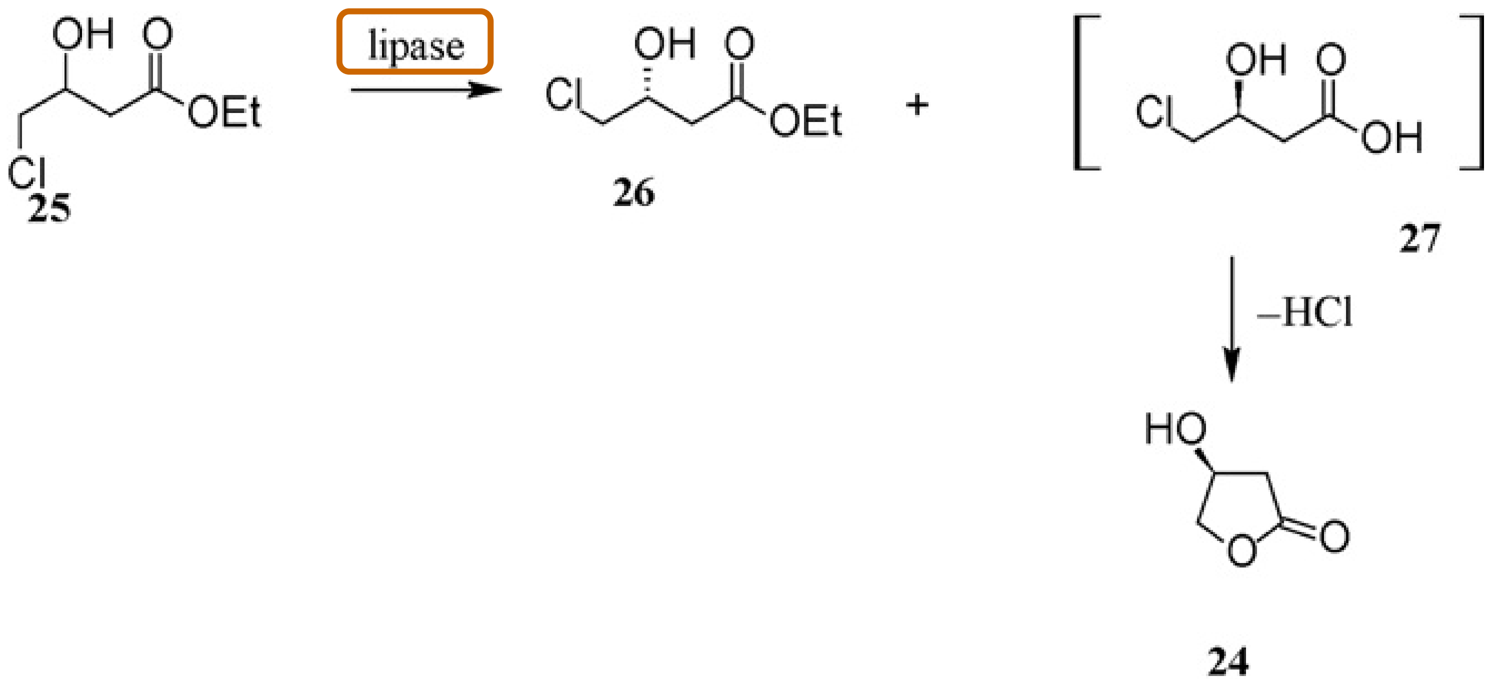
**21a-b**

**22a-b**

# Lipase approach

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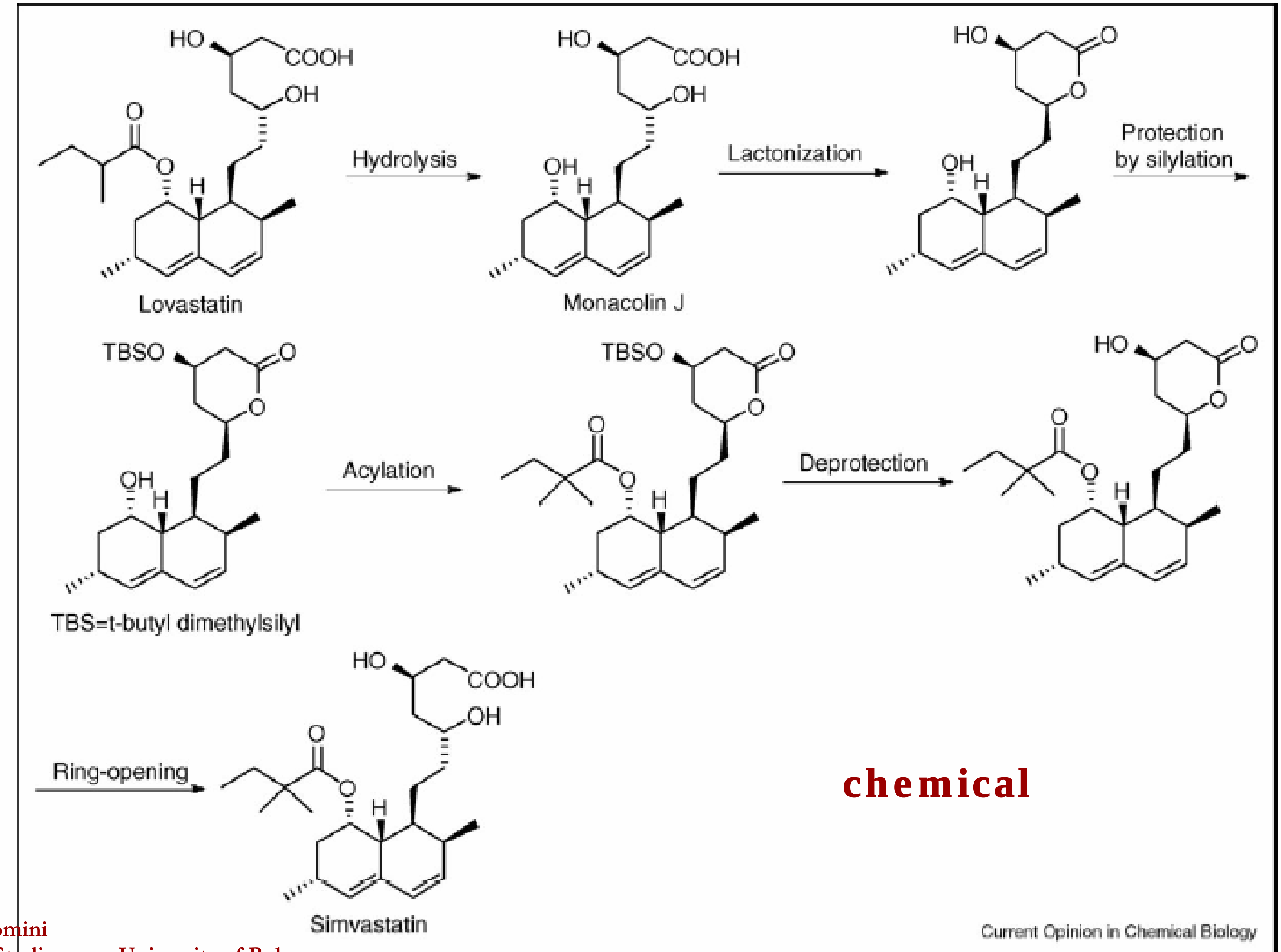
# Chemical *versus* chemo-enzymatic syntheses: a comparison




