CODEXIS®

Enzyme Catalysis

Easy and Efficient

Pascal Dünkelmann

Bringing Life to Chemistry™

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Potential of Biocatalysis

- Replacement of kinetic resolutions with asymmetric synthesis
- Catalytic rather than stoichiometric reagents
- Exquisite enantioselectivity; e.e.s >99.5 are routine
- Exquisite chemoselectivity
- Green processes: water as solvent, mild conditions; e.g. reductions (IPA) and oxidations (air) at RT, atmospheric pressure
- Inexpensive catalysts manufactured by low-cost fermentation
- No special equipment required to run reaction
- More reaction classes become available

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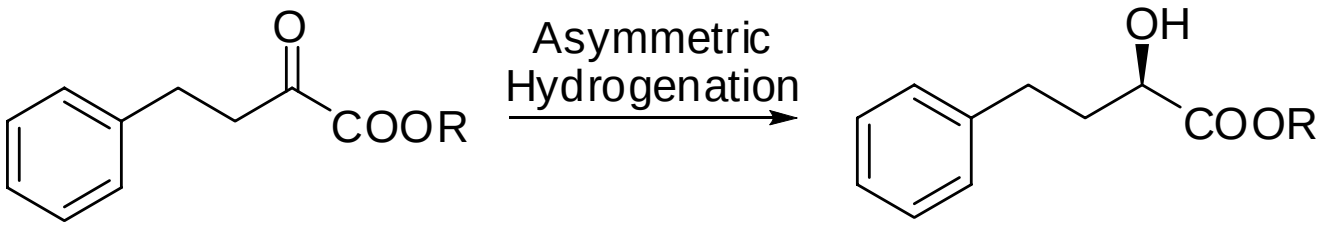
Comparison of selected metrics: Yield & Selectivity



Feature	Whole cell bioreduction	LDH/FDH Membrane	Pt-cinchona Ketoester	Pt-cinchona Diketoester
Yield (%)	99	79	46	50
Atom economy (%)	30	34	51	52
e.e. Product (%)	>99.8	>99.8	80-92	76-82

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Comparison of selected metrics: Productivity

Feature	Whole cell bioreduction	LDH/FDH Membrane	Pt-cinchona Ketoester	Pt-cinchona Diketoester
Space-time yield (mol/L/d)	1.7-0.64	1.0	24	12
Ketone conc (%)	1.9	1.9	>20	>20
kg materials/ kg product	105	82	58	40
kg solvent/ kg product	42	25	14	16

Conclusion: “Green-ness” of biocatalysis is lacking here due to low substrate concentration and isolation of dilute product from water

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Codexis Biocatalysis Paradigm

1. **Design the desired conceptual biocatalytic chemical process**
   - **Make it "green-by-design"**
2. **Evolve the biocatalyst for fitness to enable the desired process**

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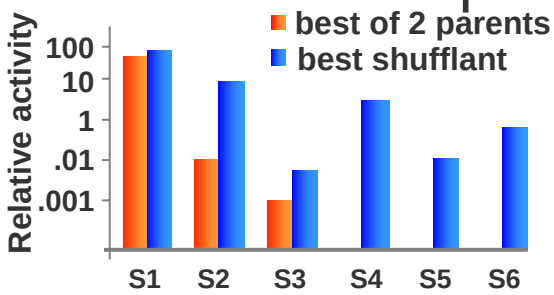
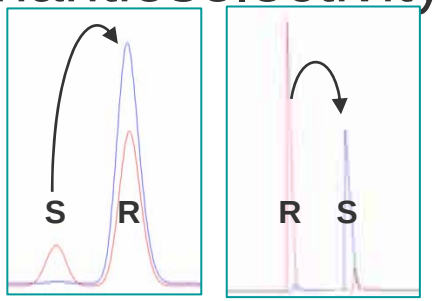
Evolutionary Criteria

Substrate Specificity



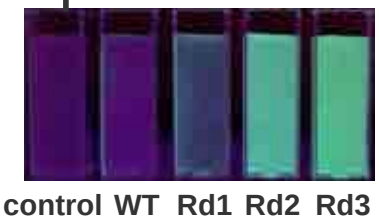
Selectivity

Enantioselectivity



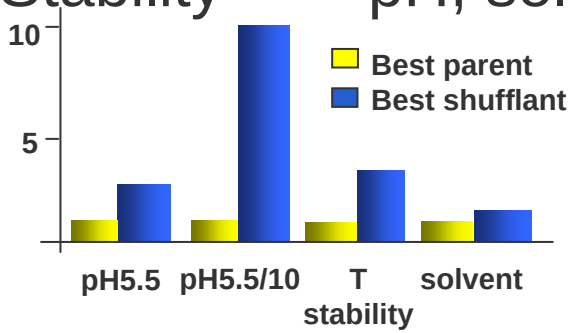
Substrate/Product Tolerance

Expression



Stability

pH, solvent



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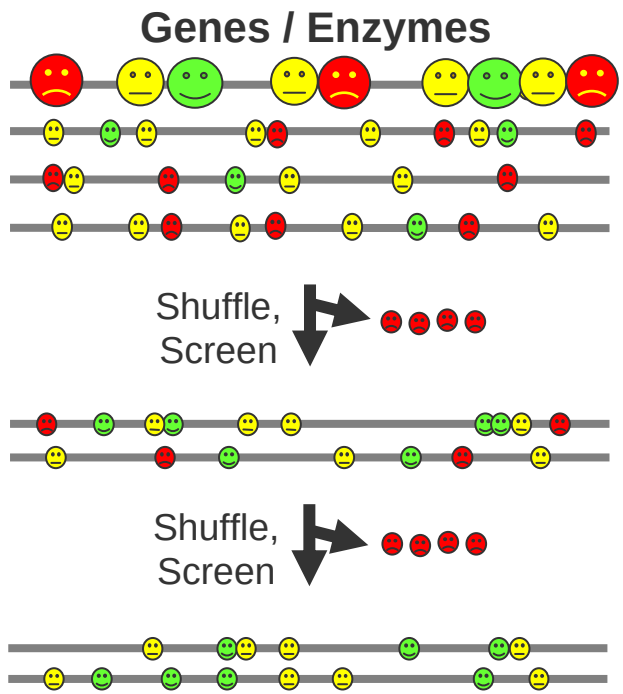
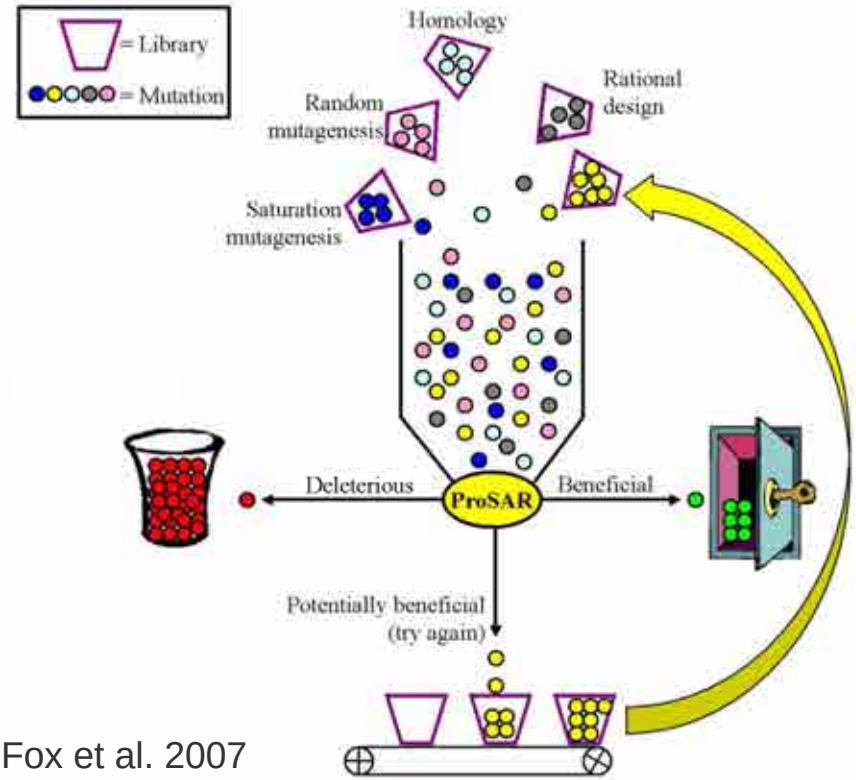
Legacy DNA shuffling



Stemmer 1994

Sorting gene-by-gene

ProSAR-Driven Semi-Synthetic Shuffling



Fox et al. 2007

Sorting mutation-by-mutation

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Nature Biotech. March, 2007

Improving Catalytic Function by ProSAR-Driven Enzyme Evolution

- 4000-fold improved over WT
- 37 mutations from WT
- Random mutation, rational design, shuffling and ProSAR used in concert
- Most beneficial mutations were not in top 10 hits and would have been missed without PROSAR

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Codex™ Diagnostic Panels

Codex™ Diagnostic Panels:

- Functionally diverse biocatalyst variants of a wild-type biocatalyst pre-evolved to:
  - to be chemical process robust (organics-stable, thermostable)
  - to be readily manufactured at commercial scale
  - to accept wide range of substrates
  - to produce different stereo-isomers
- Combinatorial design for structure-function analysis (Codexis' ProSAR™)
- Arrayed on three 96-well plates

