Evolving Enzymes for Highly Efficient Chemical Processes

Pascal Dünkelmann

CODEXIS

Talk Outline

- **Introduction to Codexis and our biocatalysis paradigm**
  - "What do we do & how do we do it?" or "What is directed evolution and what are the benefits?"
- **Real examples of process improvement on pharmaceutical targets**
  - Atorvastatin
  - Panel Plates as an investigational tool
  - Ezetimibe
  - Montelukast
- **Summary**

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Codexis' Biocatalysis Paradigm

- **Design the desired conceptual biocatalytic *chemical* process**
  - Make it "green-by-design"
    - High substrate loadings / low organic solvent
    - Minimise / eliminate "nasty" reagents
    - High selectivity / low by-products & less purification

- **Evolve the biocatalyst for fitness to enable the desired process**
  - Iteratively improve the performance of the biocatalyst via genetic modification (Molecular Breeding™)

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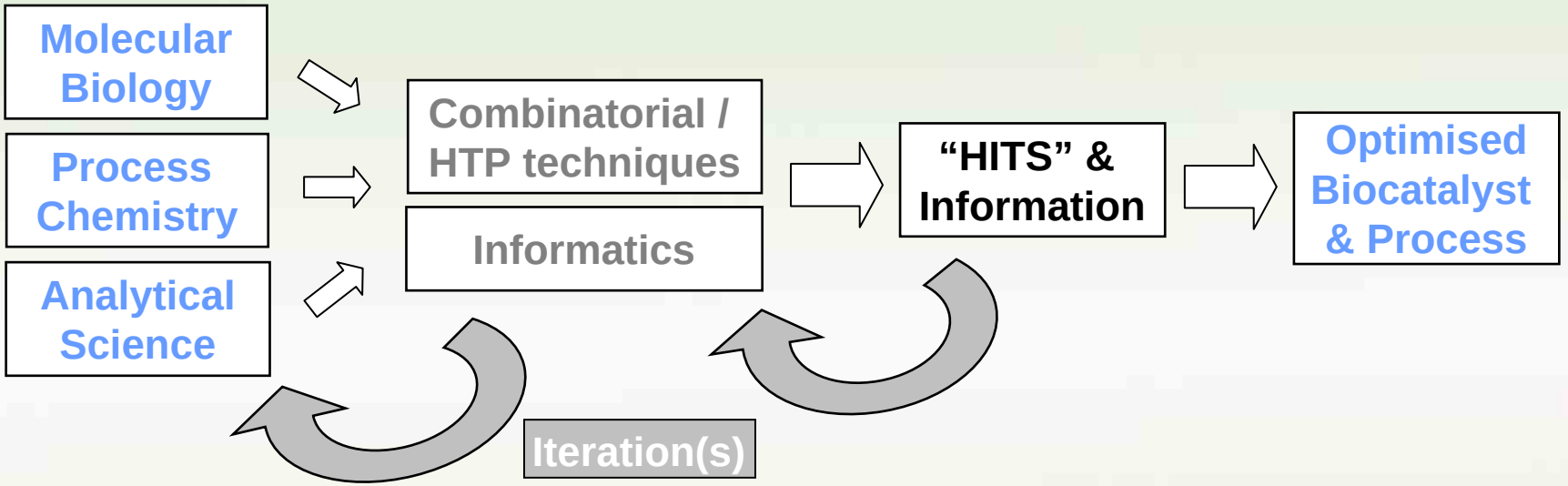
Codexis' Biocatalysis Paradigm

- Bring together several scientific specialties to design and evolve a proprietary biocatalyst and a chemical process in tandem



- Allows for unconventional biocatalytic processes which solve specific problems

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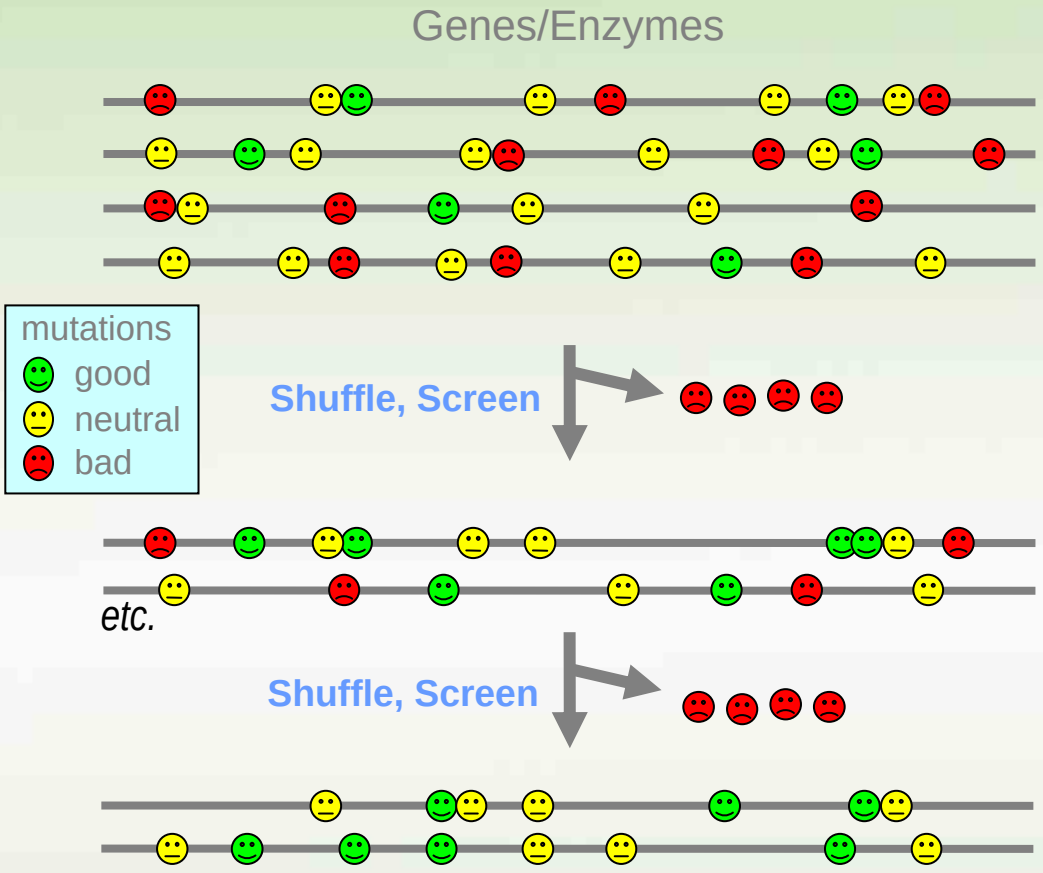
How MolecularBreeding™ Improves Genes



- Random fragmentation provides many potential crossover points
- Recombination in correct order provides a high % active enzymes
- Population based shuffling combines fragments from multiple parents
- Screening identifies genes with novel combinations of beneficial mutations and/or fewer deleterious mutations
- Rapid large improvements are obtained

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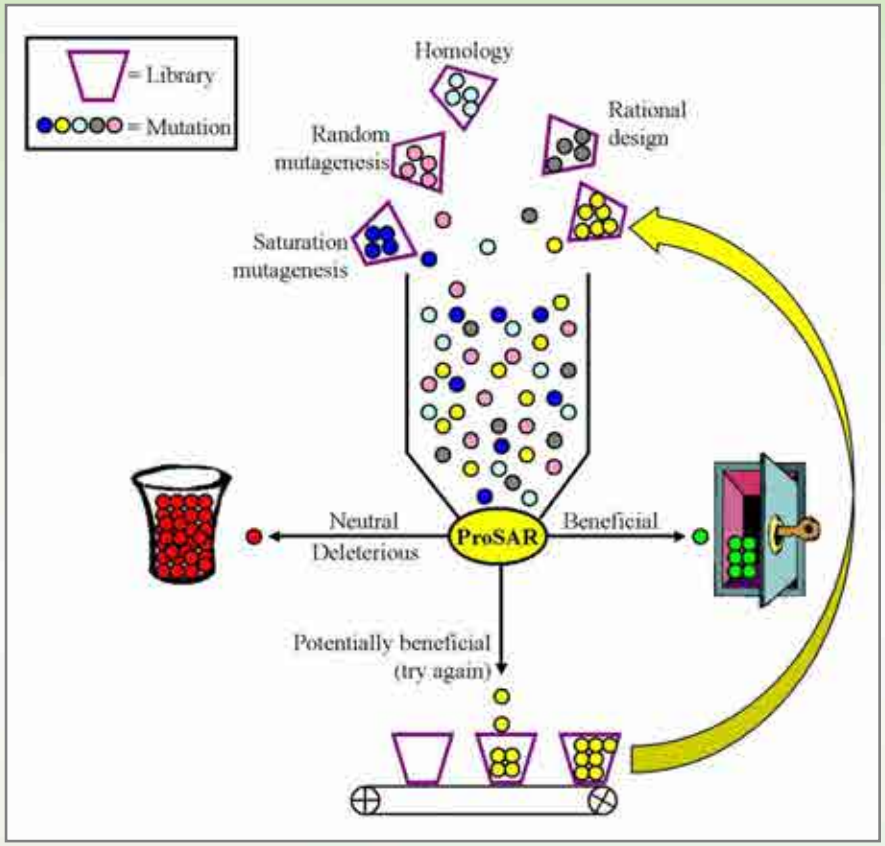
The ProSAR-Driven Methodology