**chemical**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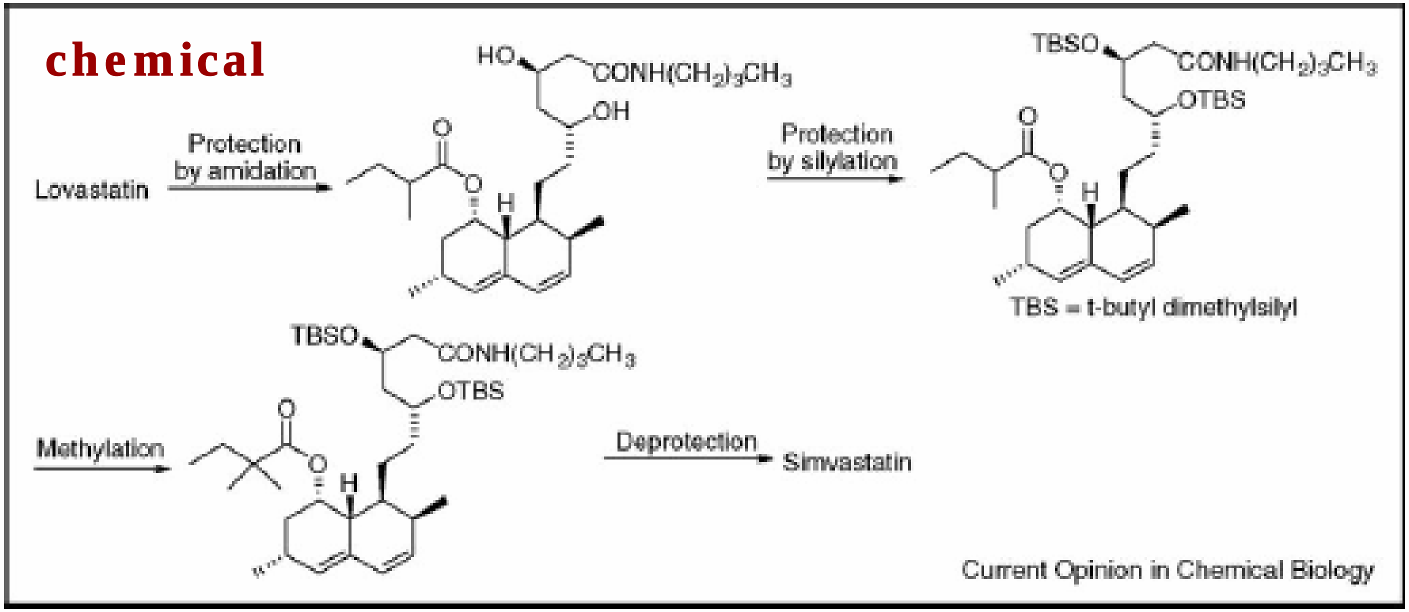
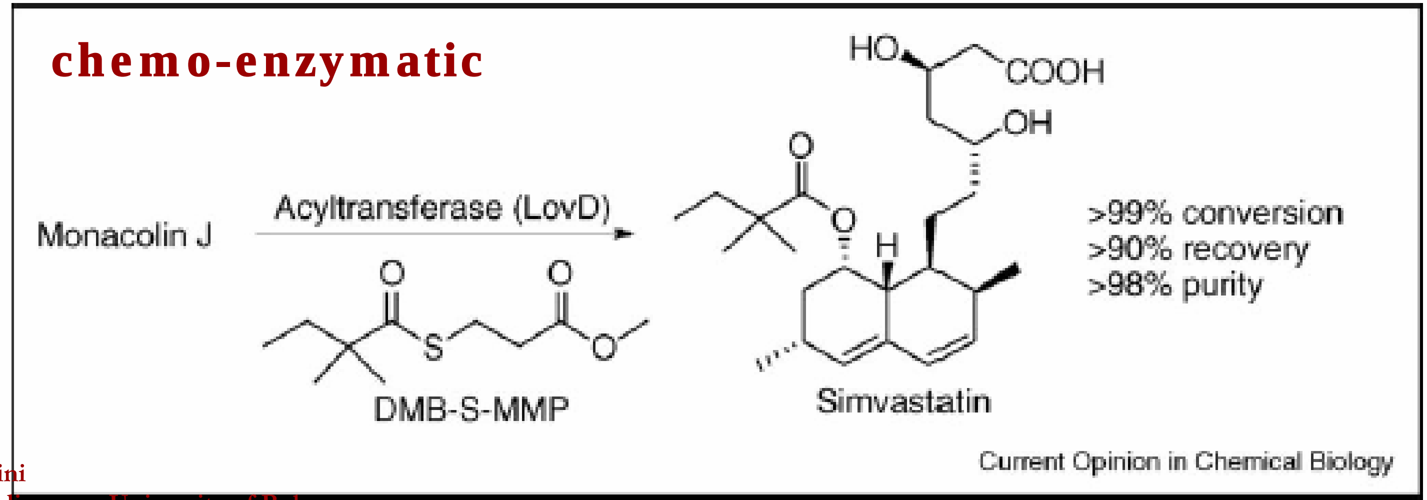
# Chemical *versus* chemo-enzymatic syntheses: a comparison

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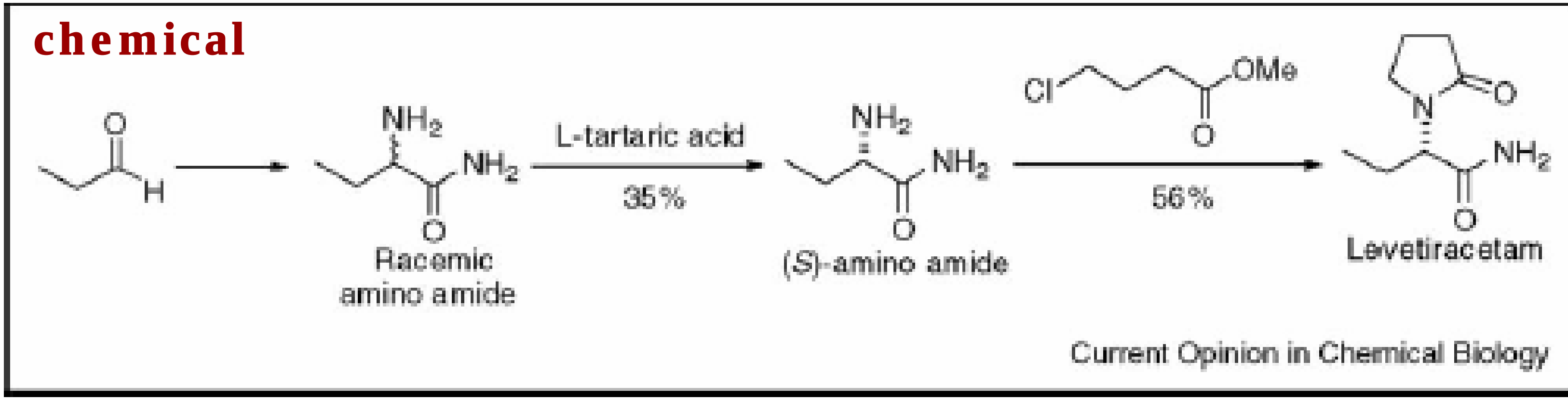
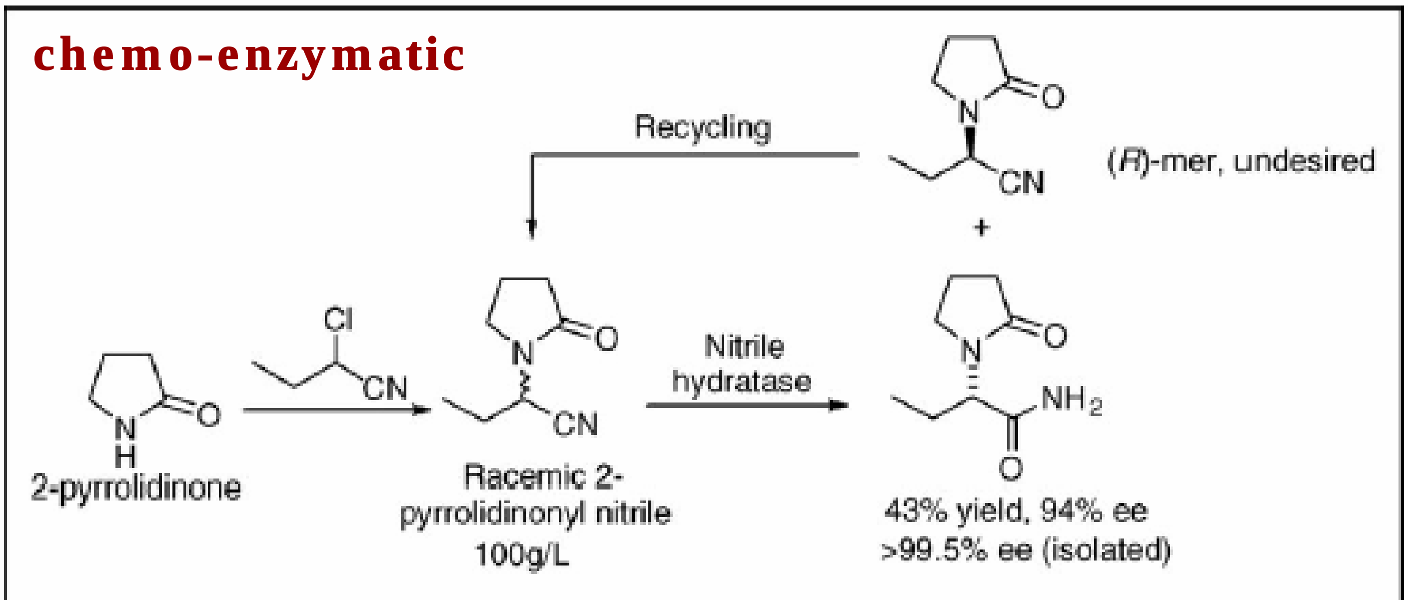
# Chemical *versus* chemo-enzymatic syntheses: a comparison

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Current Opinion in Chemical Biology 2009, 13:43–50

# Enzymes in Organic solvents

Maggiore stabilità termica degli enzimi in solvente organico (thermal unfolding, thermal deamidation)