- A diagnostic tool for PROtein Sequence-Activity Relationships
- Provide a jumpstart for directed evolution by DNA shuffling

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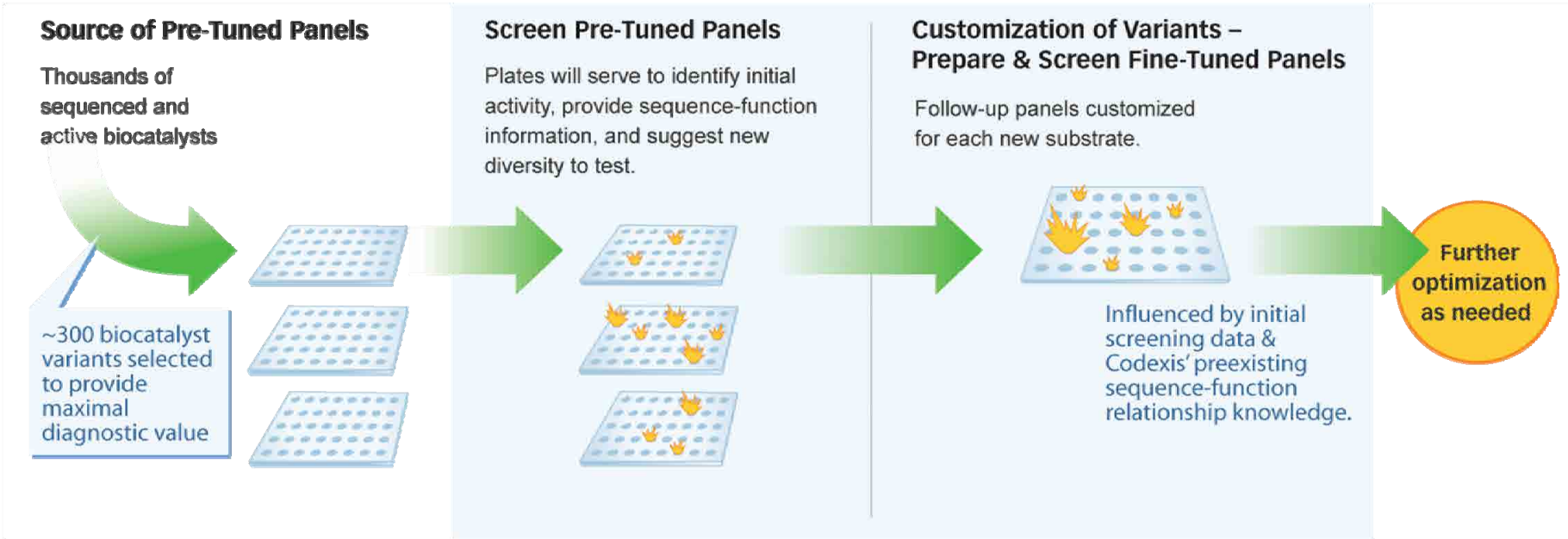
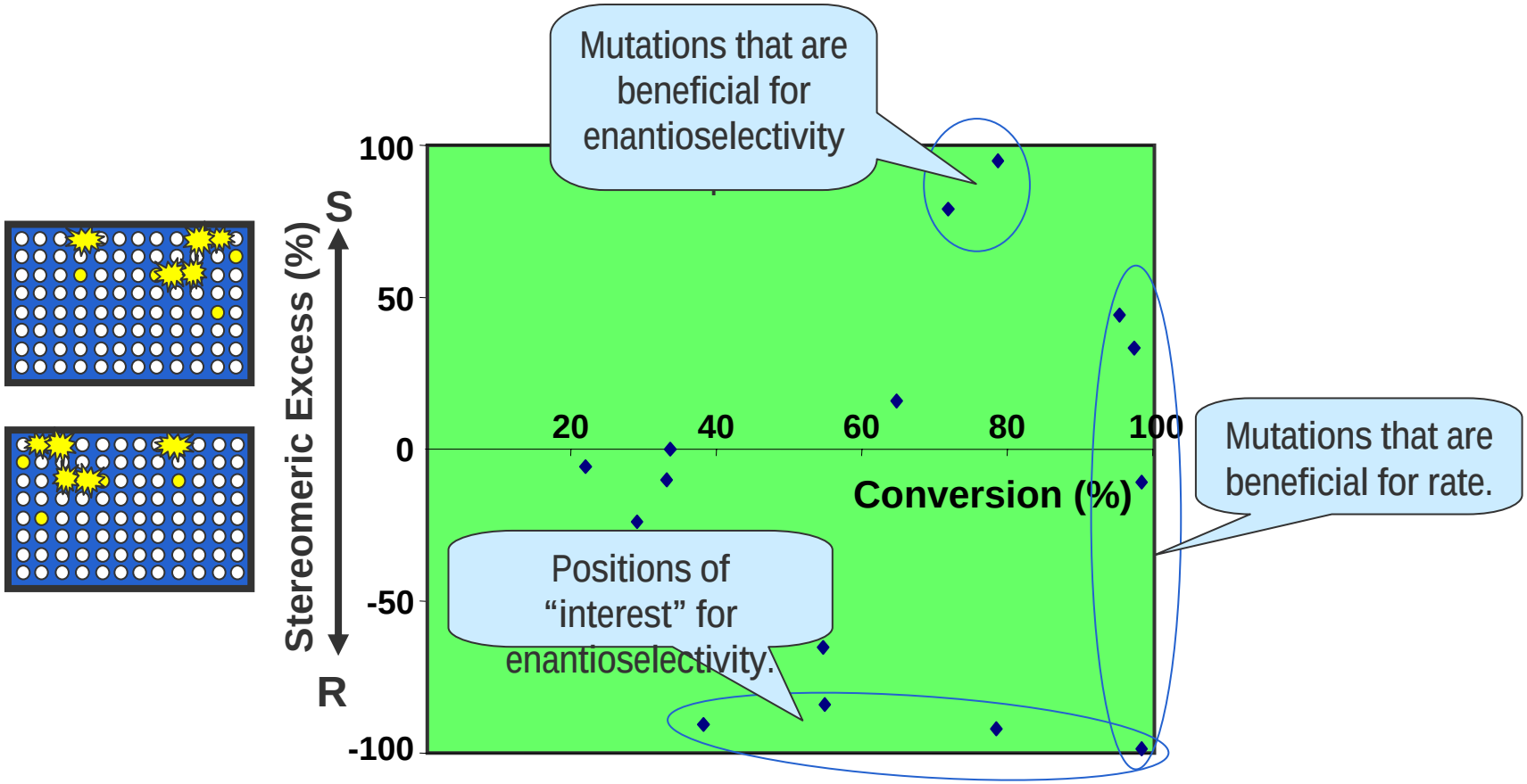
Screening a Codex Panel

CODEX PANEL OVERVIEW – PRE-TUNED AND FINE-TUNED PANELS



Using the KRED Panel for Ezetimibe Intermediate



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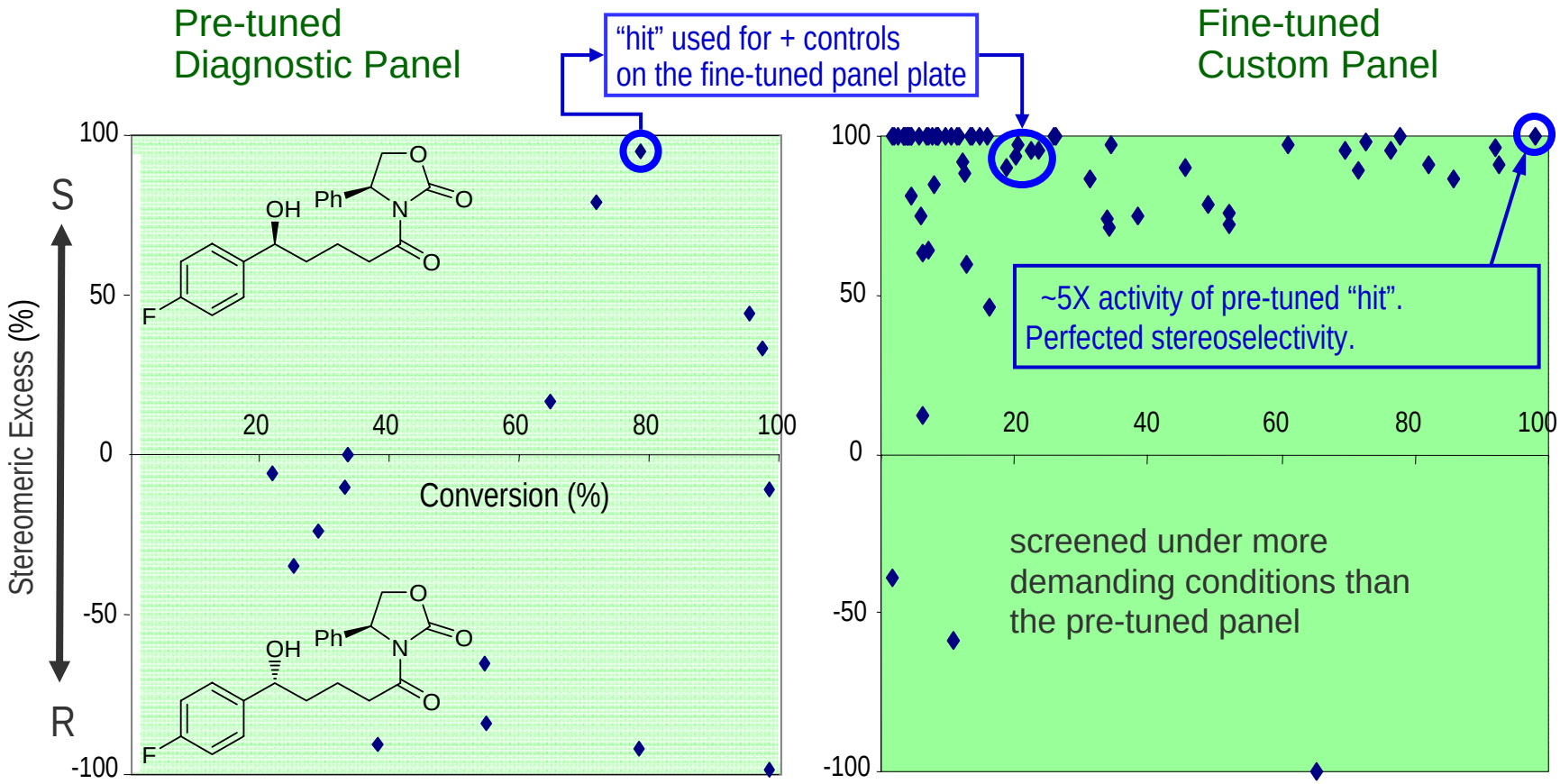