- At any point there are approximately 50 mutations under investigation in the hopper
- These are tested in combinatorial libraries and the function and the sequences of the resulting proteins are analyzed
- Mutations are parsed into four classes:
  1. "Beneficial" are fixed into the population
  2. "Potentially beneficial" are sent back into the hopper for retesting
  3. "Deleterious" are discarded
  4. "Neutral", which have no effect on protein function, are discarded
- The amount of diversity under investigation is maintained by adding novel diversity discovered in rational design, homologous sequences, saturation or PCR mutagenesis libraries

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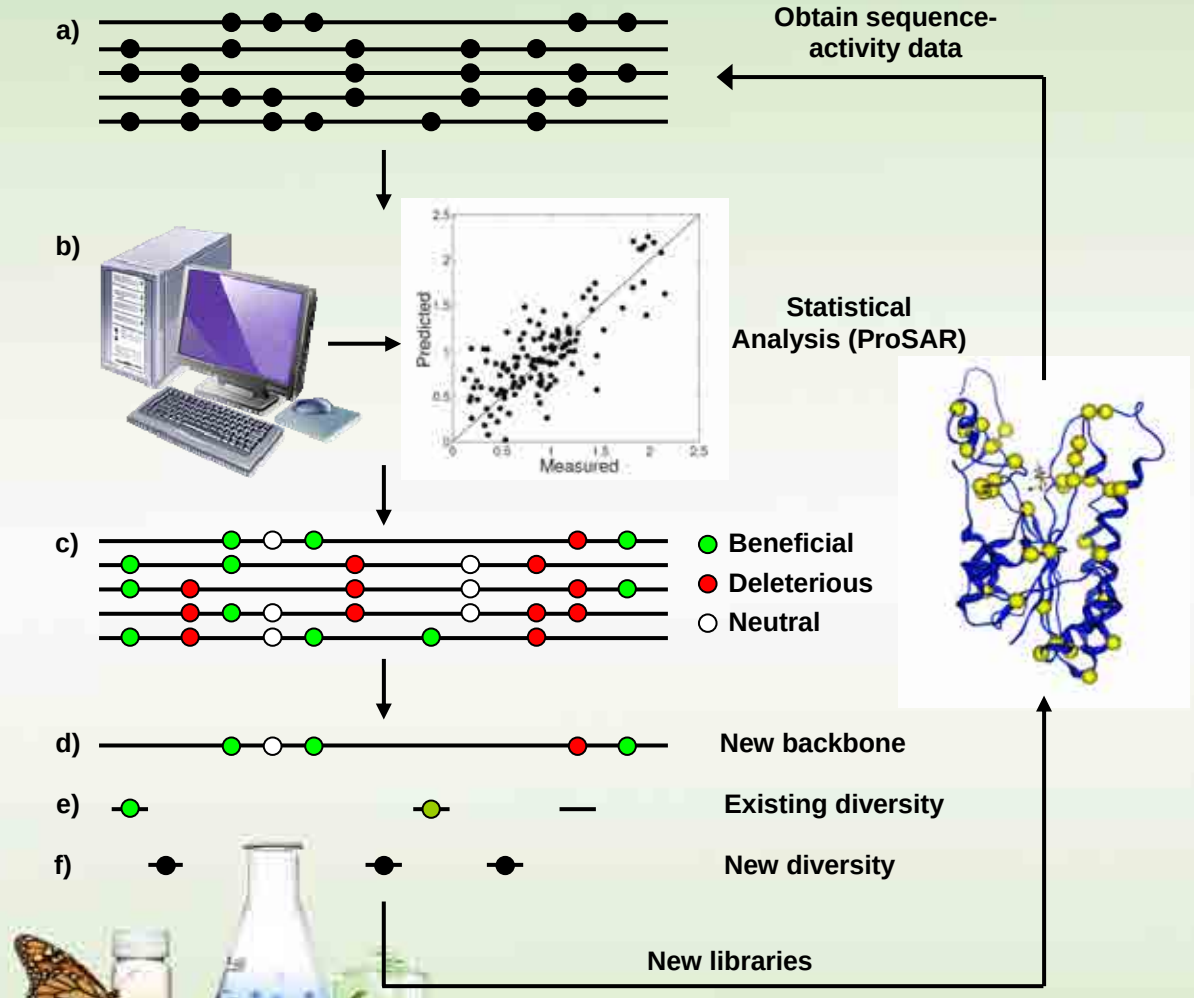
State-of-the-Art Enzyme Evolution



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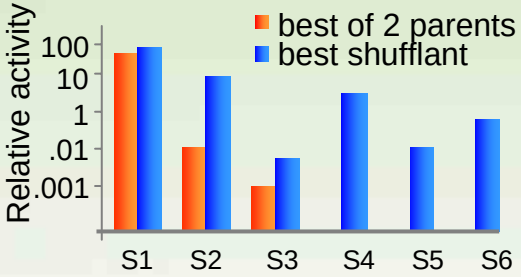
Directed Evolution - Benefits

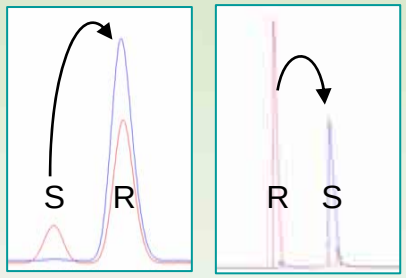
Substrate Specificity



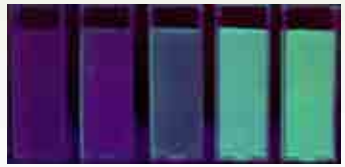
Selectivity

(impurities)

Enantioselectivity



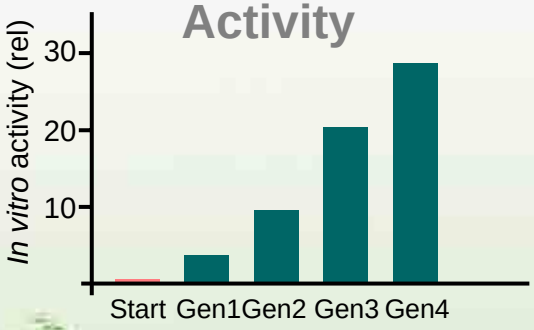
Expression



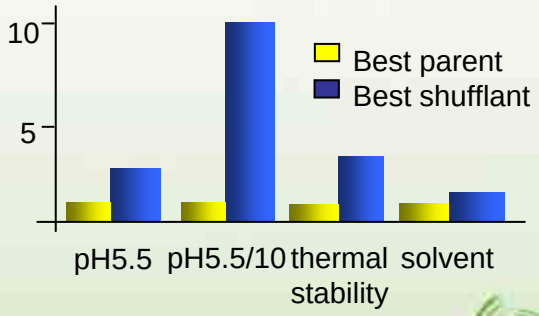
Substrate/Product Tolerance

(conversion)

Activity



Stability - pH, solvent



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Real Examples (i) – Atorvastatin

4 Enzymes developed to provide a new, environmentally friendlier and more efficient / cost-effective process

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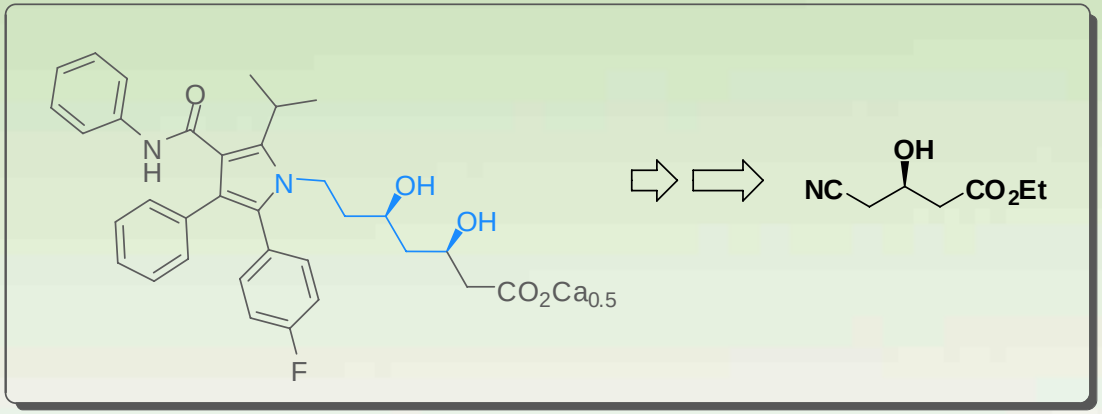
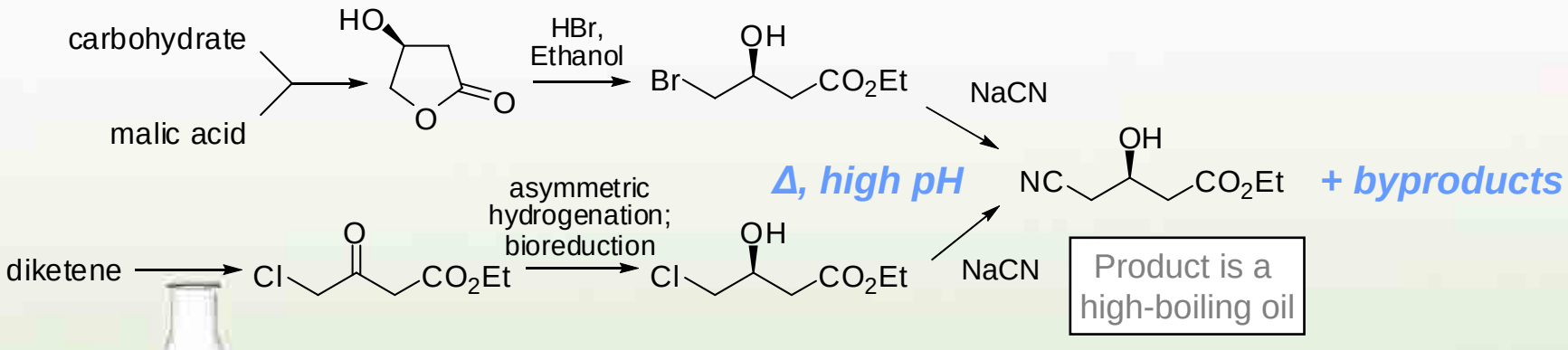
Hydroxynitrile Starting Material for Atorvastatin