Aumentata solubilità dei substrati

Separazione piu' facile dei prodotti di reazione

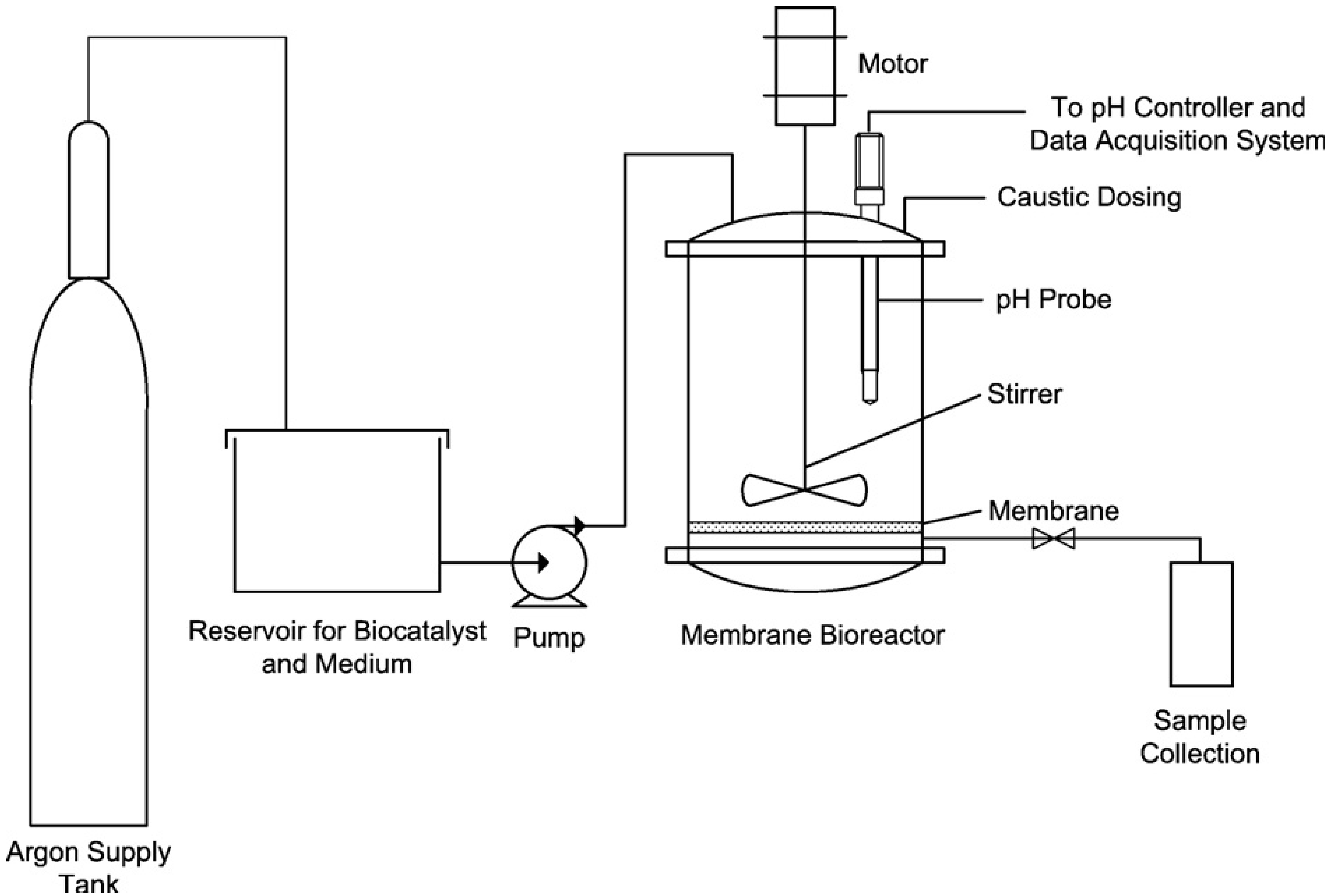
Multi-phase reactors

Spostamento dell'equilibrio delle reazioni

Selettività diversa rispetto al solvente acquoso "medium engineering"

**Lipasi** **Deidrogenasi**

# Membrane Reactor





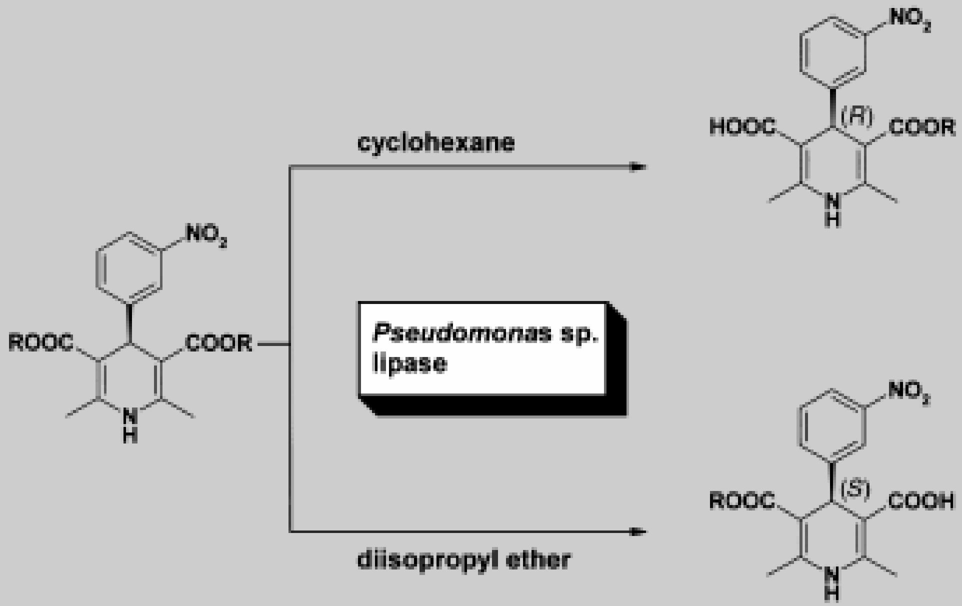

Biotechnology Advances 27 (2009) 286–296

# Solvent effects on enantioselectivity

ANGEWANDTE CHEMIE

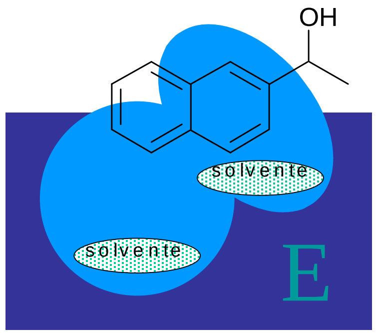
**Properties and Synthetic Applications of Enzymes in Organic Solvents**

Giacomo Carrea* and Sergio Riva*



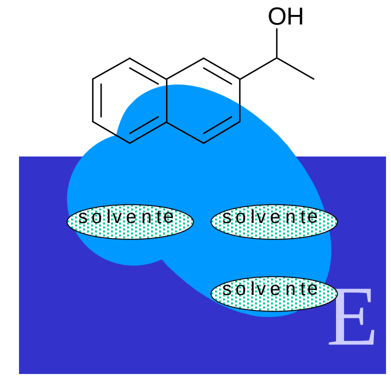

Enzyme selectivity in organic solvents can differ from that in water and can change, or even reverse, from one solvent to another.

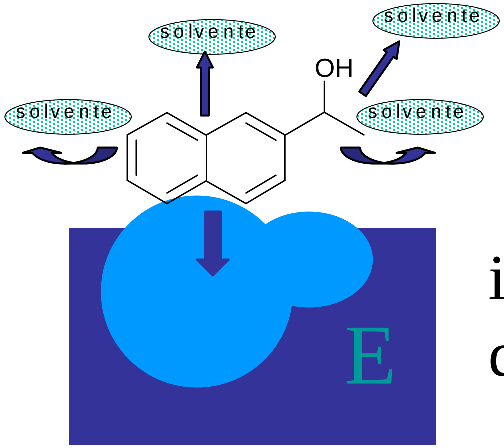
*Angew. Chem. Int. Ed.* 2000, *39*, 2226–2254




la presenza di molecole di solvente
nel sito attivo dell'enzima

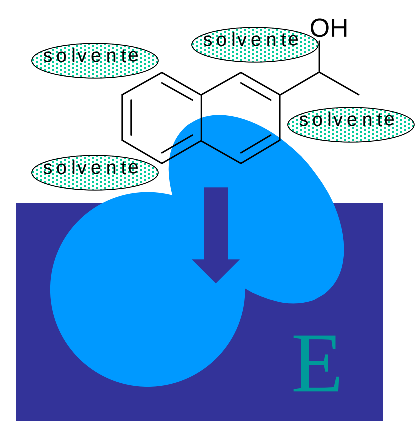
modifica del sito attivo
da parte del solvente