Fine-tuned panel "hit" required 500x further activity improvement

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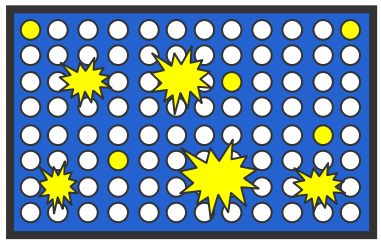
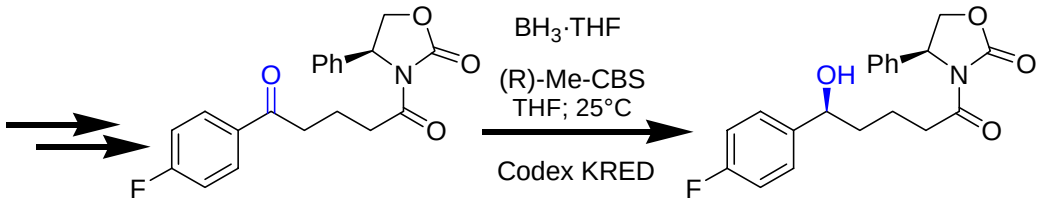
Process Comparison



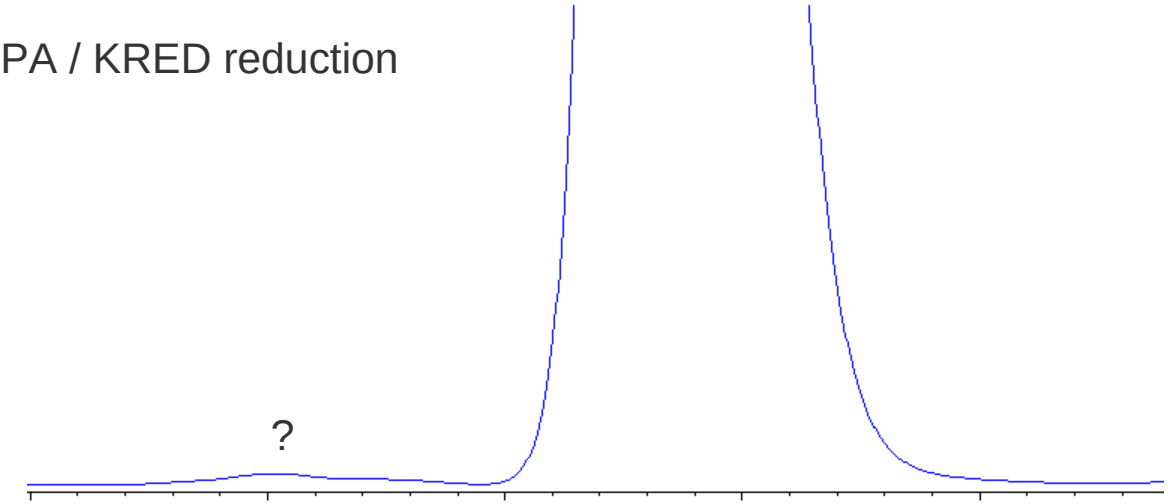


Chiral intermediate for ezetimibe

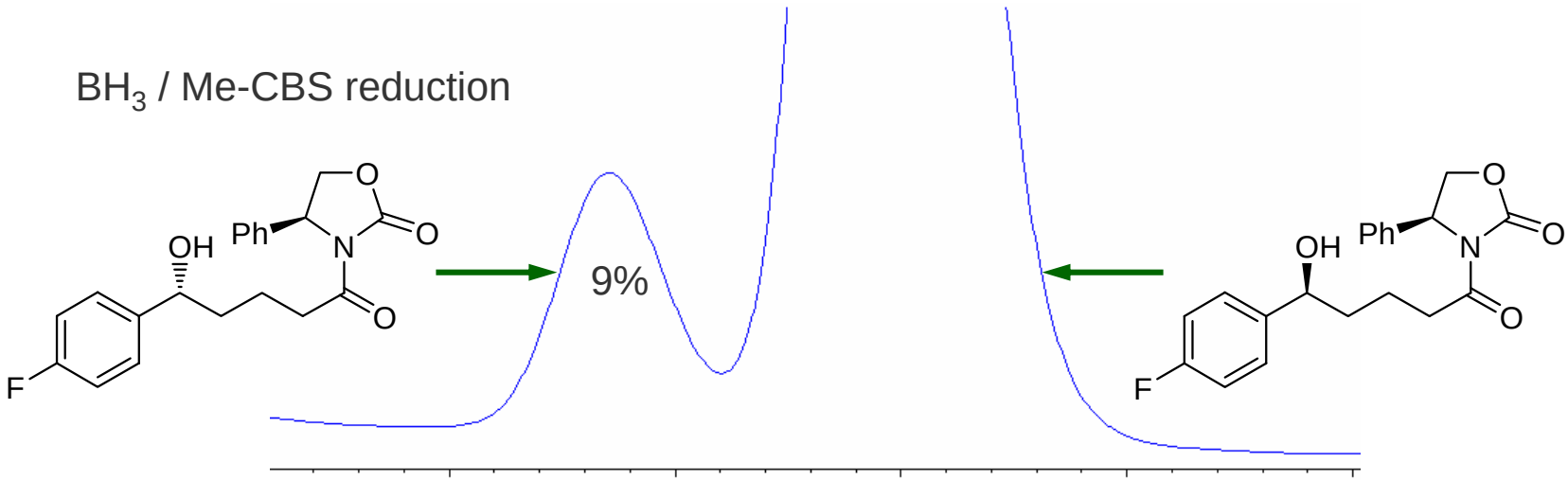
Chemical Process	KRED Process
Uses Me-CBS and BH_3 Both are toxic and hazardous. Me-CBS is expensive and used at high loading	Catalytic: Runs at 100 g/L: Environmentally friendly, Greatly reduced hazard (water, IPA at RT), Economically superior
Stereoselectivity is inadequate and highly "touchy" (over-reduction). Sensitive to moisture and reagent quality. Stereopurity upgrade required.	Reduction using glucose, GDH in H_2O, Produces >99.9% e.e. alcohol
Complicated Work-up	Straightforward work-up In tech transfer

Stereopurity of Codexis' Chiral Alcohol for Ezetimibe

Codexis IPA / KRED reduction



BH_3 / Me-CBS reduction



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The Azetidinone Intermediate for Carbapenem Antibiotics

Replacing Noyori's Ru-BINAP

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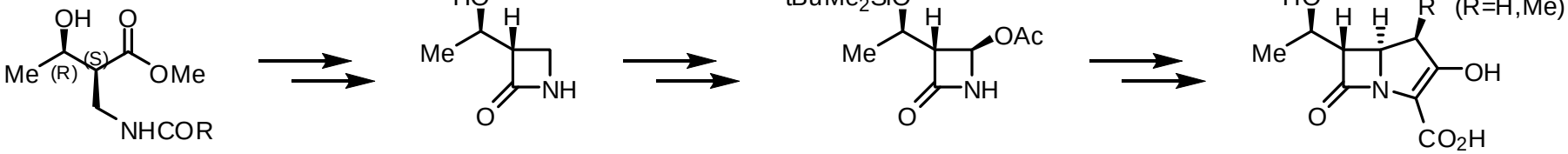
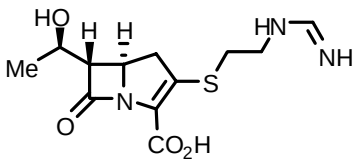
Azetidinone Intermediate for Carbapenem Antibiotic Nucleii



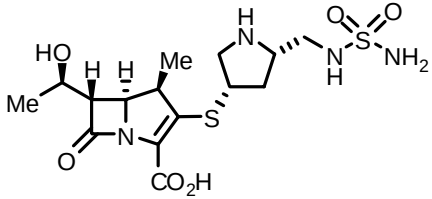
"azetidinone"