Existing processes

- Forcing conditions for cyanation resulted in base-catalyzed side reactions



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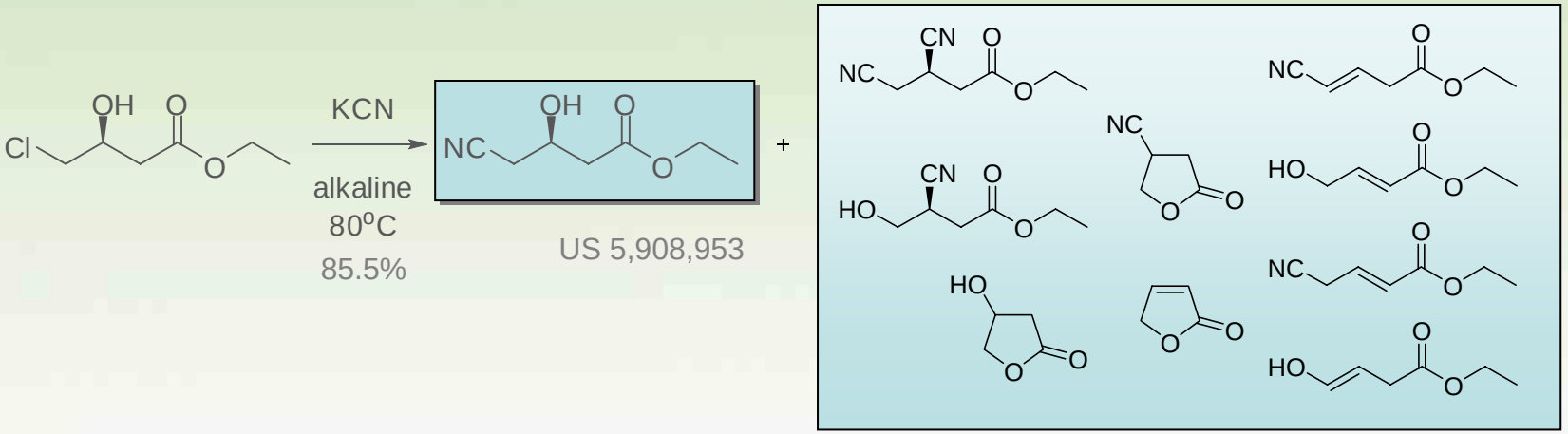
By-Products in Uncatalyzed Cyanation



- High vacuum fractional distillation from close-boiling by-products is required.
- Cleanup does not address the root of the problem.
  - ➢ A catalyst for cyanation at neutral pH was needed to solve this problem

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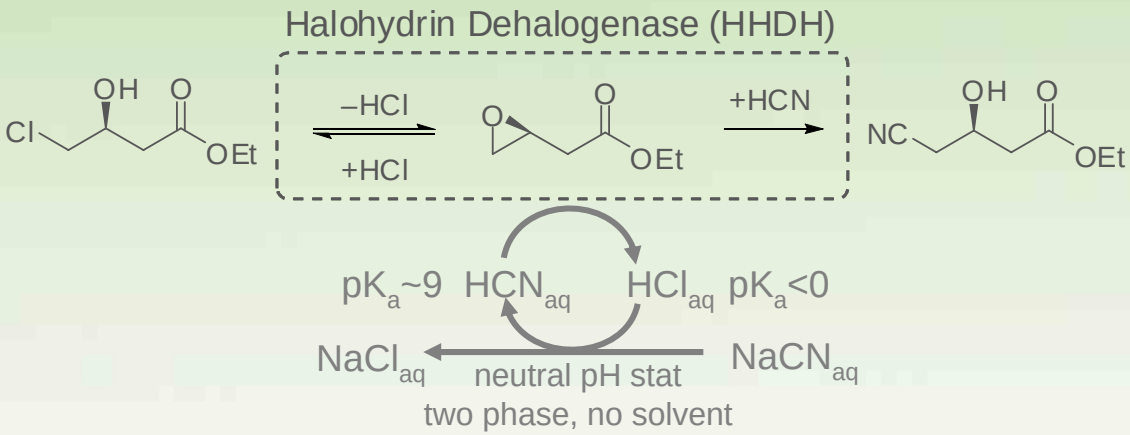
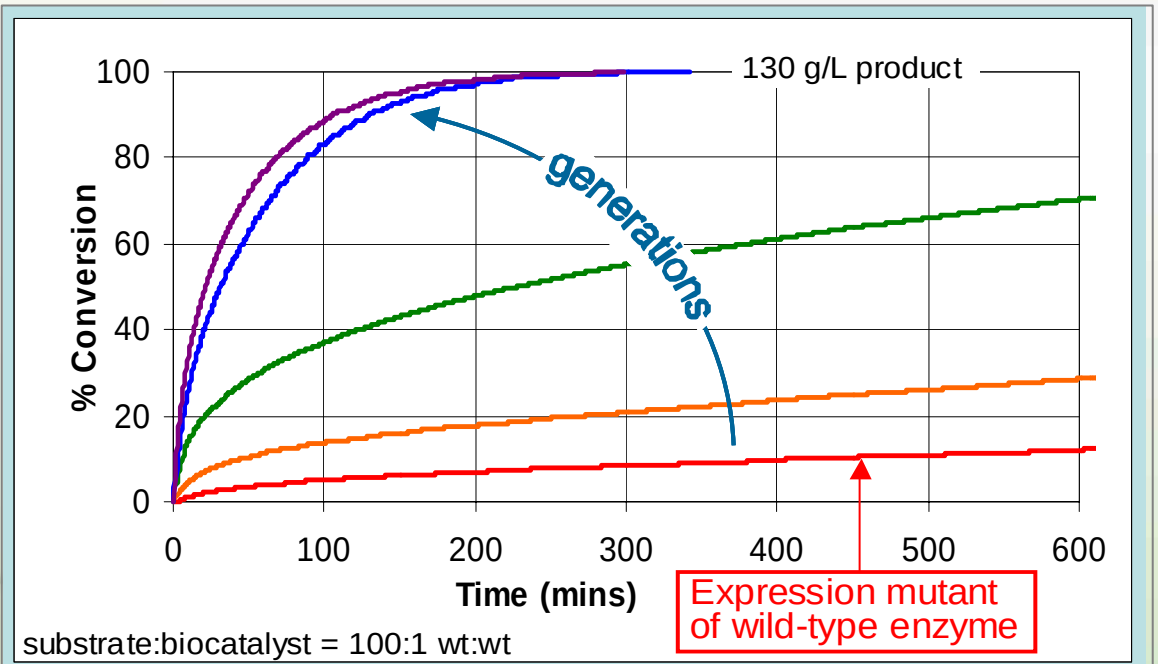
Biocatalytic Cyanation



Replaced natural "reagent" for the enzyme – CN for Cl



Improvements in:

- Activity / Productivity
- Relieved product inhibition
- Stability in process

➢ ~4000x improvement in volumetric productivity per biocatalyst loading

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HN Process Improvements

- **Shuffling also solves processing / scale-up issues often associated with natural enzymes:**

natural enzymes

organic layer (product)

aqueous layer

shuffled biocatalysts

7 g/L KRED/GDH; 80 g/L Substrate	**<1 g/L KRED/GDH; 160 g/L Substrate**
Reaction time: 24 hrs	**Reaction time: 10 hrs.**
LAB Phase separation: >1 hour	**PLANT Phase separation: ~1 minute**
Isolated yield: ~80%	**Isolated yield: >95%**