il substrato si desolvata prima
di entrare nel sito attivo

il substrato entra all'interno dell'enzima
come cluster soluto solvente

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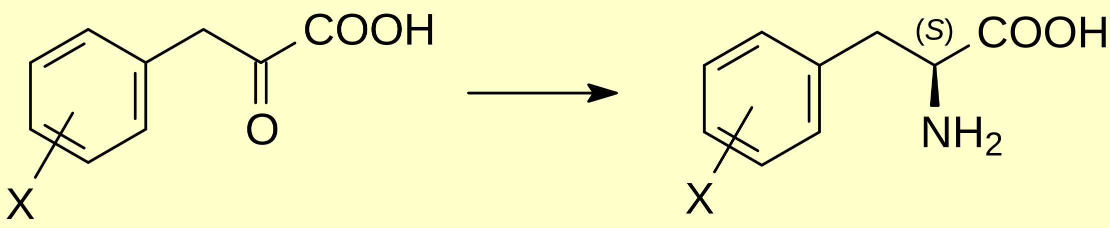
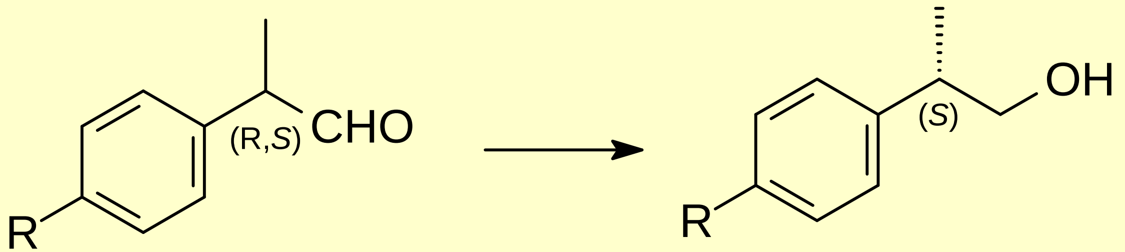
# Dehydrogenases in Organic Solvents

## Engineered Phenylalanine Dehydrogenase (PheDH-N145A)



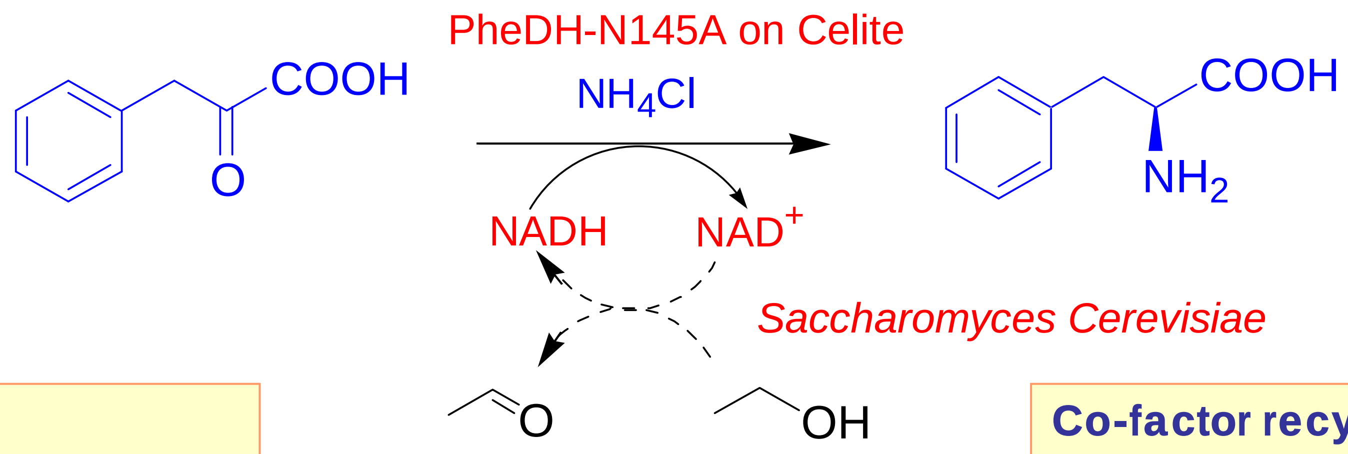

G.Cainelli, P.C.Engel, P.Galletti, D.Giacomini, A.Gualandi, F.Paradisi, *Org. Biomol. Chem., 2005, 3, 4316*
Patent WO 2006015885 A1 20060216 CAN 144:252784 2006.

## Horse Liver Alcohol Dehydrogenase (HLADH)




D. Giacomini, P. Galletti, A. Quintavalla,a G. Gucciardob, F. Paradisi *Chem Commun*, 2007, 4038
Patent Pending, PCT/EP2007/063844, RM2006A000686, University of Bologna

# Engineered Phenylalanine Dehydrogenase in Organic Solvents




**Enzyme**

Engeenered Phenyl Alanine Dehydrogenase (PheDH-N145A) immobilised on Celite®521

**Co-factor recycling**

coupled enzyme process with Saccharomyces Cerevisiae and ethanol as co-substrate

## Reaction Medium

**Homogeneous system**

aqueous buffer (TRIS pH 8.5) with miscible organic solvents

**Biphasic system**

aqueous buffer (TRIS pH 8.5) and immiscible organic solvents

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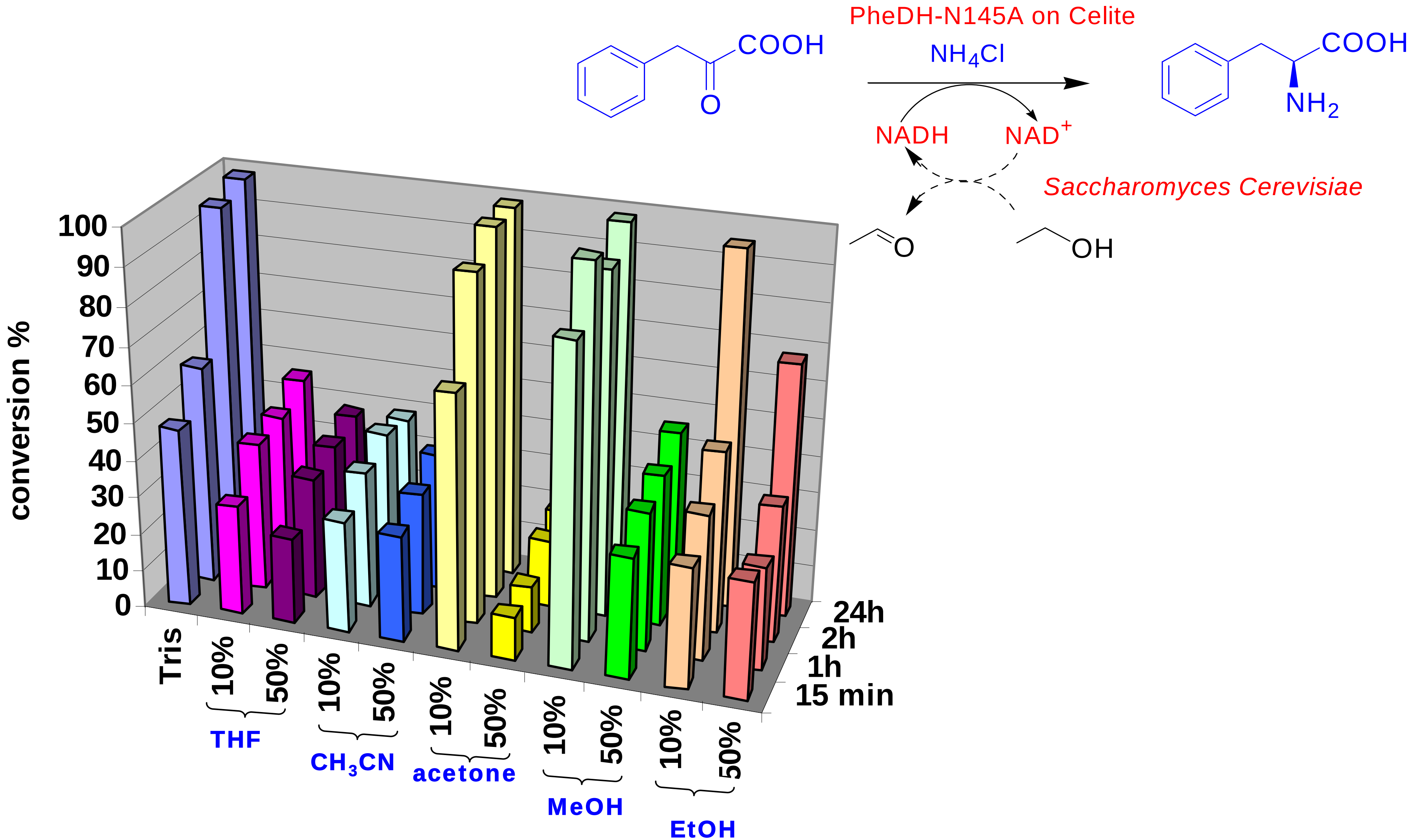
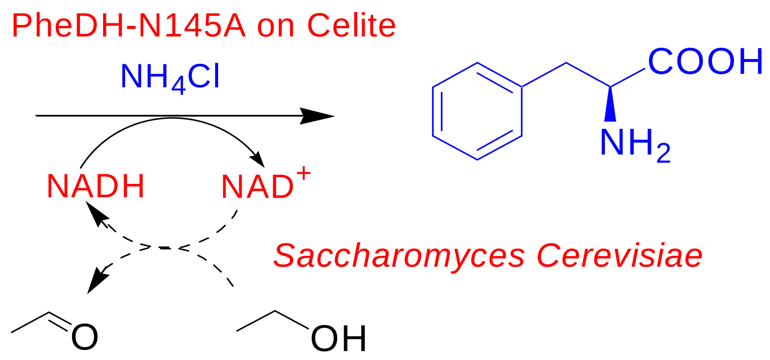
# Homogeneous medium: buffer and THF, acetone, or methanol