"AOSA"
item of commerce

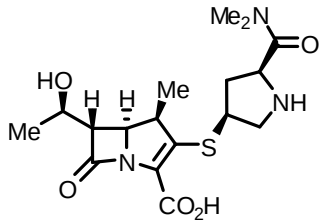
carbapenem nucleus
intermediates



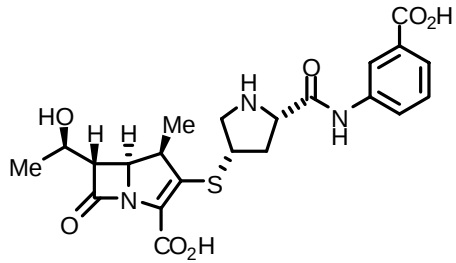
imipenem



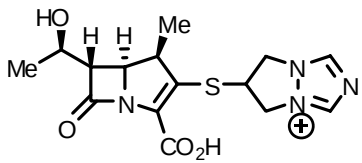
doripenem



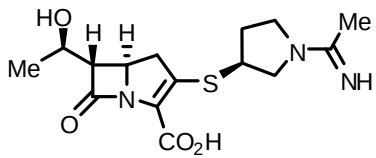
meropenem



ertapenem



biapenem



panipenem

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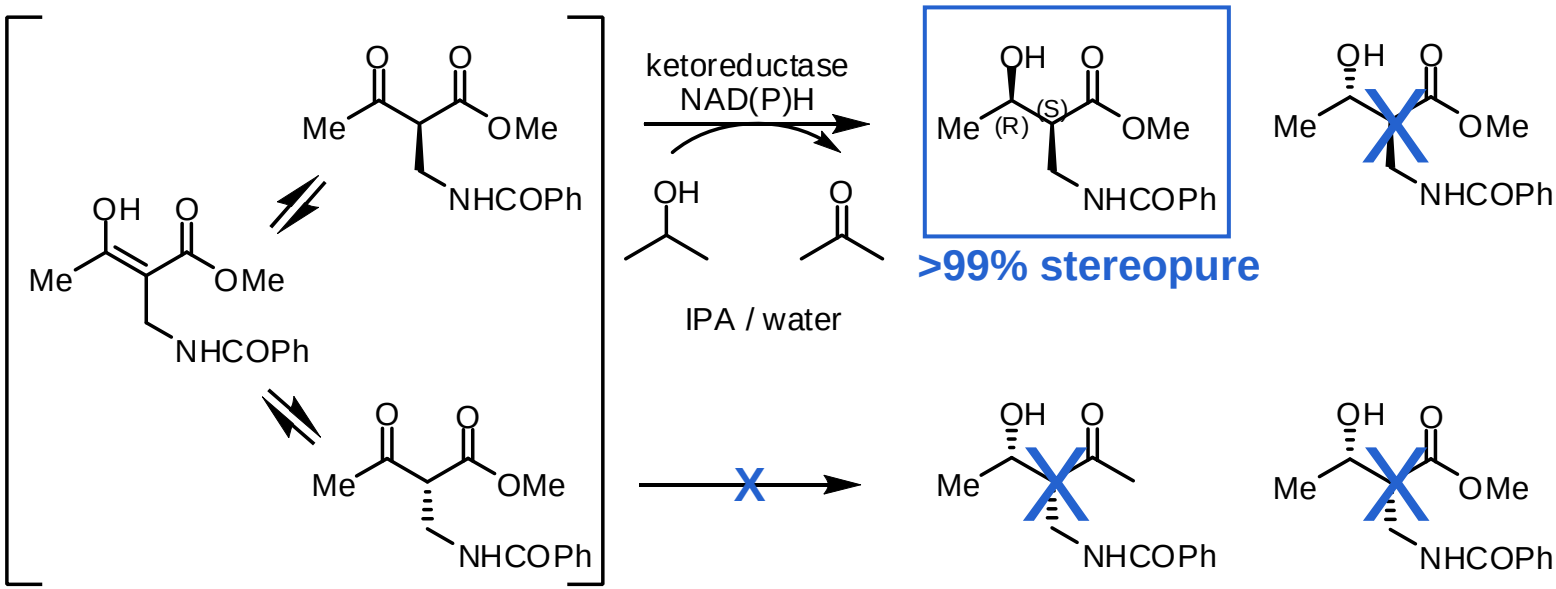
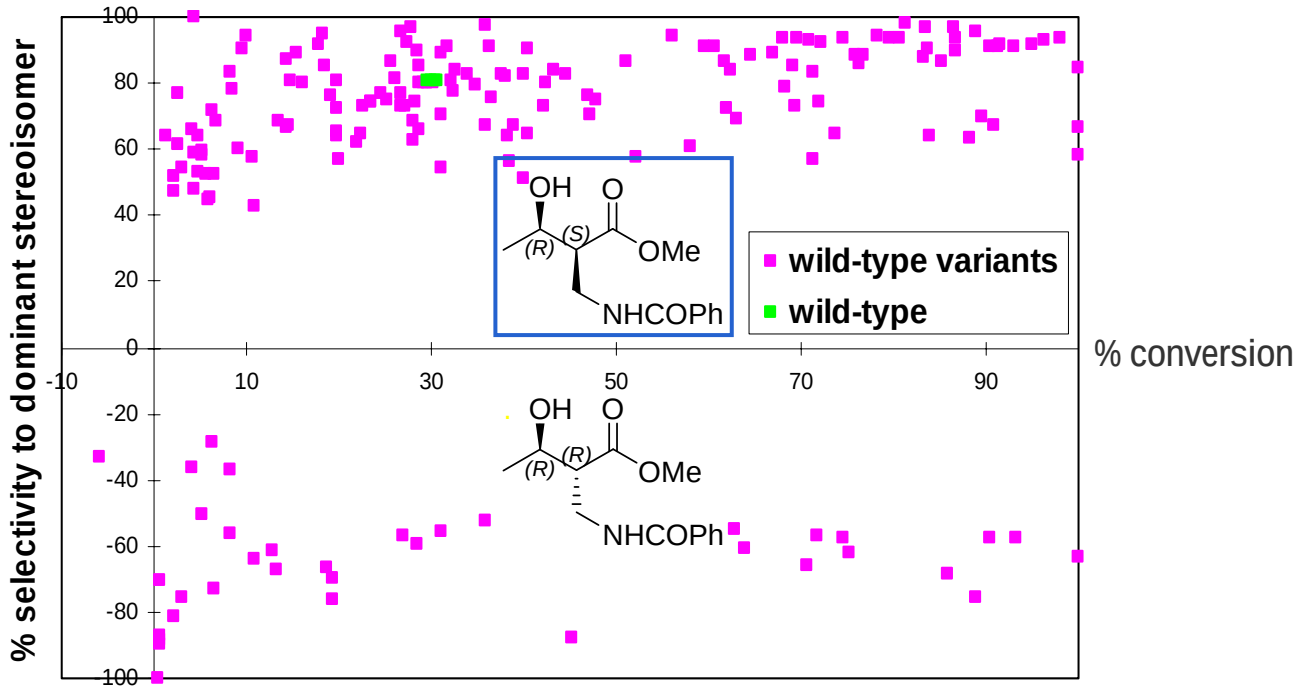
Desired Selectivity



More highly stereospecific/diastereoselective reduction with IPA in water enabled by a custom-evolved ketoreductase

Codex KRED Panel Screen



The Panel data shows diversity for higher activity and higher stereoselectivity over the wild type

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Rapid KRED Evolution

- Screened KRED panel
- Based on ProSAR, generated and screened one semi-synthetic library
- Rationally constructed 4 variants of the shuffled hit with combinations of 3 mutations previously identified as sometimes beneficial in evolution of this wild-type's variants

Parameter	Target	wild-type	final biocatalyst
[substrate] (g/L)	≥ 200	20	**250**
[enzyme] (g/L)	≤ 1 g/L	5	**1**
NADP (g/L)	≤ 0.1	0.1	**0.01**
Time (hrs)	≤ 24	24	**24**
Conversion (%)	>90	<5	**100**
Stereopurity (%)	>99	80	**>99**

- **The directed evolution was completed using only previously known mutations**
- **From initiation to tech-transfer, the project took 3 months**

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The Chiral Alcohol Intermediate for Montelukast