Science & Technology July 10, 2006 ; Volume 84, Number 28; pp. 24-27

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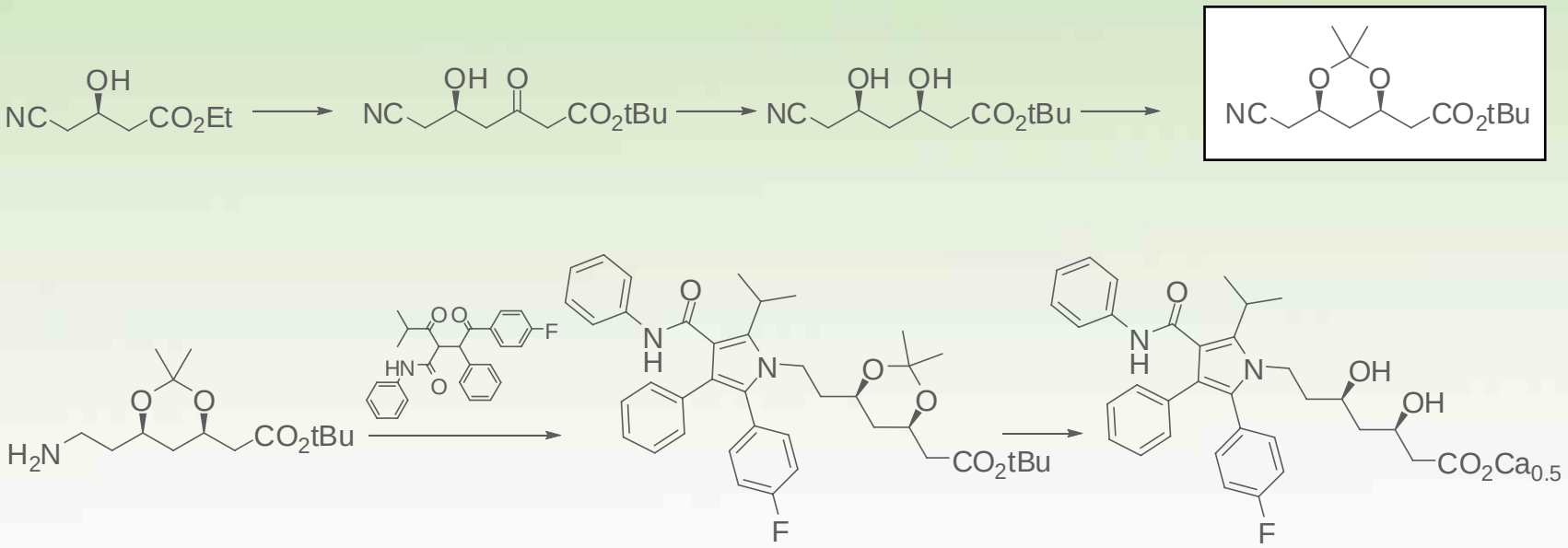
TBIN - Key Intermediate for Atorvastatin



- TBIN is the first crystalline intermediate in the manufacture of Atorvastatin
- Diastereomeric purity of the statin side-chain is upgraded at this point

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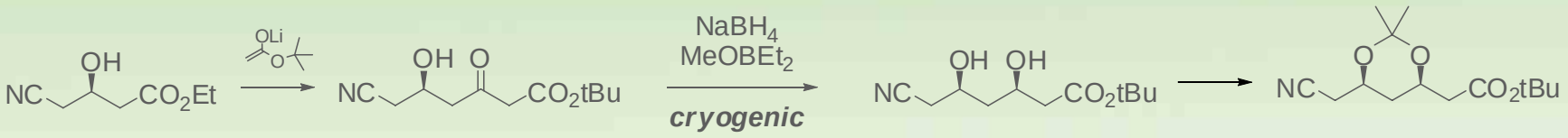
TBIN – Original Process



HN

"chiral diol"

TBIN (ATS-8)
first crystalline intermediate

- **Original Reduction Step:**

  Stoichiometric borohydride reduction of stoichiometric boronate complex

  - Cryogenic conditions are required for adequate diastereoselectivity.
  - Imperfect diastereoselectivity (3-4% diastereomer at –70°C)
  - Wrong diastereomer must be purged downstream; yield losses likely
  - Cumbersome quench & vacuum distillation(s) to remove/recover $MeOBEt_2$
  - Stoichiometric borate waste stream.

US 6,596,879 to Warner-Lambert

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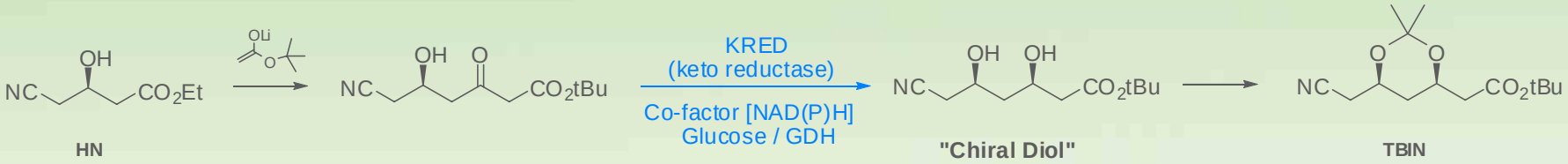
Codexis' Ketone Reduction Alternative



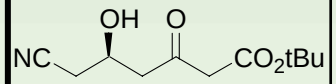
- **Desired reduction step:**

  Catalytic transfer hydrogenation under ambient conditions.

  - Perfect diastereoselectivity at the incipient chiral center
  - Hydride source is cheap and renewable
  - High yield of crystalline TBIN
  - Simple extractive work-up
  - Benign waste stream

Identification of Starting Enzyme

Diastereoselectivity

Substrate \ Enzyme	003	006	008	009	010	011	012
	nd	>99% trans	>99% cis	nd	nd	>99% cis	nd

*nd: not detected

CDX008 as starting point required ~200x improvement in activity/stability to enable commercial process

Activity

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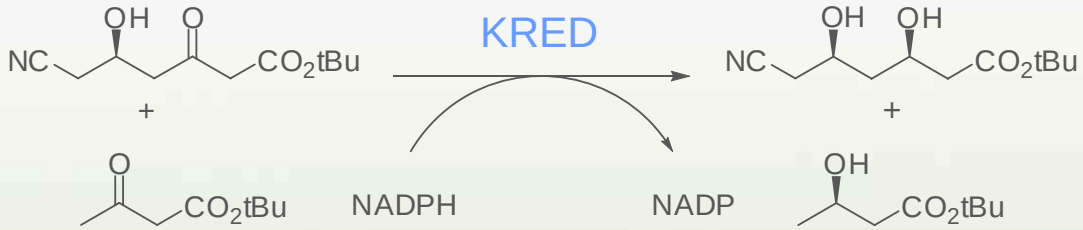
TBIN - Enzyme Evolution Strategy

Libraries of enzyme variants are expressed in *E. coli* and evaluated in HTP using a tiered approach:

- 1st tier to identify active and stable variants (fluorescence – NADPH / 340nm)
- 2nd tier to confirm activity (on HK) and selectivity (LCMS – differentiate products)
- 3rd tier to confirm *in process* improvements (20 ml scale - chemistry)

Note: HK contains 10-20% tert-butyl acetoacetate (BAA) which is a substrate for some ketoreductases, hence 2nd tier screen



General KRED activity in tier 1 detects improvements for both ketone substrates

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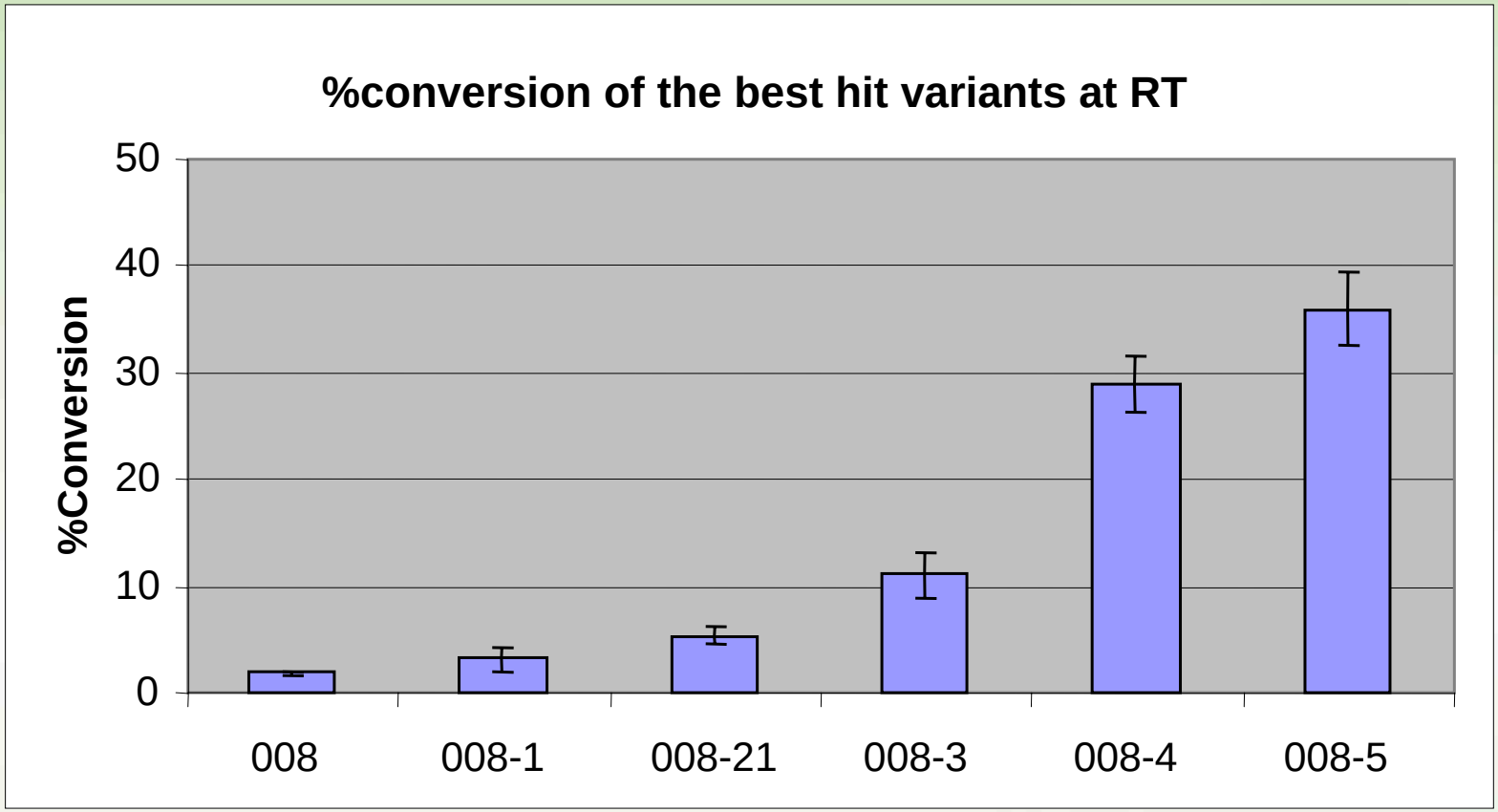
Activity Improvement of CDX008