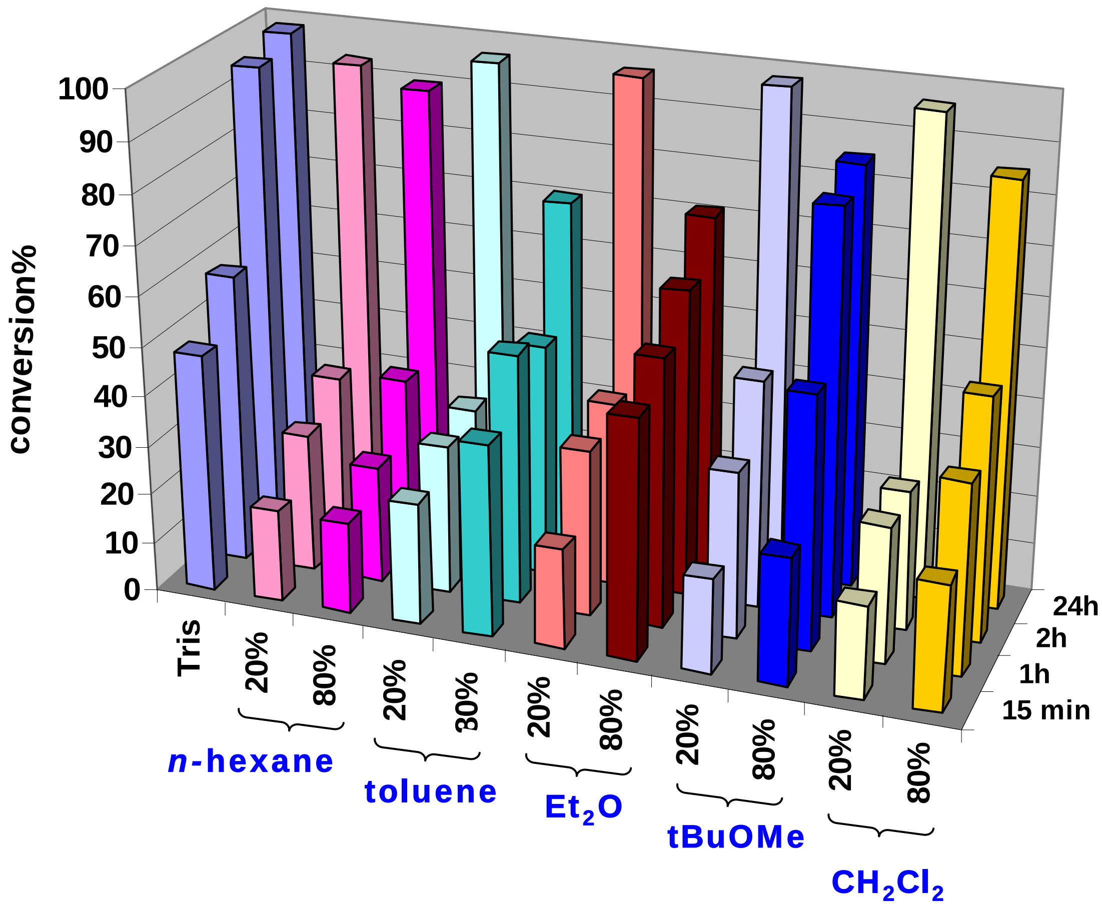





G.Cainelli, P.C.Engel, P.Galletti, D.Giacomini, A.Gualandi, F.Paradisi, *Org. Biomol. Chem., 2005, 3, 4316*

# Biphasic medium : buffer with *n*-hexane, toluene, $Et_2O$, *t*BuOMe, or $CH_2Cl_2$

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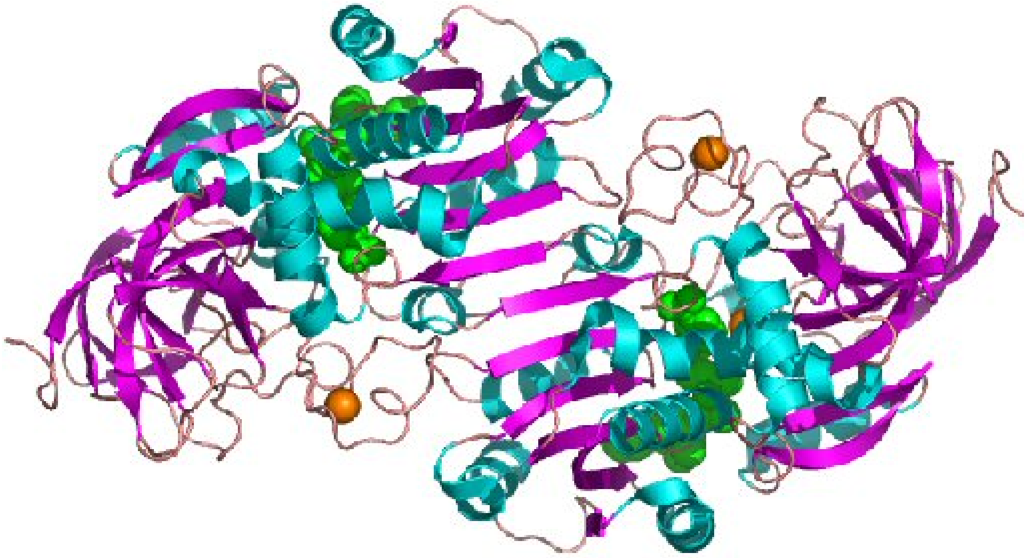
# Alcohol Dehydrogenases

Yeast Alcohol Dehydrogenases



Horse Liver Alcohol Dehydrogenases

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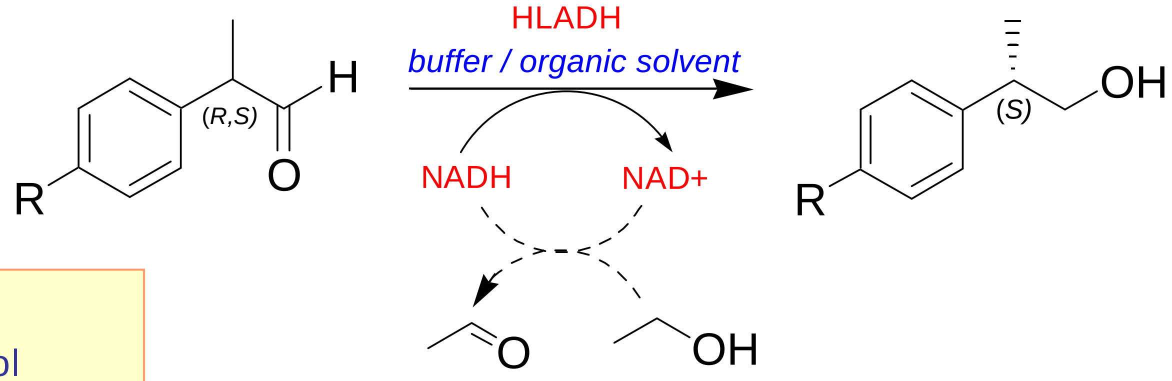
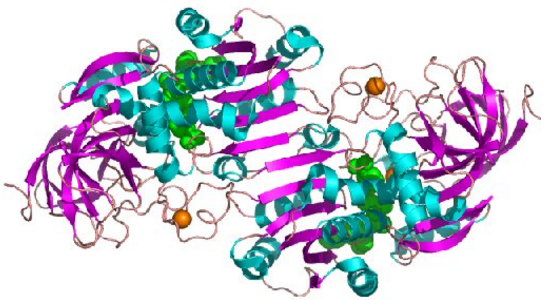
# Horse Liver Alcohol Dehydrogenase in Organic Solvents




**Enzyme**

Horse Liver Alcohol Dehydrogenase (HLADH)