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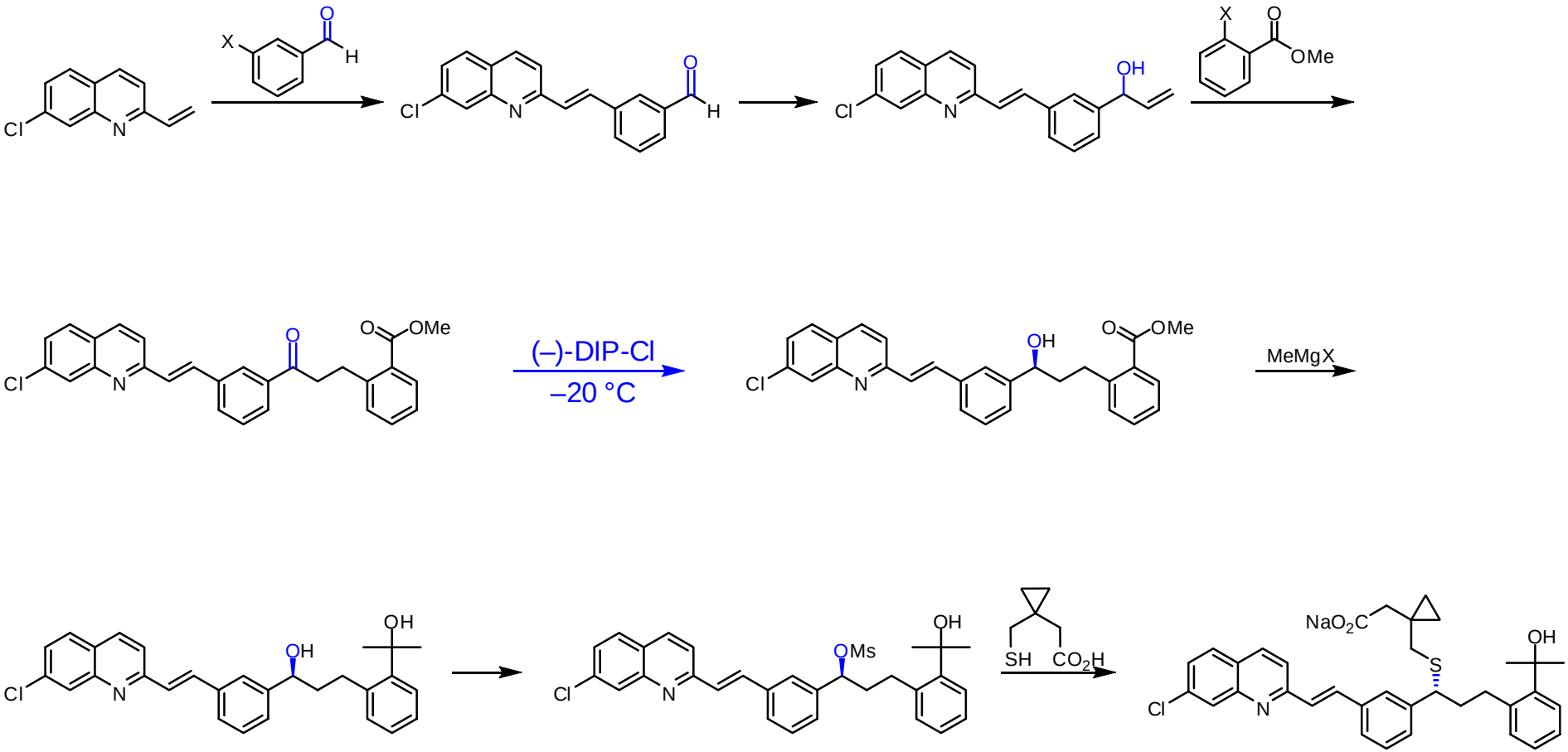
Merck's Route to Montelukast



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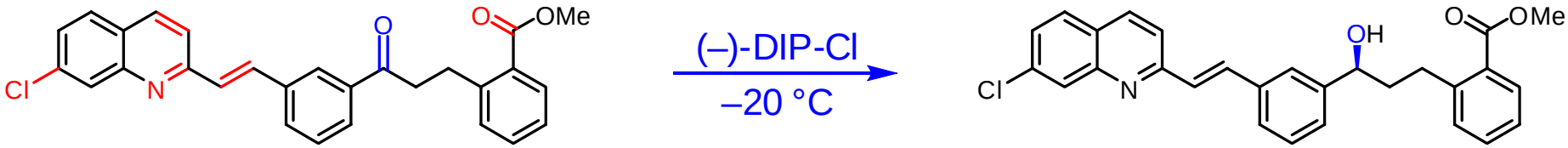
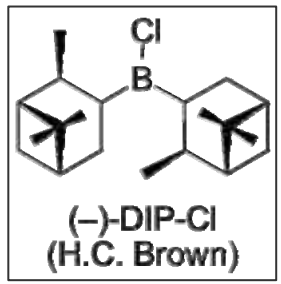
Merck's Reduction Reaction



- Merck's process[1] uses a mild and selective reagent, (–)-DIP-Cl that is a corrosive solid, acidic (DOT); causes burns; moisture-sensitive
- "To obtain both completion and high enantioselectivity, 1.8 equiv. of the reagent was necessary"
- Produced 97% e.e. alcohol. Isolated 87.1% yield, 99.5% e.e.
- Tedious work up to remove borate salts
- Merck publication: "Despite the availability of this method, alternative economical and environmentally acceptable procedures are desired"[2]



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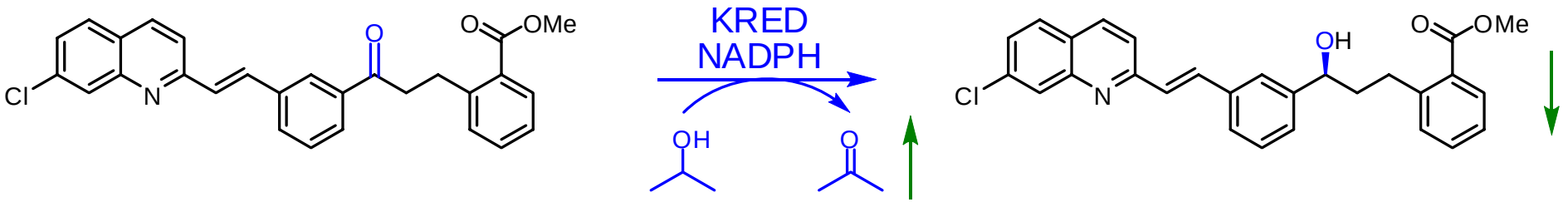
1. J. Org. Chem. 58, 3731 (1993)
2. Appl Microbiol. Biotechnol. 49, 709 (1998)

Codexis' Biocatalytic Reduction



- No commercially available/wild type enzyme exhibits any activity
- Activity was identified in a collection of Codexis' previously evolved KREDs, but ~2000x activity improvement was needed to meet commercial process targets
- Improvement was accomplished by Codexis' directed evolution technology
- Process requirements for evolutionary fitness:
- >50% IPA to shift equilibrium reaction.
- 50 °C: for acetone removal to shift equilibrium reaction
- <<0.01 g/L solubility in 1:1 IPA/water at room temperature

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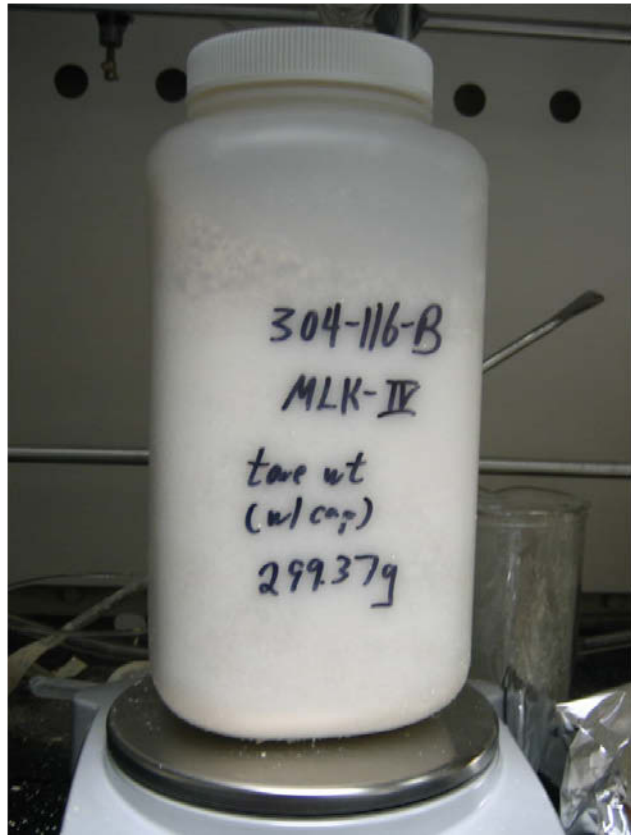
Codexis‘ Process

Slurry-to-Slurry reaction of nearly insoluble substrate to nearly insoluble product