- ~20-fold improvement over Wild Type by 5 rounds of shuffling

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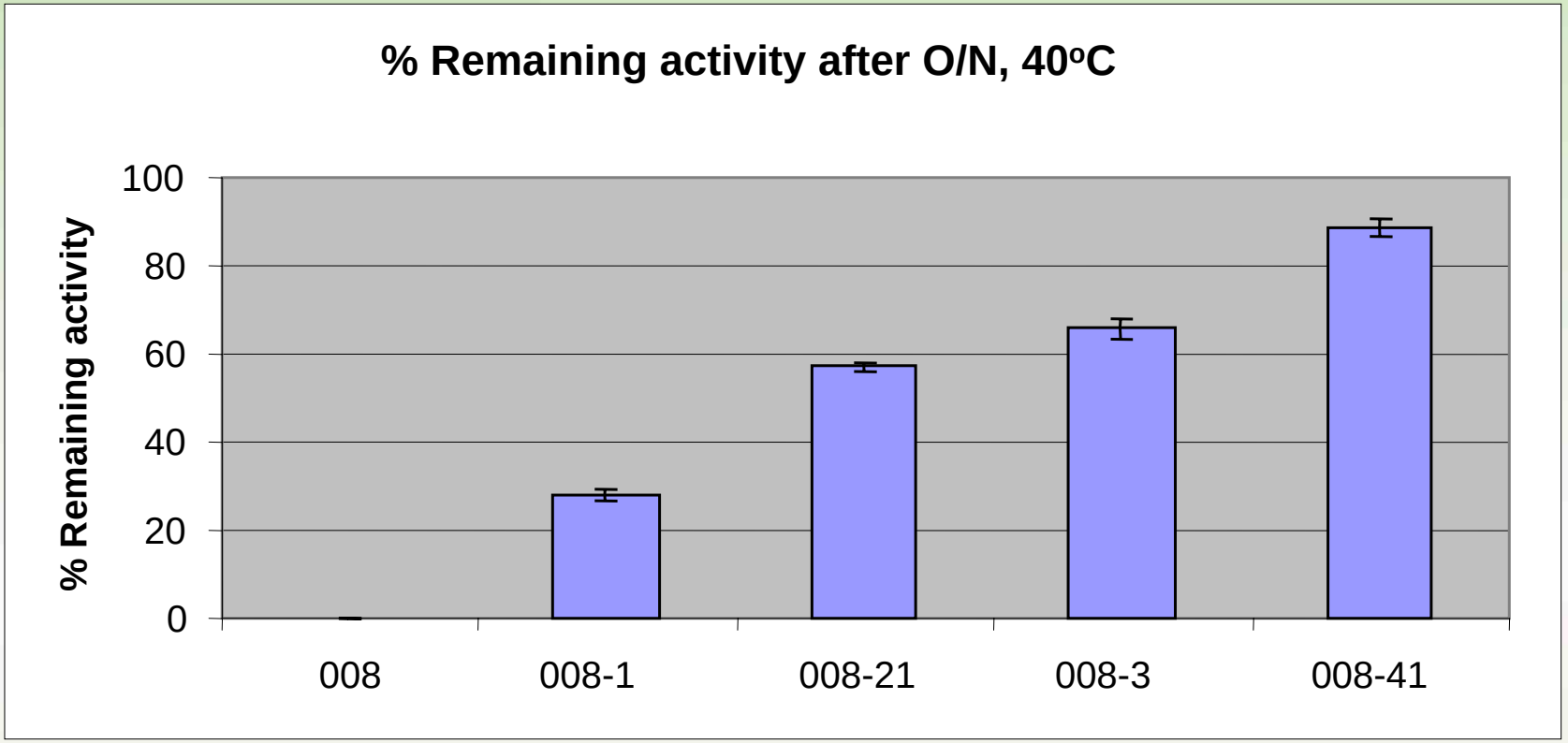
CDX008 Thermostability Improvement



~85% residual activity after O/N heat treatment (40°C)

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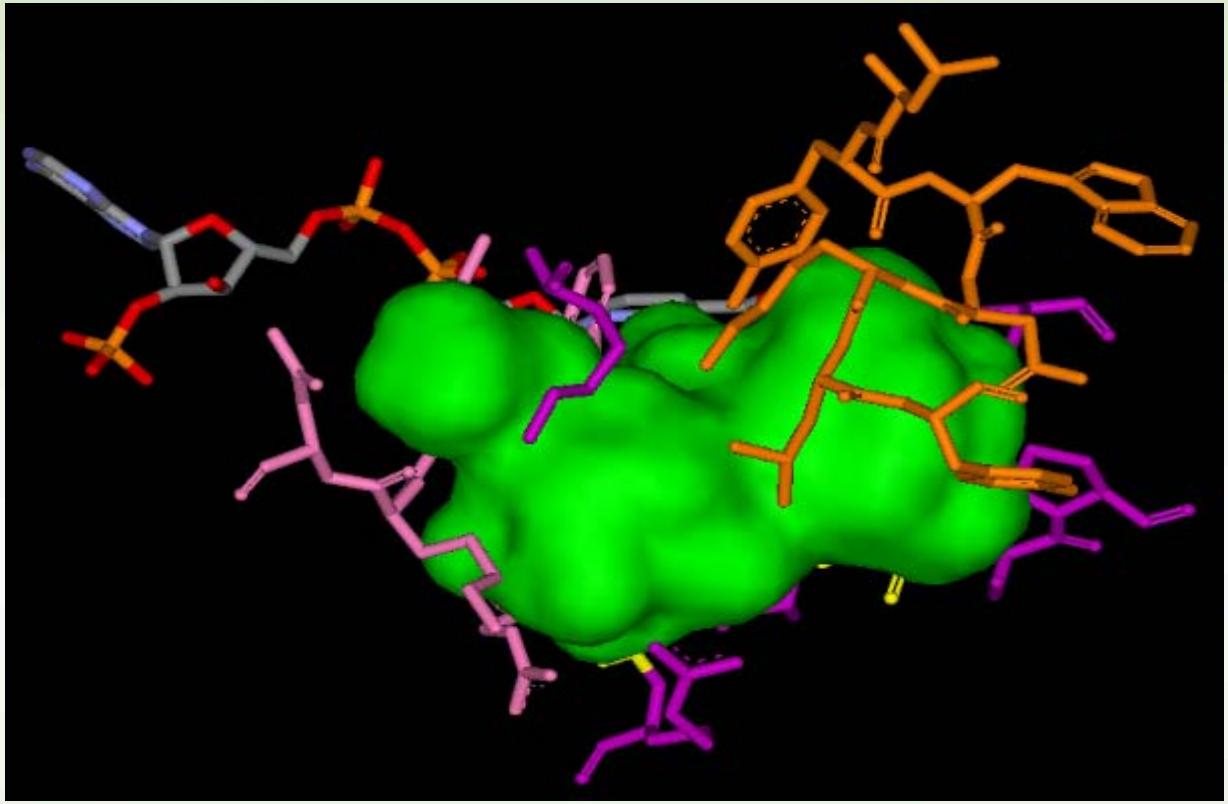
Active Site - Candidate Positions for Randomization