**Co-factor recycling**

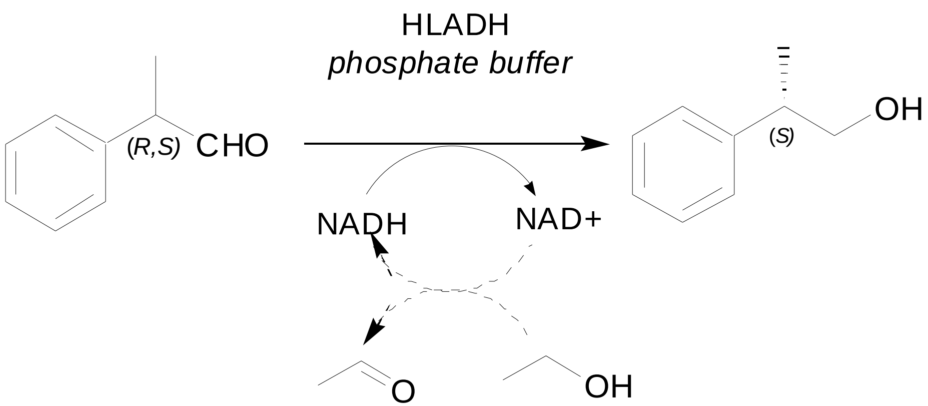
Coupled substrate process with one single enzyme (HLADH) and ethanol as co-substrate

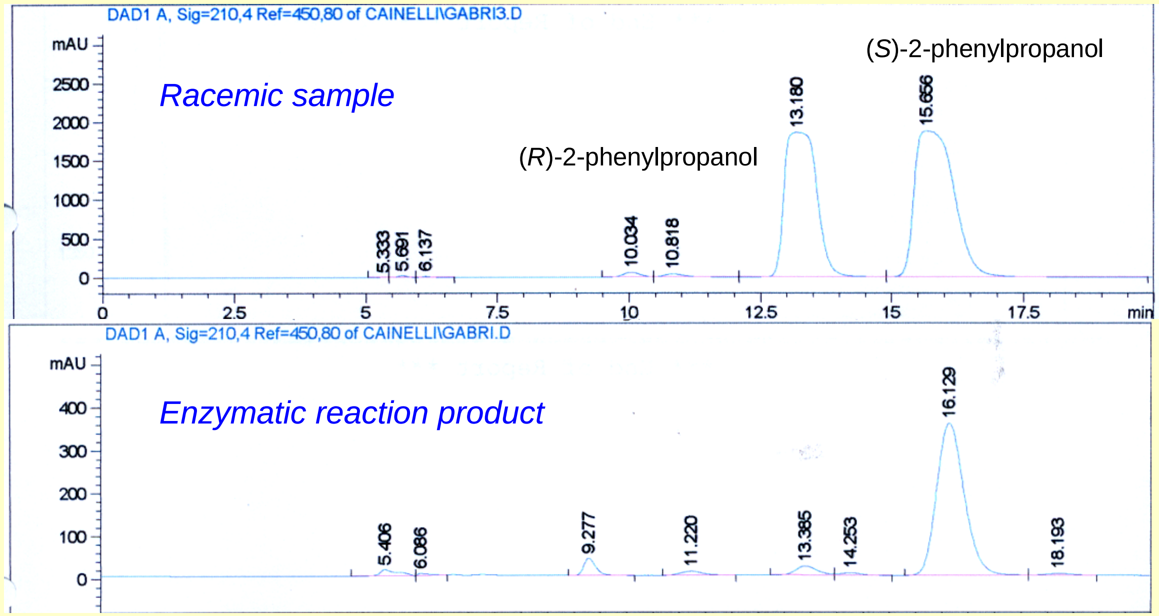
## Reaction Medium

**Homogeneous system**
aqueous buffer (phosphate pH 7.5) with miscible organic solvents

***Biphasic system***
aqueous buffer (phosphate pH 7.5) and immiscible organic solvents

# Enantioselective enzymatic reduction: 2-Phenylpropanal

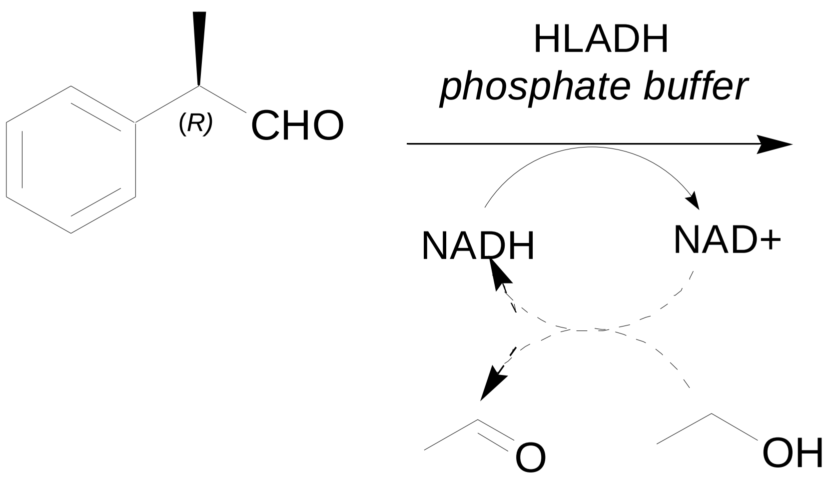



Chiralcel OF, 0.5 mL/min, hexane/iPrOH 93/7

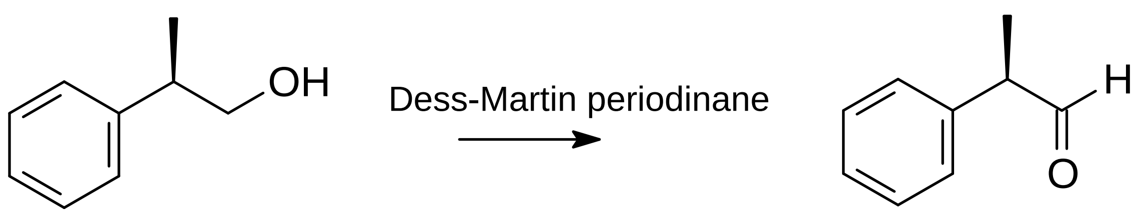
D. Giacomini, P. Galletti, A. Quintavalla, G. Gucciardo, F. Paradisi *Chem Commun*, 2007, 4038
D.Giacomini, P. Galletti, F. Paradisi *Innovation Technology* 2008, 78.

# Enantioselective Bioreduction with Stereoinversion



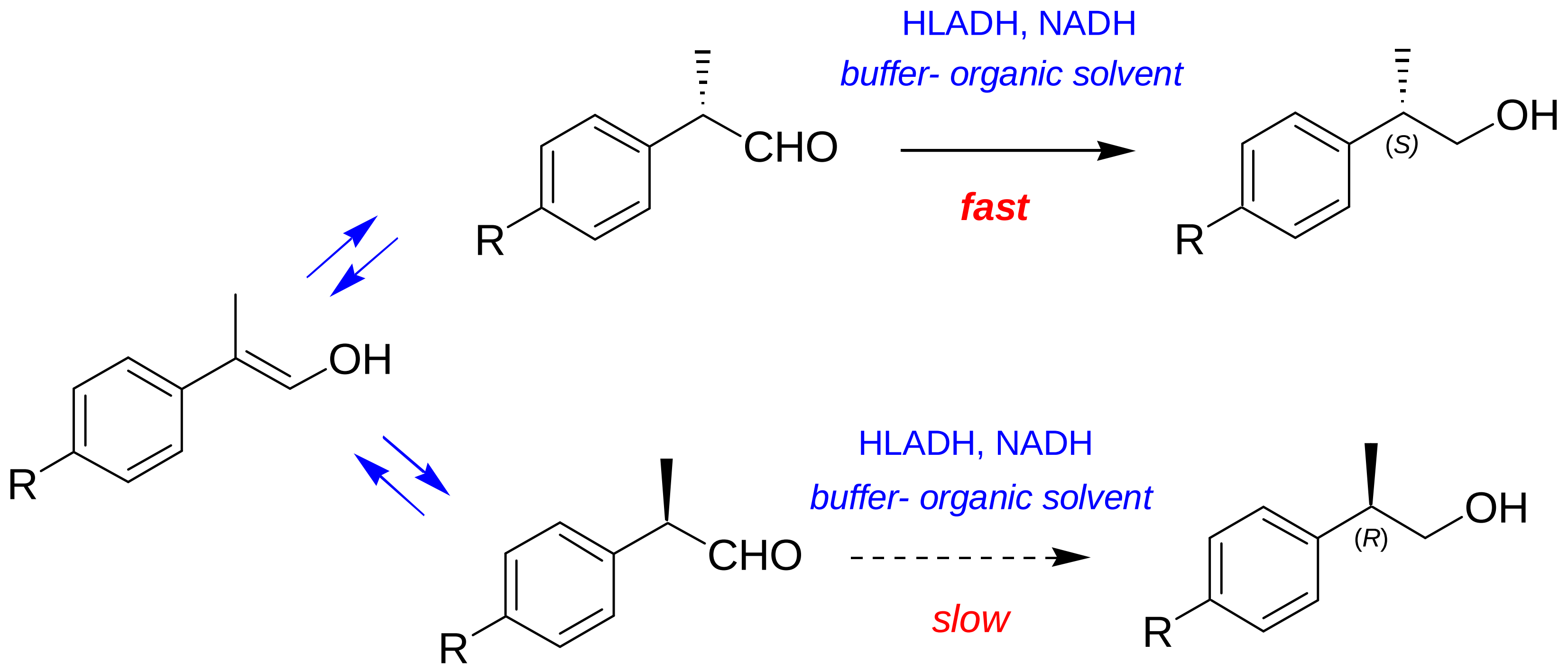

NADH stoich: 96% Y (*S*)/(*R*) 96:4

NADH recycled: 95% Y (*S*)/(*R*) 95:5




R. Berardi, G. Cainelli, P. Galletti, D. Giacomini, A. Gualandi,L. Muccioli and C. Zannoni,
*J. Am. Chem. Soc.*, 2005, *127*, 10699–10706