10 L reaction slurry → filter, wash, dry → 1 kg product
on spec, incl. use test to API

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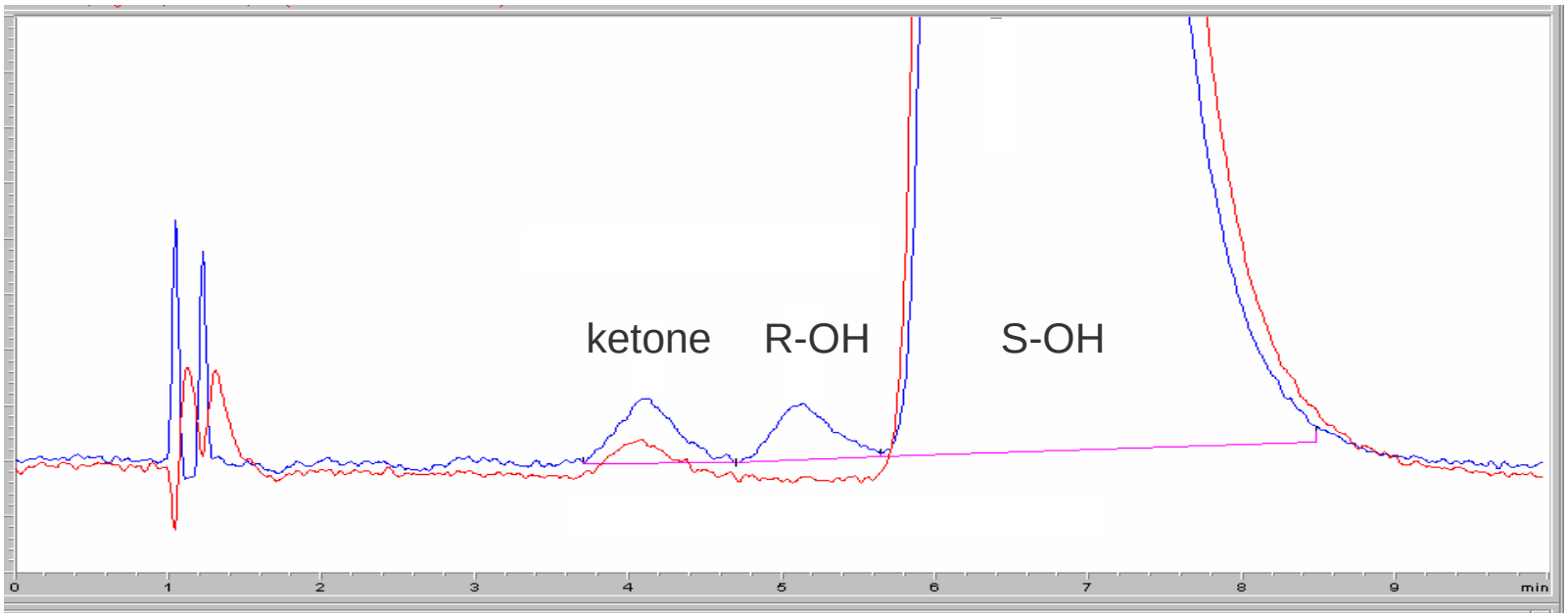
Perfect Enantioselectivity

blue: **crystalline** product from (–)-DIP-Cl reaction, 99.4 % e.e.

red: precipitate filtered from KRED reaction slurry;

R-enantiomer is never observed



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Process Performance

	(-)-DIP-Cl Process	Biocatalytic Process
Ketone Concentration	100 g/L	100 g/L
Chiral Agent/Ketone	1.8	catalytic
Temperature	-25 deg C	45 deg C
Conversion	not provided (87% isolated yield)	99.3%
Product Isolation	extraction with high dilution	direct filtration
Enantiomeric Excess	99.2% (after crystallization)	>99.9% (without crystallization)
Solvent/Product (L/kg)	30–50	6
Solvents used	dichloromethane, THF	iPrOH, toluene, water
Other waste	borate salts, other inorganics, 3.6 equiv. of α-pinene	biodegradable enzyme, water

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Other Available Enzyme Classes

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Enzyme Experience

• Ketoreductases (KRED)	chiral alcohols
• Transaminases (TA)	chiral amines
• Acylases (ACY)	chiral alcohols or amines (by resolution)
• Ene Reductases (ERED)	α and/or β chiral ketones, esters, nitriles
• Halohydrin Dehalogenases (HHDH)	chiral epoxides, vicinal cyanohydrins, amino alcohols, diols
• Nitrilases (NIT)	chiral carboxylic acids
• Cytochrome P450 (human and bacterial)	metabolites, hydroxylation, dealkylation, heteroatom oxidation

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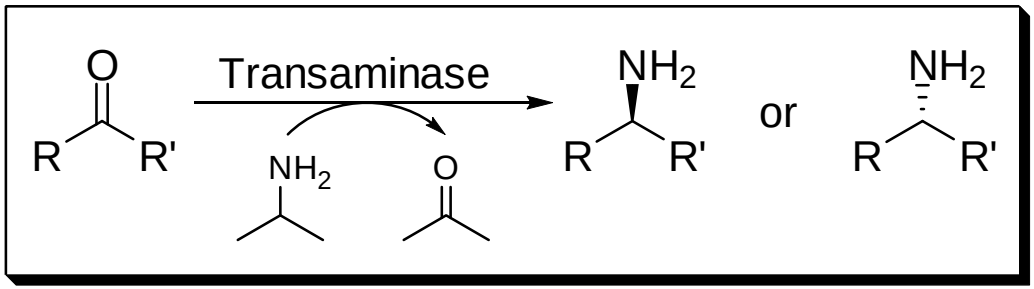
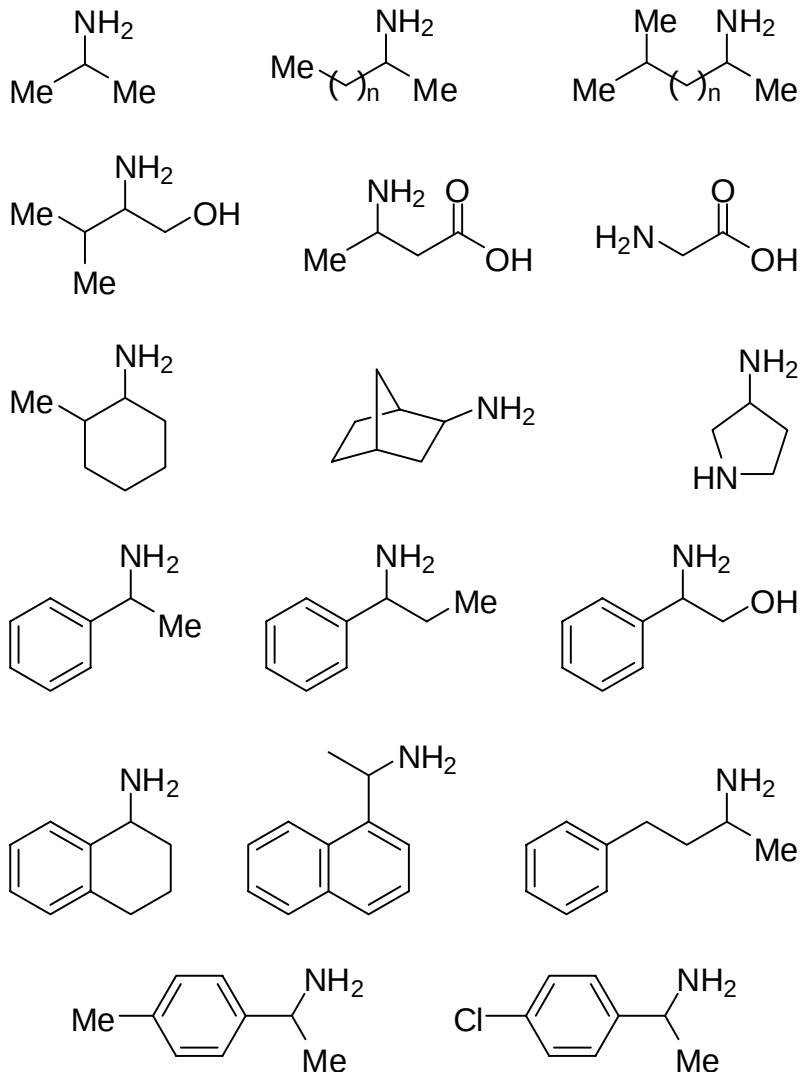
Transaminases (TAs)



Chemical Process	Codex TA Panel
• Diastereomeric salt resolution • Reductive amination • Reduction of enamines • Simulated Moving Bed	• Versatile catalyst for a broad range of substrates • Standard methods of use • High volumetric through-put
Issues: •Low yielding •Expensive catalysts •Need for dedicated equipment	Solutions: •Economically attractive processes •High yielding •Standard catalyst manufacturing



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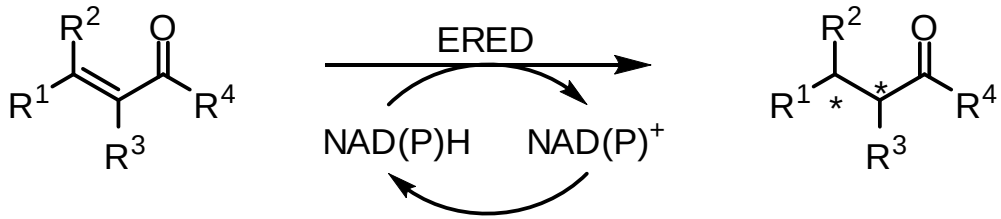
Ene Reductases (EREDs)

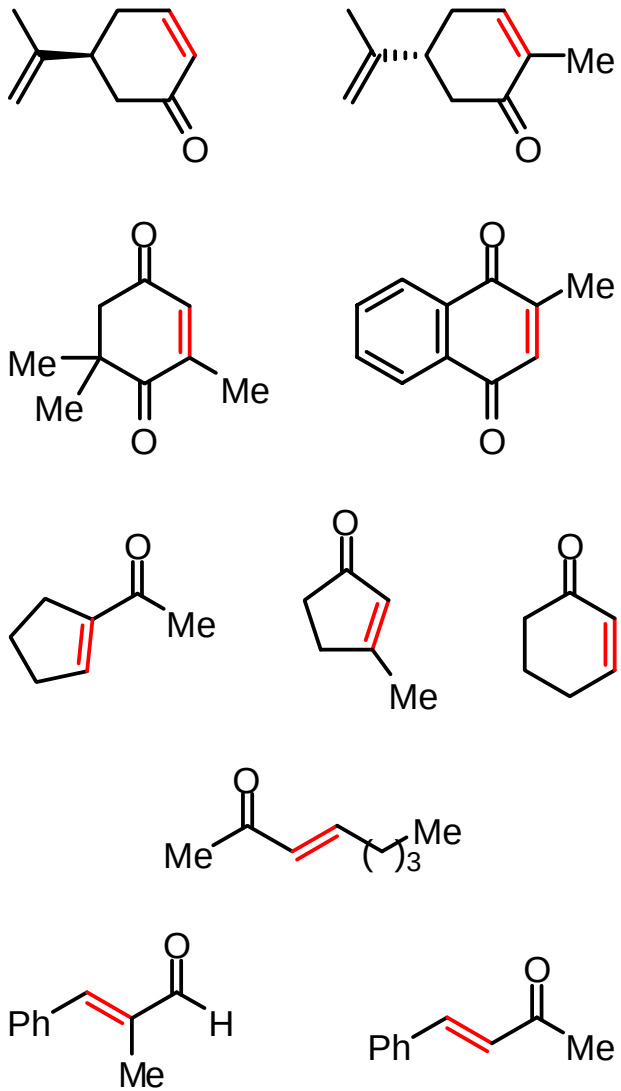


The ERED Codex for:

Chiral reduction of α,β-carbonyls:
- Esters
- Nitriles
- Ketones/aldehydes
- Nitro compounds

Co-factor recycling with either IPA/KRED or glucose/GDH

Variants are highly stable to organic solvents (e.g. IPA) and elevated temperatures



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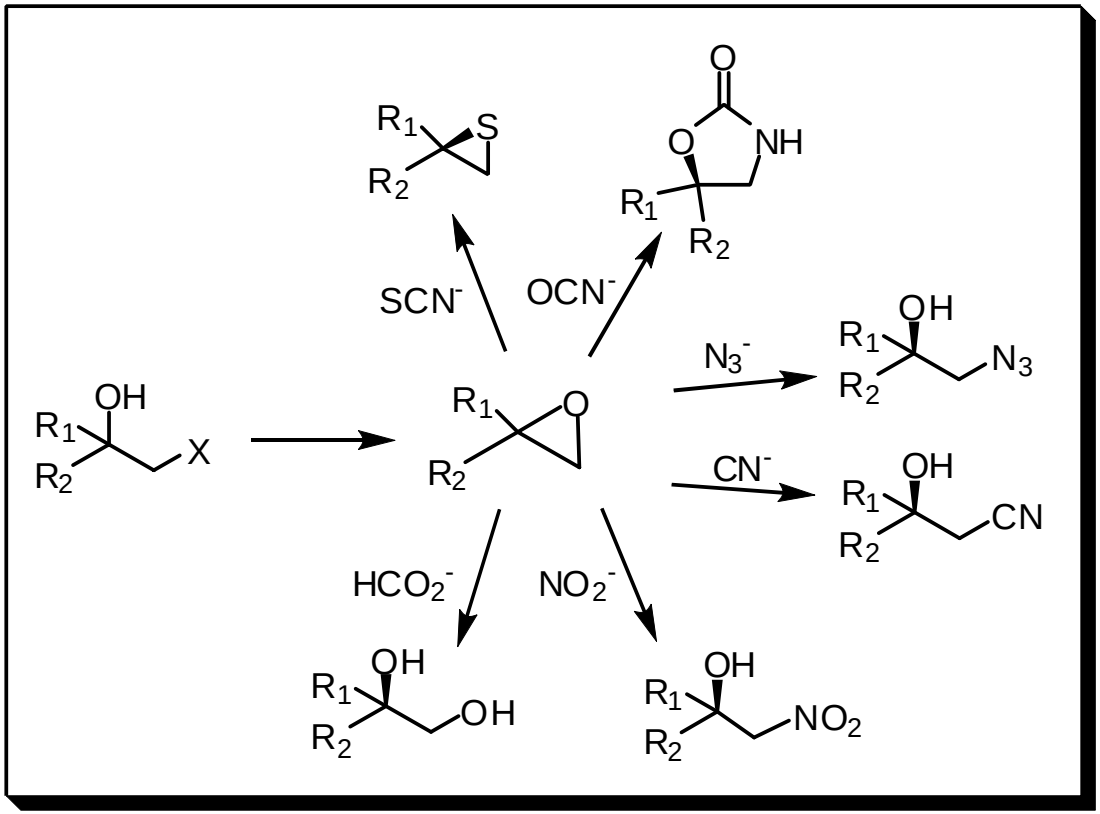
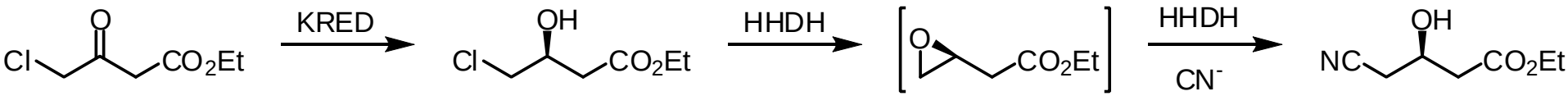
Halohydrin Dehalogenases (HHDHs)



Codex HHDH Panel
Broad range nucleophiles
α,α –disubstitution
HHDH Opportunity: •Hetero atom substitution allowed •Regioselectivity tuneable •Cheap nucleophiles



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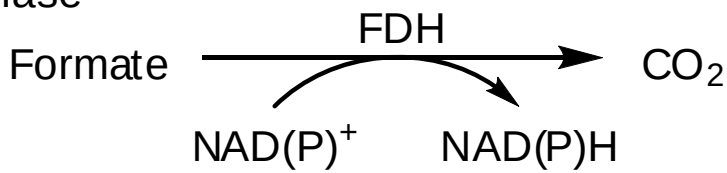
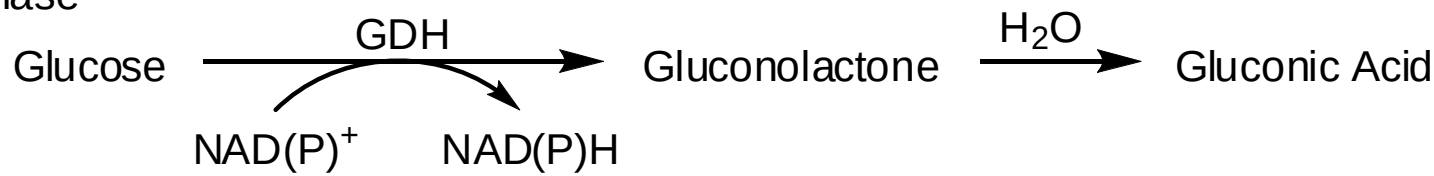
Cofactor Recycling

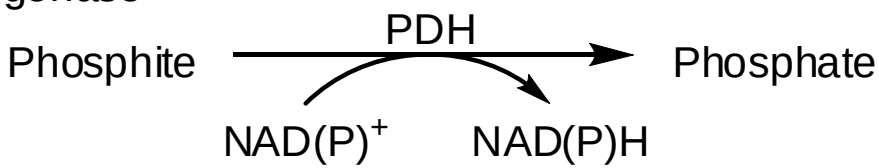
Formate Dehydrogenase



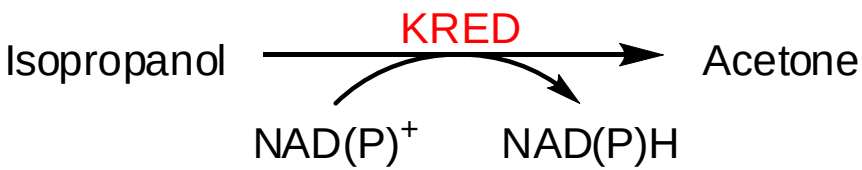
Glucose Dehydrogenase



Phosphite Dehydrogenase



Ketoreductase



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And More…

- Nitrilases: Mild hydrolysis of nitriles to carboxylic acids/amides; Panel available soon
- Hydrolases: in collaboration with Novozymes
- Cytochrome P450 enzymes: for metabolite synthesis and lead diversification
- Other reactions can be developed, *e.g.* amine oxidases…

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Boceprevir and Telaprevir

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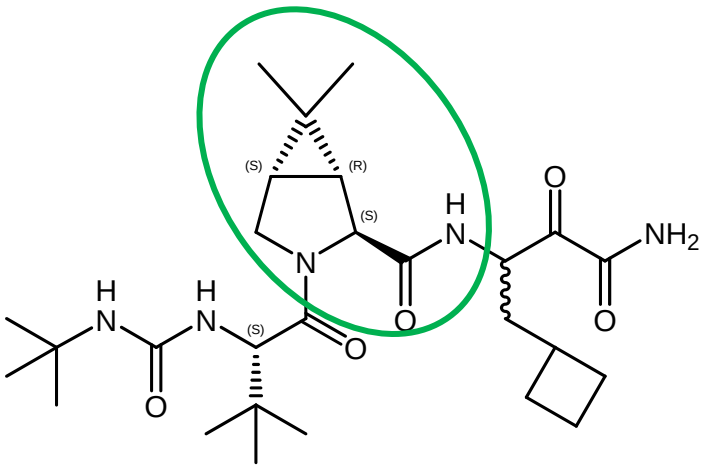
Boceprevir and Telaprevir