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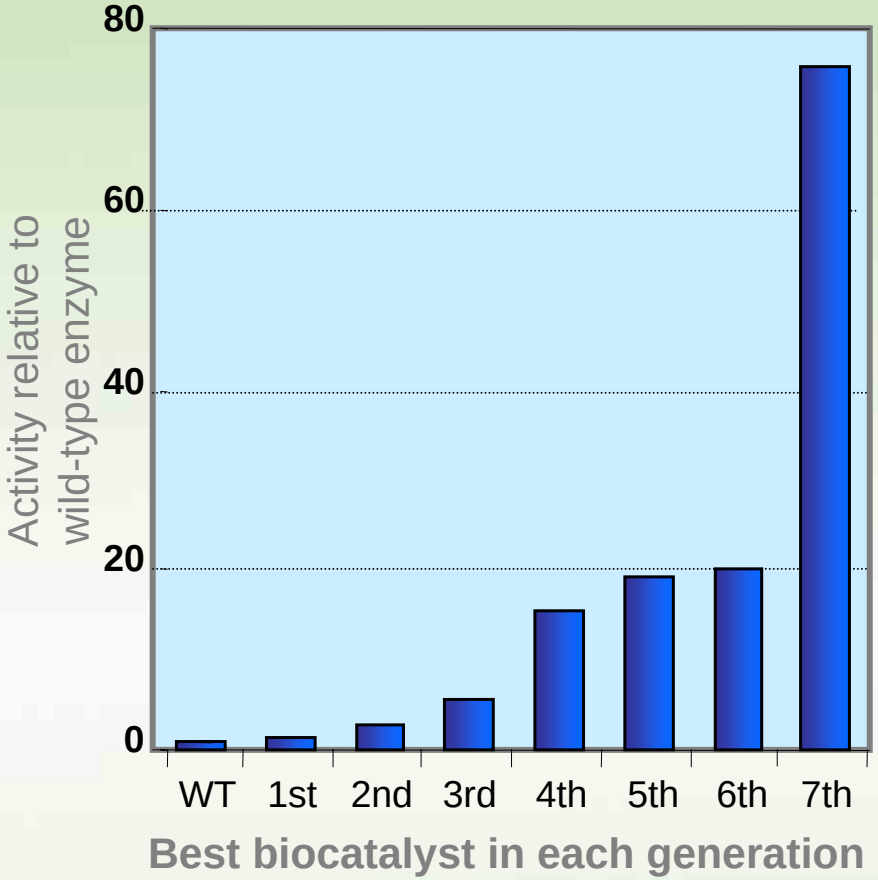
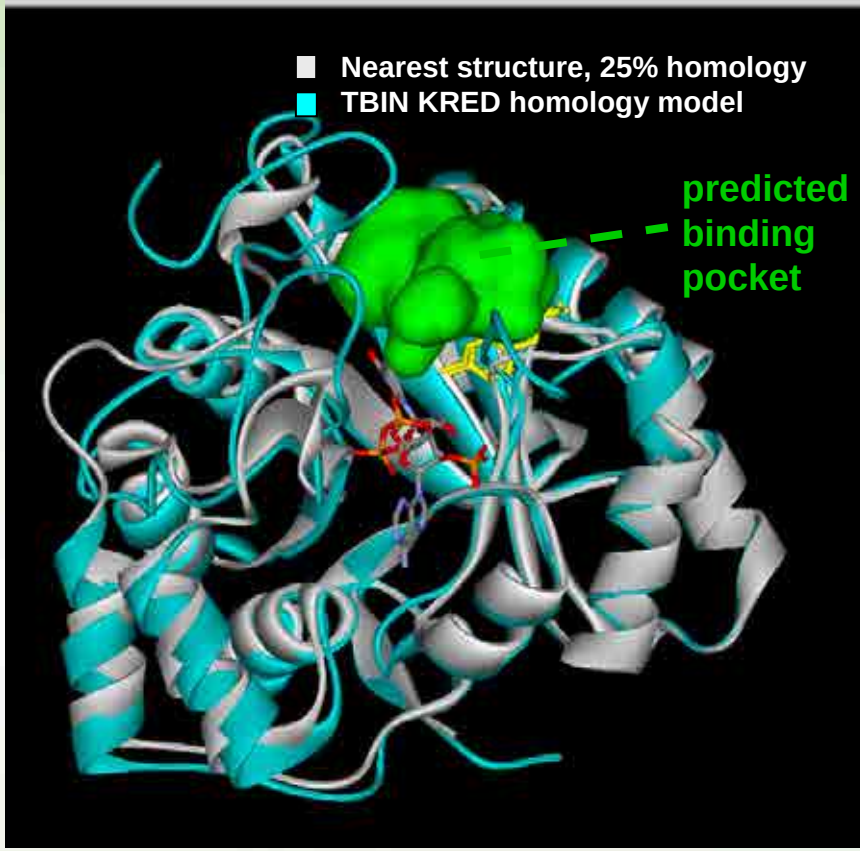
KRED Evolution for TBIN





7th Round evolvant met productivity targets

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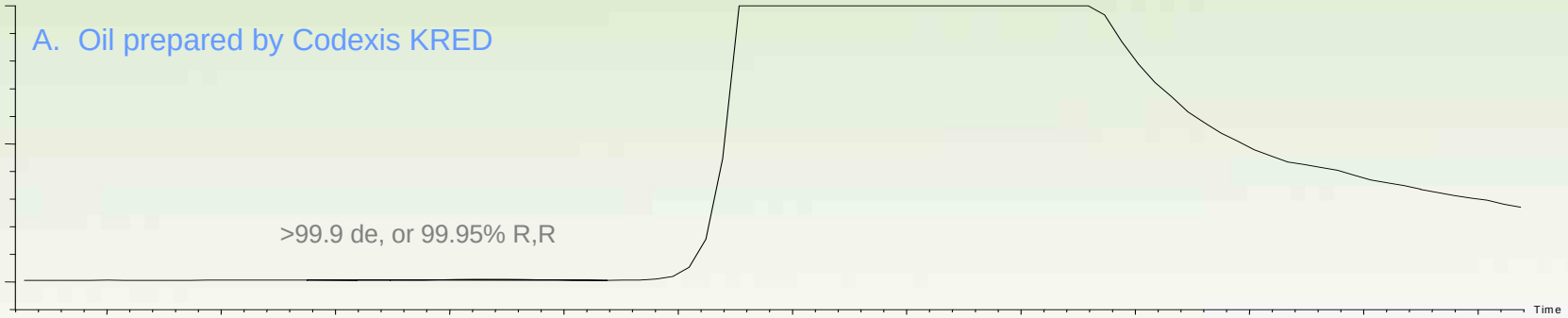
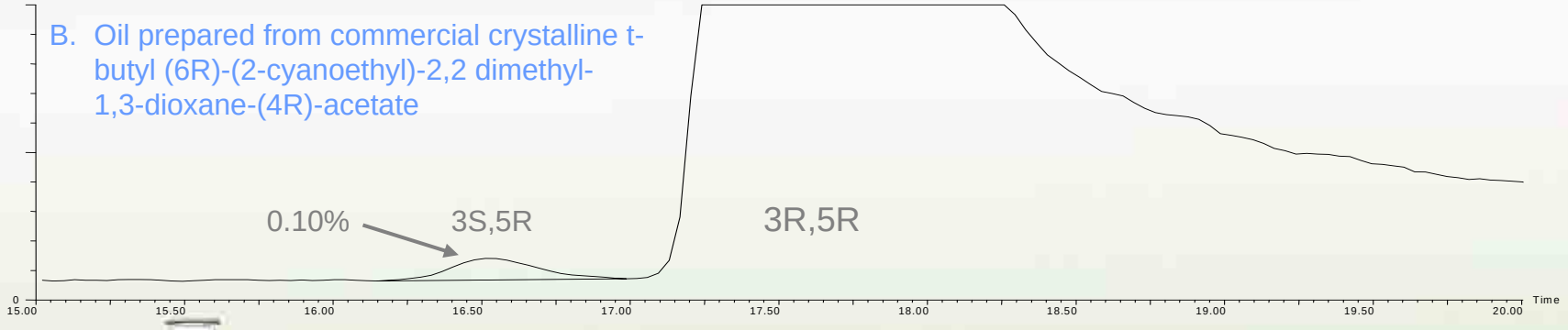
Diastereopurity of Codexis' C_7 diol

LC/MS/MS chromatograms of t-butyl 6-cyano-(3R,5R)-dihydroxyhexanoate samples





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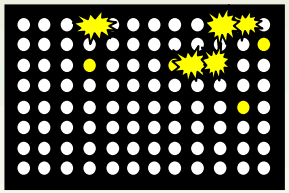
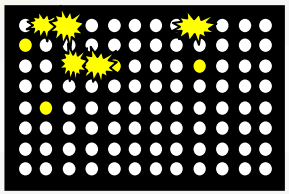
Codex™ Panel Plates as an Investigational Tool