# Dynamic Kinetic Resolution process via keto-enol tautomerism

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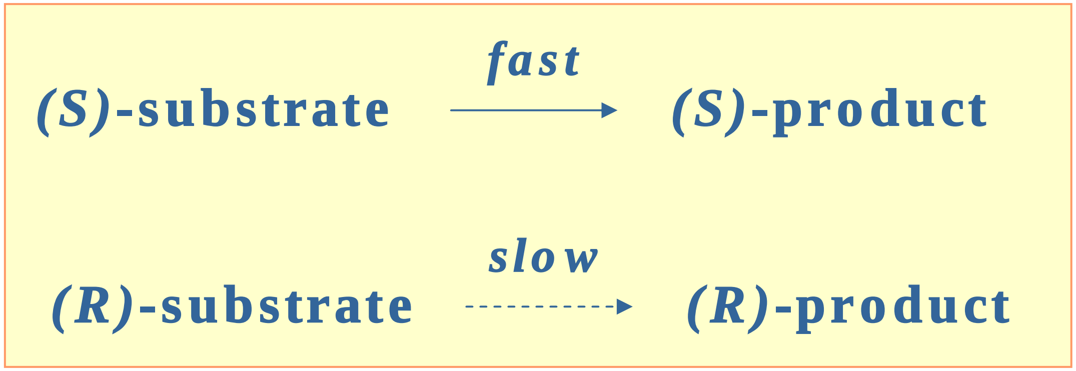
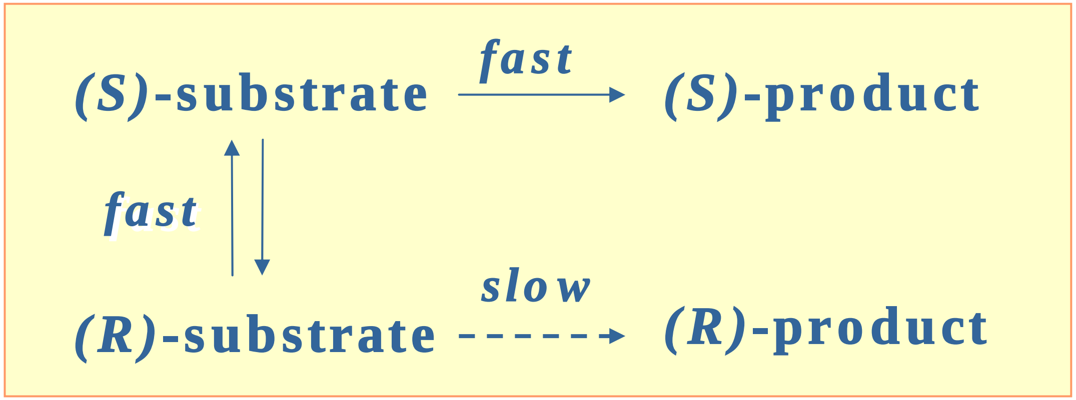
# Dynamic Kinetic Resolution: Beyond the Kinetic Resolution




**Kinetic Resolution**

max.theoretical yield 50%




**Dynamic Kinetic Resolution**

max.theoretical yield 100%

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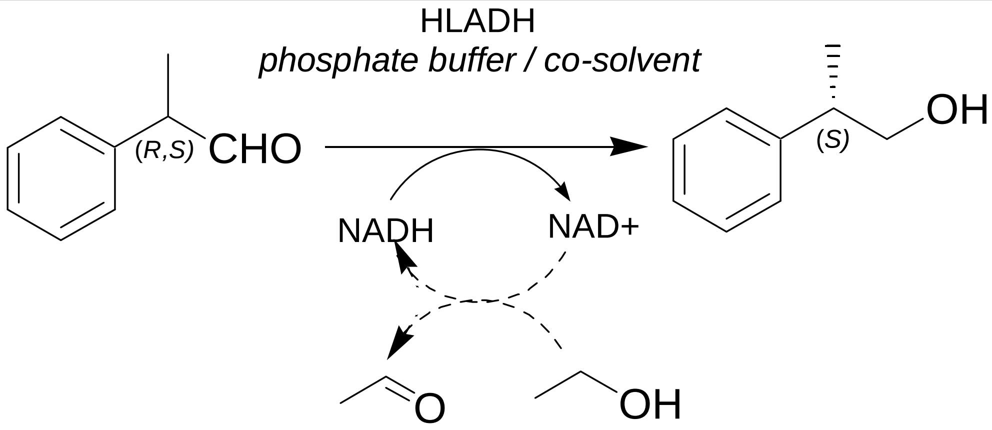
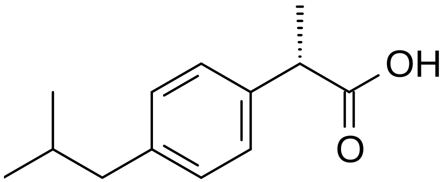
# Dynamic Kinetic Resolution with organic co-solvents



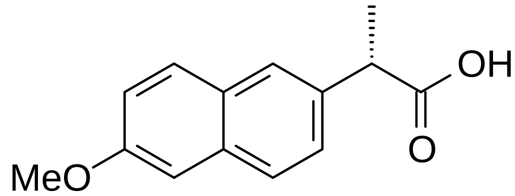

| entry | co-solvent (%) | NADH recycle | time (h) | HLADH (mg/ml) | yield % | S% | R% |
|---|---|---|---|---|---|---|---|
| 1 | $CH_3CN$ (10) | no | 5 | 0.01 | 71.9 | 89 | 11 |
| 2* | $CH_3CN$ (16) | yes | 96 | 0.09 | 90.3 | 94 | 6 |
| 3 | THF (10) | no | 5 | 0.01 | 54.7 | 96 | 4 |
| 4 | THF (10) | yes | 5 | 0.01 | 54.1 | 95 | 5 |
| 5 | *n*hexane (90) | no | 5 | 0.2 | 69.9 | 74 | 26 |
| 6 | *n*hexane (95) | no | 5 | 0.2 | 80.3 | 64 | 36 |
| 7 | *n*hexane (95) | yes | 5 | 0.2 | 68.0 | 72 | 28 |
| 8 | *n*hexane (99) | yes | 5 | 0.2 | 54.6 | 90 | 10 |

* 1 mmol scale

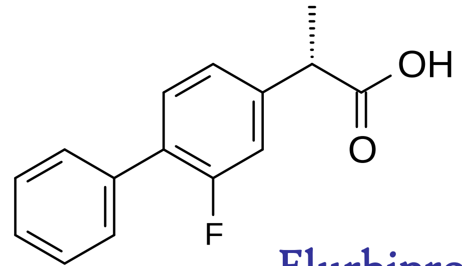
# Profens: a family of (S)-enantiomers dedicated to inflammation



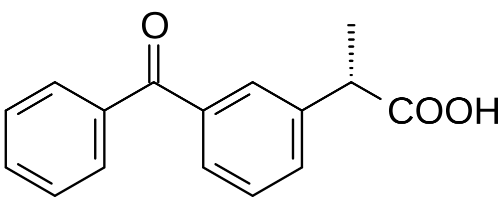

Ibuprofen



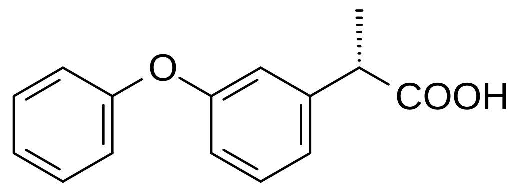

Naproxen




Flurbiprofen

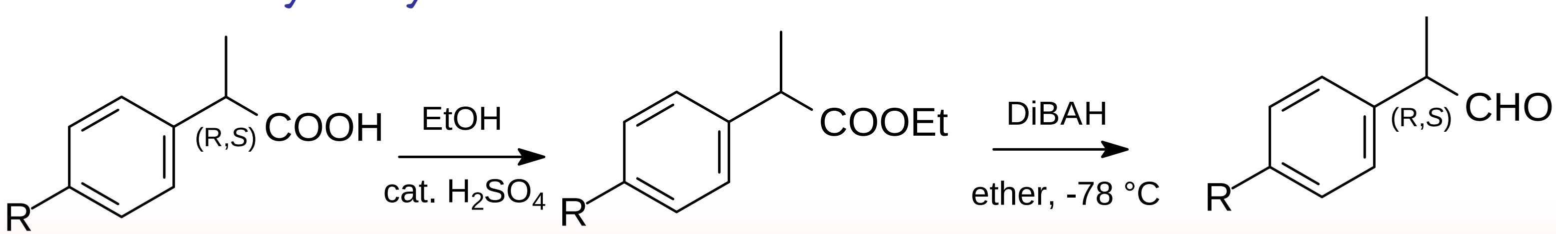



Ketoprofen




Phenoprofen

## Profen-aldehydes synthesis

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# References


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A.J.J. Straathof, S. Panke, A.Schmid The production of fine chemicals by biotransformations
Current Opinion in Biotechnology 2002, 13:548–556