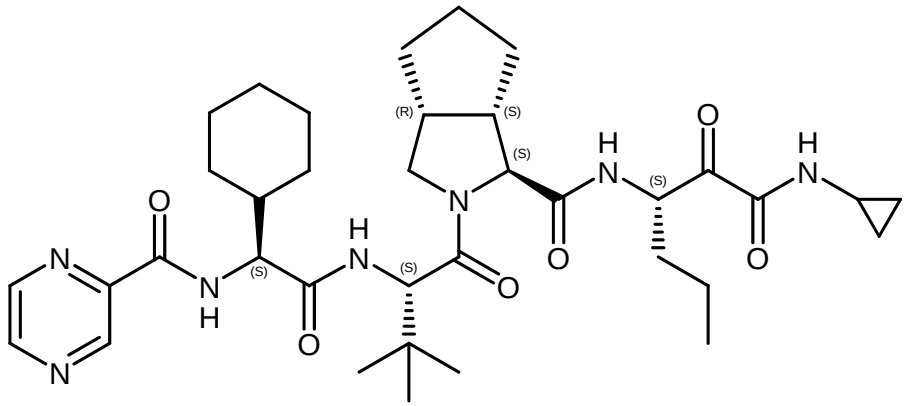
Hepatitis C Protease Inhibitors in Phase III Clinical Trials

Candidates for a first-in-class Hepatitis C therapy



boceprevir

Schering-Plough



telaprevir

Vertex

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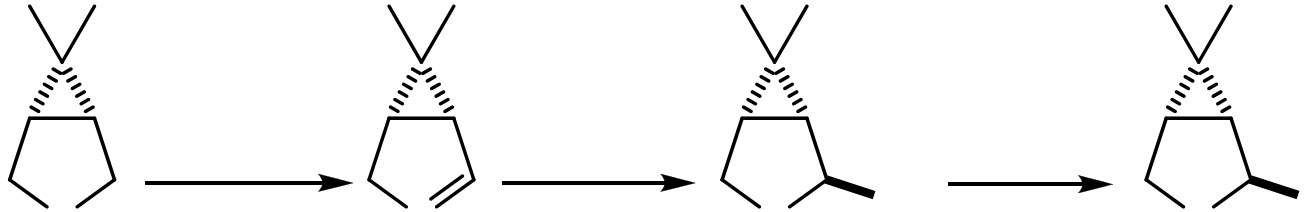
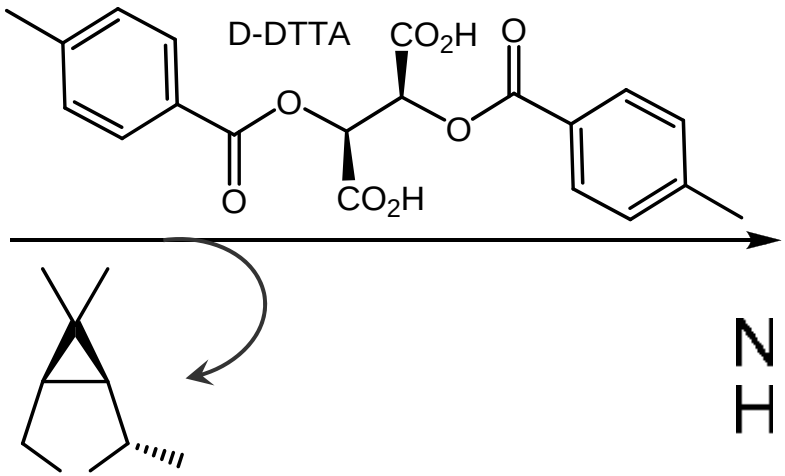
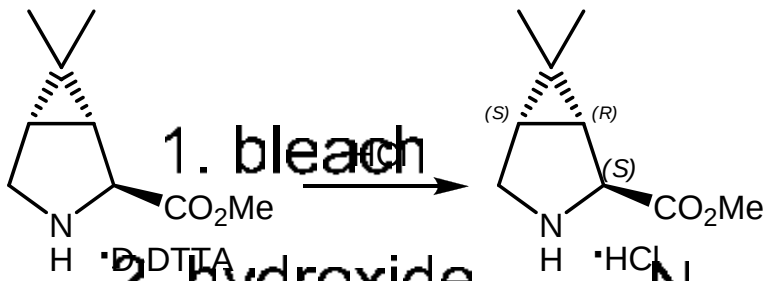
Established Route for Boceprevir's Bicyclic Proline Analog

3 steps from chrysanthemate
30% yield with MW loss



symmetric **racemic** **racemic-trans:** **racemic**
kinetic product
major thermodynamic product



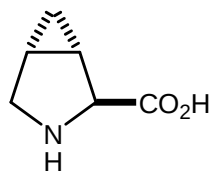


1. bleach
2. hydroxide

40–45% from racemic

~96–99% e.e.

WO 2007/075790
US 4,183,857, for racemic



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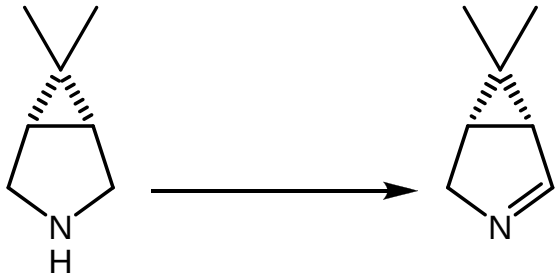
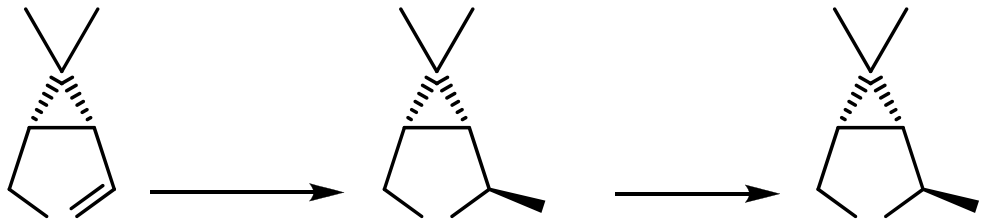
CODEXIS®

Creation of 3 Chiral Centers with 1 Enzyme

- Desired: <u>enantioselective</u> desymmetrization to create first two centers:



- Take advantage of facial selectivity in cyanide addition :



CODEXIS®

Preferred Enzymatic Process

- Fed-batch reaction, feeding a neutral solution of the amine and bisulfite
  - Minimizes bisulfite inhibition and oxidation
  - Add $Na_2S_2O_5$ to the amine in solution to get to neutral pH
  - The salt solution contains protonated amine and sodium cations with bisulfite and sulfite anions (bisulfite pKa ~7)
- Overall reaction:

- Flow through air with agitation adequate to provide a practical mass-transfer limited reaction rate
- After the reaction, NaCN is added to form the amino nitrile in the same pot

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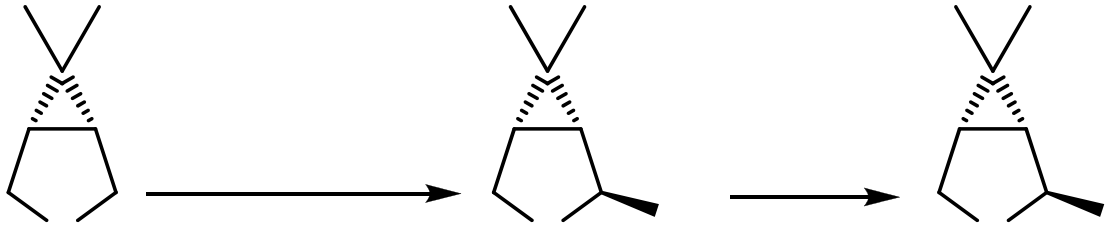
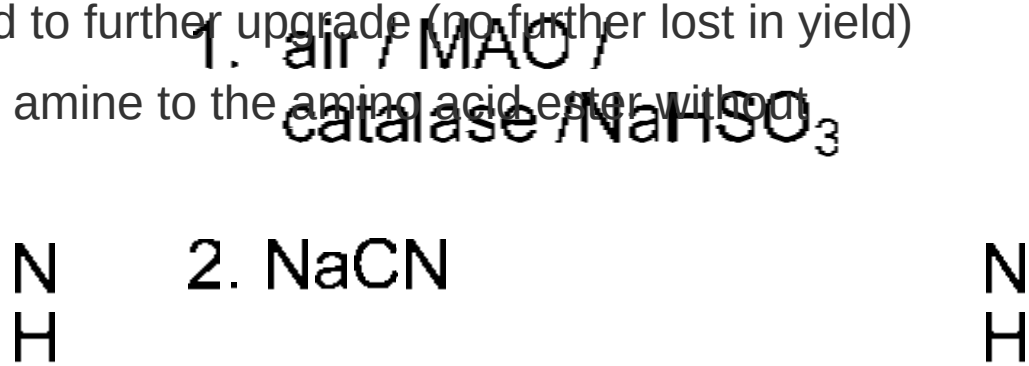
Process for Boceprevir's Bicyclic Proline



Major Advantages:

- No accumulation of noxious imine (it is isolated in established route)
- Overall yield is at least 2.5x higher than the established route
- >99% optical purity; no need to further upgrade (no further lost in yield)
- Can be telescoped from the amine to the amino acid ester without intermediate isolations



CODEXIS®

Corporate Profile

- **Privately-held company based in California, USA**
- **Incorporated in February 2002**
- **Employees: ~300**
- **Sites in California, Germany, Singapore and Hungary**
- **Alliance Partners: Pfizer, Merck, BMS, Lilly, Arch, Shell, Cargill, Schering Plough**
- **Track record: >40 projects completed, >10 commercial products**

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