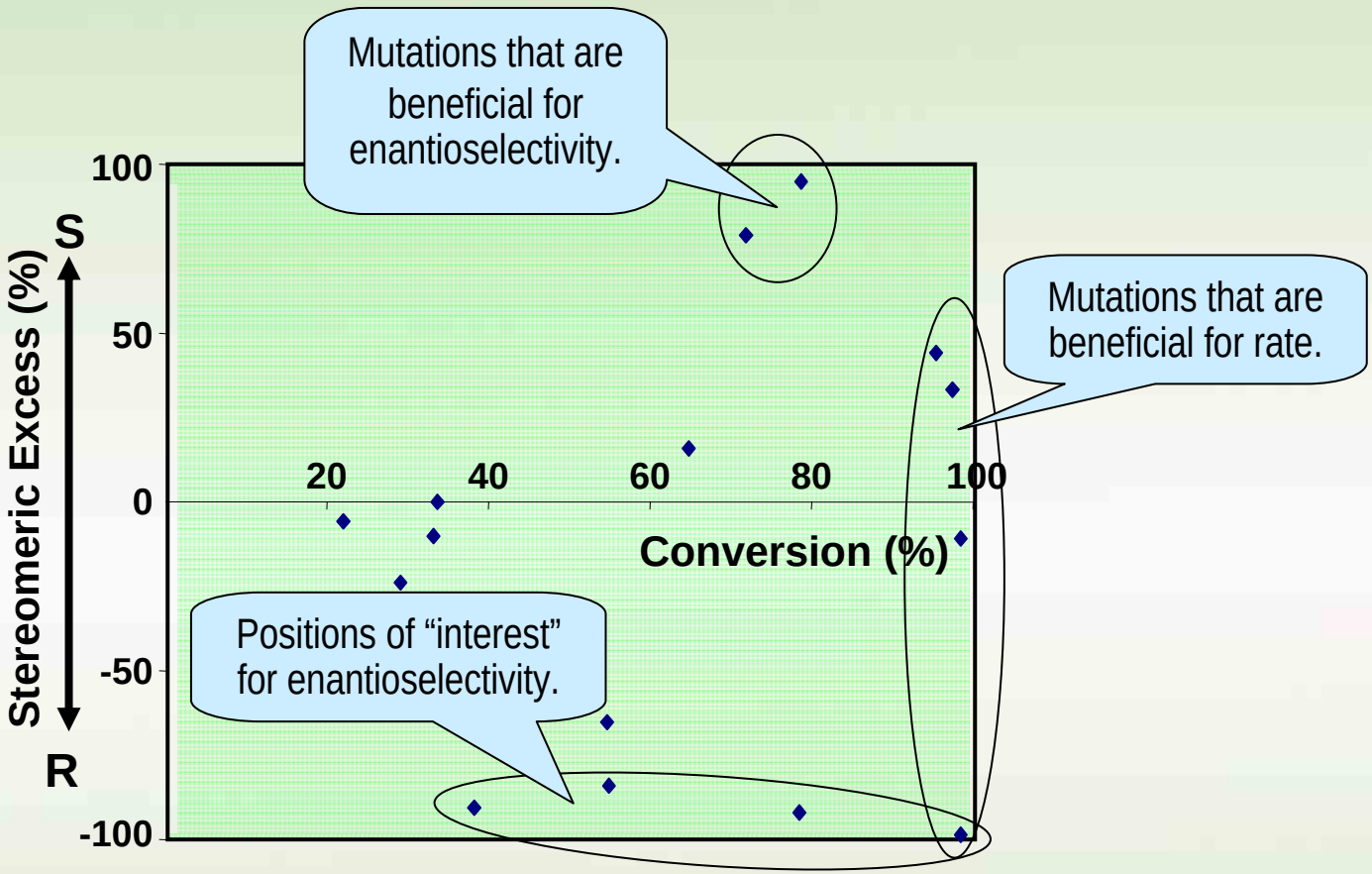
Previously selected recombinant diversity allows identification of starting points for evolution,
potentially short-cutting project timelines by months

CODEXIS®

Case Study – Codex™ Panel Result







- Based on the sequence-activity data from the pre-tuned panel, a fine-tuned panel was designed and screened.

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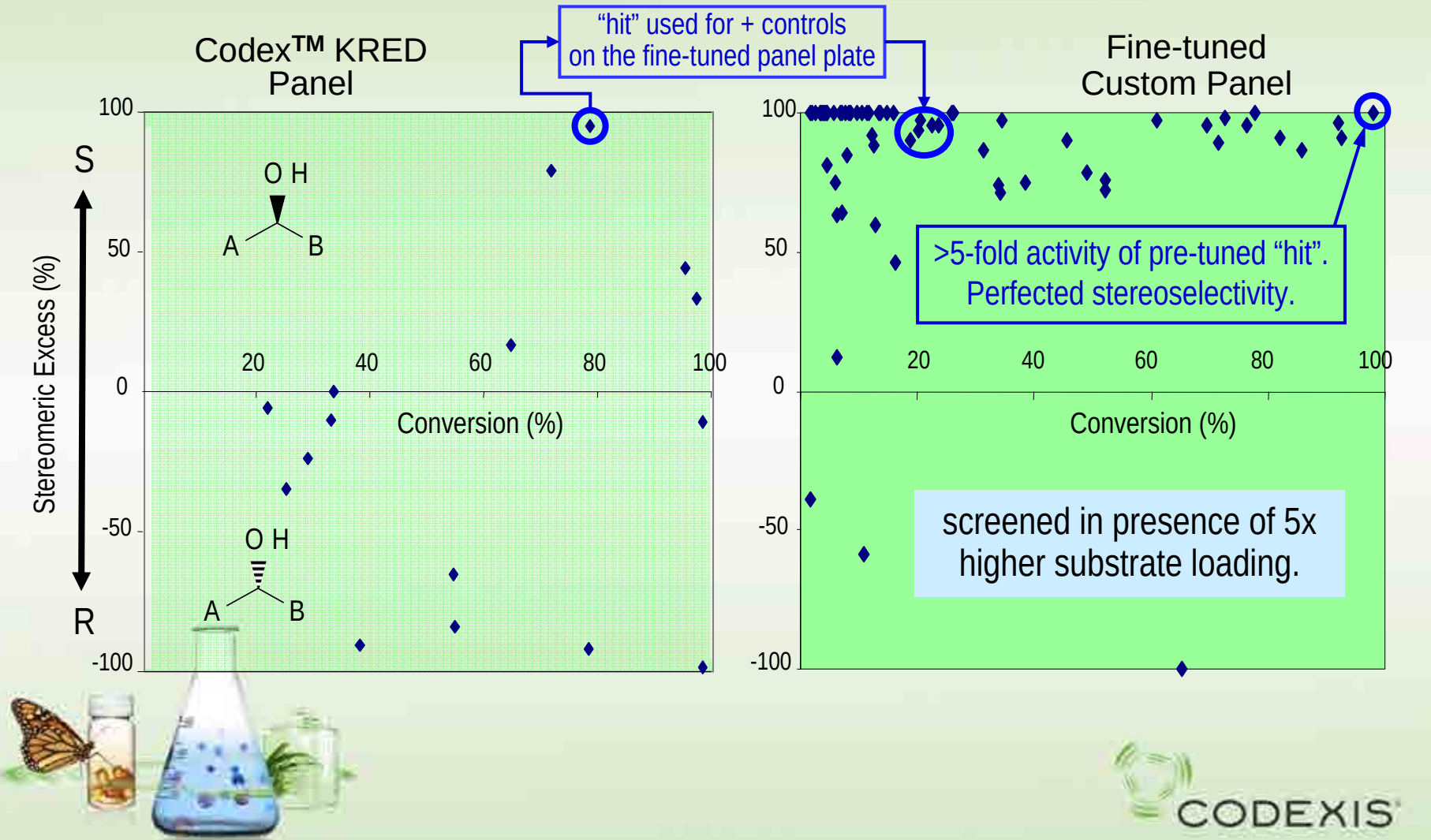
Case Study – Fine-tuned Panel Result



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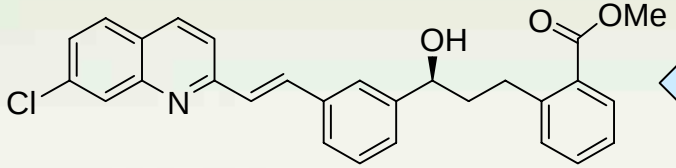
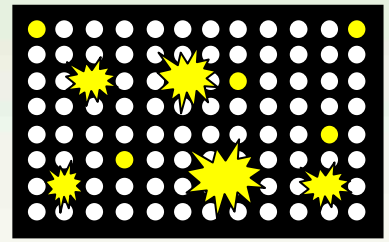
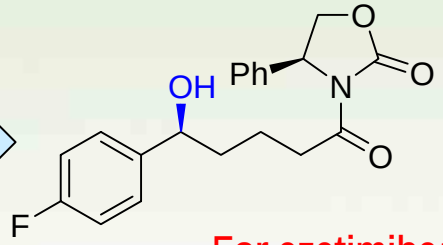
Utility of KRED Codex™

The Codex™ KRED Panel provided starting enzymes for the development of new, biocatalytic processes for the following intermediates:

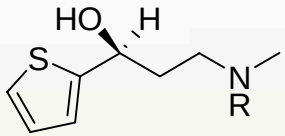


For montelukast:
Natural enzyme: no activity
Current: commercialized

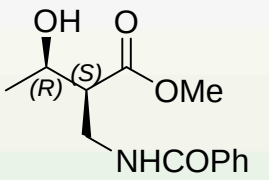




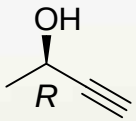
For ezetimibe:
Natural enzyme: no activity
Current: tech. transfer



For duloxetine:
Natural enzyme: no activity
Current: commercialized



For penem antibiotics:
Natural enzyme: 80% d.e.
Current: >99.9% d.e.
tech. transfer



Chiral Raw Material::
Natural enzyme: inactivated by substrate
Current: tech. transfer