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Tetrahedron: Asymmetry 2004, 2727

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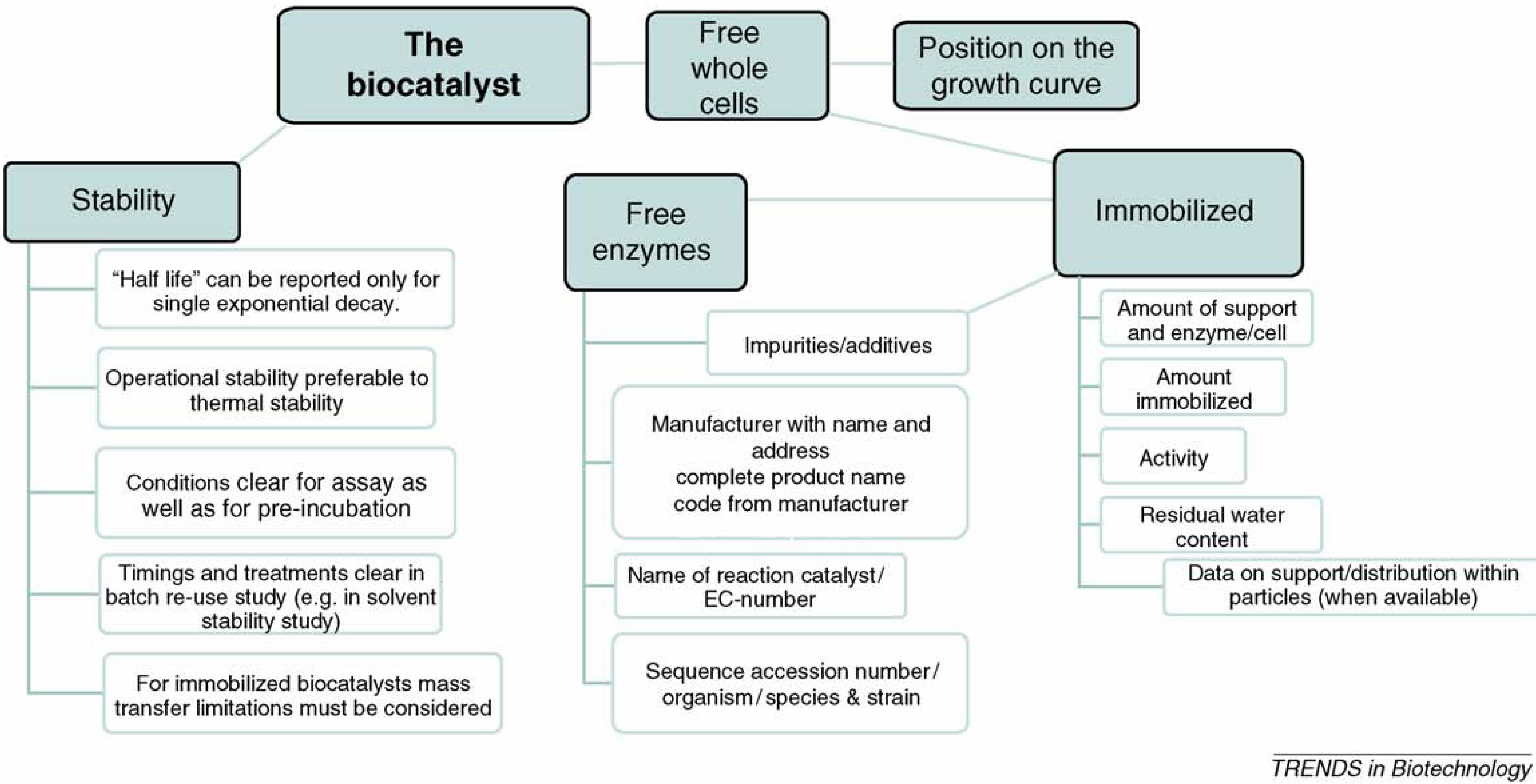
Aus. J. Chem. 2004, 57,281-289.

Patel, Coordination Chemistry Reviews 252 (2008) 659–701

# Enzymes in Industry





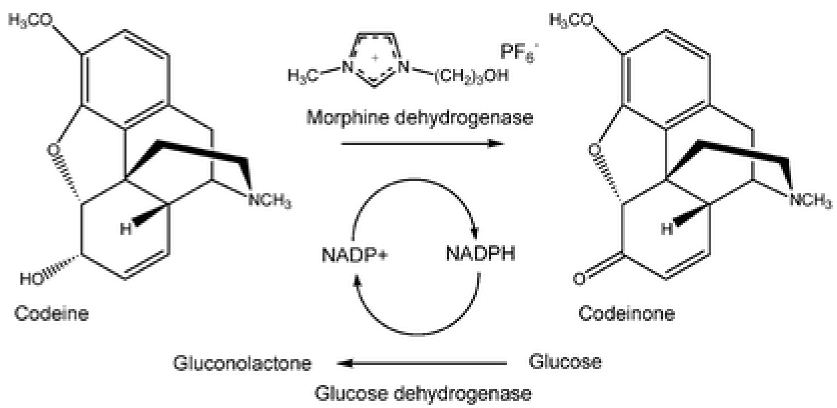

Trends in Biotechnology 2010, 171

# Enzymes in non-conventional media :

# Ionic Liquid

**Cofactor-dependent enzyme catalysis in functionalized ionic solvents†**

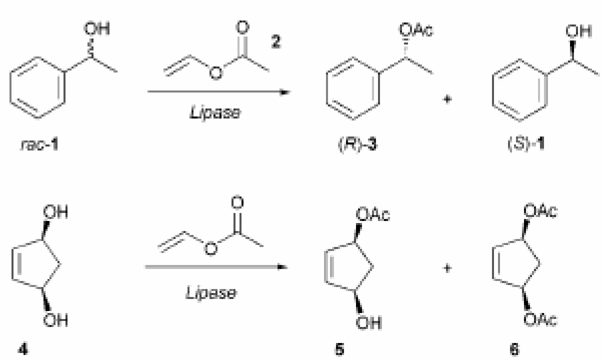
Adam J. Walker*[a,b] and Neil C. Bruce[a,b]
[a] *CNAP, Department of Biology (Area 8), University of York, PO Box 373, York, UK YO10 5YW. E-mail: aw41@york.ac.uk; Fax: +(44)1904 328786; Tel: +(44)1904 328777*
[b] *Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, UK CB2 1QT*





*Chem. Commun.*, 2004, 2570 - 2571

# Fluorous phase




*Scheme 1.* Lipase-catalyzed kinetic resolution of racemic 1-phenylethanol (1) and desymmetrization of *meso*-2-cyclopentene-1,4-diol (4).

*Table 1:* Kinetic resolution of 1-phenylethanol (1) catalyzed by Novozym 435.

| Solvent | $t$[a] [h] | Conv. [%] | *ee* (*S*)-1 [%] | *ee* (*R*)-3 [%] | Initial rates[b] [nmol min$^{-1}$ mg$^{-1}$] | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $a_w < 0.01$ | $a_w \approx 0.43$ | $a_w \approx 0.58$ | $a_w \approx 0.75$ |
| R-32 | 5 | 50 | >99 | >99 | n.d.[c] | n.d. | n.d. | n.d. |
| R-227ea | 3.5 | 49 | 96 | >99 | n.d. | n.d. | n.d. | n.d. |
| R-134a | 4 | 49 | 96 | >99 | 325 | 387 | 407 | 358 |
| hexane | 8 | 46 | 85 | >99 | 227 | 241 | 260 | 228 |
| MTBE | 35 | 49 | 96 | >99 | 51 | 64 | 68 | 60 |

[a] The time point when no further reaction was evident (that is, the rate of the reaction was approaching zero). [b] Initial rates are given in units of nmol of product 3 per minute per mg of enzyme and were determined from the slope of the time-course measurements between 0 and 5% conversion for an approximate water activity ($a_w$). [c] n.d. = not determined.

*Angew. Chem. Int. Ed.* 2004, 43, 5519–5523