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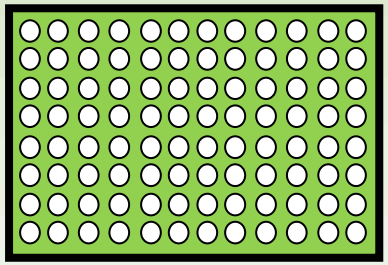
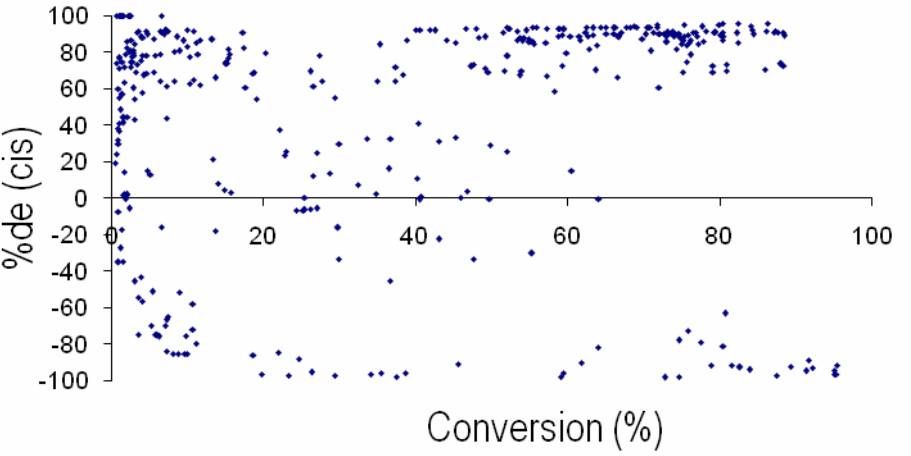
Merit of Codexis' KRED Processes

Chemical Processes	Codexis KRED Processes
Stoichiometric or Catalytic: - DIP-Cl/BH_3 (H.C. Brown) - Me-CBS and BH_3 (E.J. Corey) - Ru-BINAP (R. Noyori) - Resolution	All Catalytic: -Straightforward manufacture and use -Run at $\geq$100 g/L -Economically superior. -Scaleable.
Stereopurity: require upgrades (yield loss)	Produces >99.9% e.e. alcohol.
Hazardous and toxic: - These reagents are typically, toxic, sensitive to water and air, - Expensive.	Easy to use - Ambient conditions, run in aqueous solvents, - Degradable in waste water treatment plant.
Generally, tedious work-up procedures required.	Straightforward work-up.

Ene Reductase Codex™



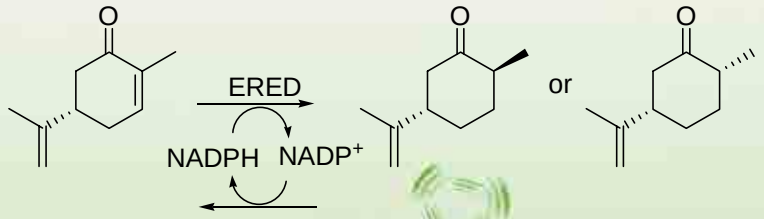


The ERED Codex is useful for:

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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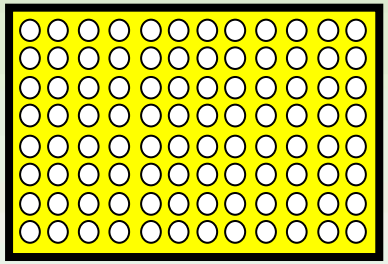
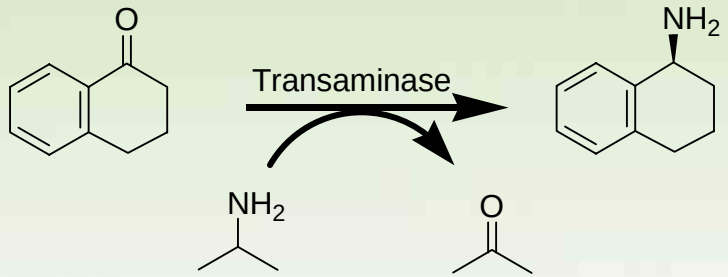
Transaminase Codex™ Panel





Chemical Process	Codex TA Panel
• Diastereomeric salt resolution, • Reductive amination, • Reduction of eneamines, • 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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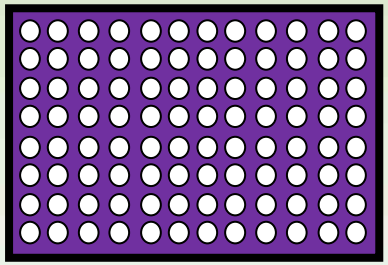
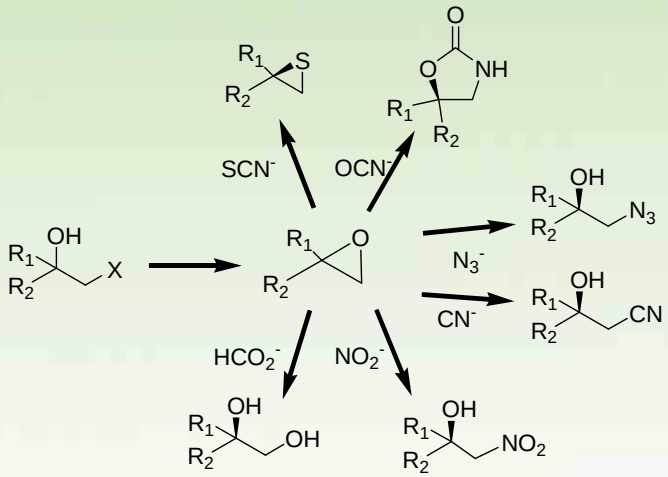
Halohydrin Dehalogenase Codex™





Chemical Process	Codex HHDH Panel
Sharpless: Epoxidation of allylic alcohols	Broad range nucleophiles
Jacobsen HKR: no α,α -disubstitution	α,α -disubstitution
Issues: • Hetero atom substitution problematic • Regioselectivity issues • Expensive TMS-CN, TMS-N_3 • Metals used: Osmium, Cobalt, Chromium	HHDH Opportunity: • Hetero atom substitution allowed • Regioselectivity tuneable • Cheap nucleophiles
	Precedent: HN for atorvastatin

Real Examples (ii) – Ezetimibe

Enzyme & process developed to avoid use of Me-CBS

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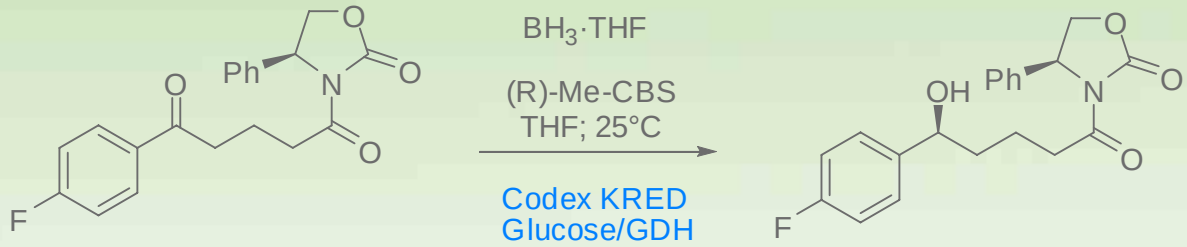
Ezetimibe Process - Differentiation



Chemical Process	Codexis 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 (toluene at RT) - Economically superior
• Stereoselectivity is inadequate and highly "touchy" (over-reduction). • Sensitive to moisture and reagent quality • Stereopurity upgrade required	• Produces >99.9% e.e. alcohol • Eliminates over reduction • Isolated yield >95%
• Complicated Work-up	• Straightforward work-up

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Real Examples (iii) – Montelukast

Enzyme & process developed to avoid use of DIP-Cl

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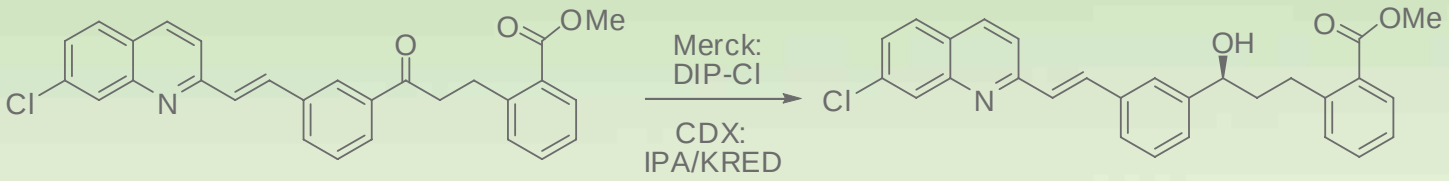
Montelukast Process - Differentiation



- Neither SM nor product are soluble - run as a slurry-to-slurry process
- No need for co-factor re-cycle or distillation

Chemical Process	Codexis KRED Process
Uses more than stoichiometric DIP-Cl/BH_3 - Corrosive, acidic solid; causes burns; moisture-sensitive - Expensive	Catalytic - Runs at 100 g/L with S/C >30: - Environmentally friendly - Greatly reduced hazard (IPA at RT) - Economically superior
Produced 97% e.e. alcohol.	Produces >99.9% e.e. alcohol.
Isolated 87.1% yield, 99.5% e.e.	Isolated yield >90%.
Tedious work-up to remove borate salts	Straightforward work-up via filtration

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Summary

- Biocatalytic options are real alternatives for process chemistry,
- Modern molecular biology tools combined with HTP screening allows for catalyst optimization on an as needed basis,
- Enzymes can be optimized to function in desired process conditions and to meet desired economic targets,
- New process and catalyst composition-of-matter intellectual property is developed.
- Combination of Molecular Biology with…..
  - Process Chemistry
  - Analytical Science
  - Combinatorial Technology

  …... provides a powerful tool for engineering of green processes

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