“From Genomes to Biotransformation Scale Up”

05 November 2014

presented at

RSC/SCI Symposium

“Challenges in Catalysis for Pharmaceuticals and Fine Chemicals”

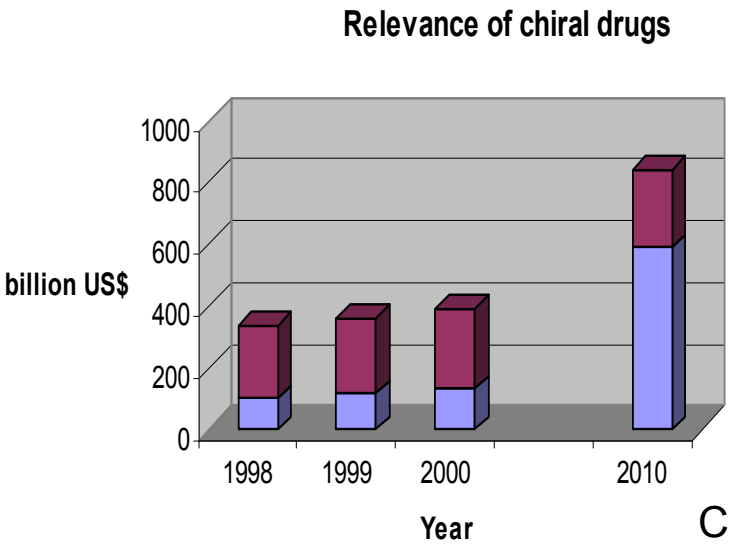
by

Dr. Stefan Mix

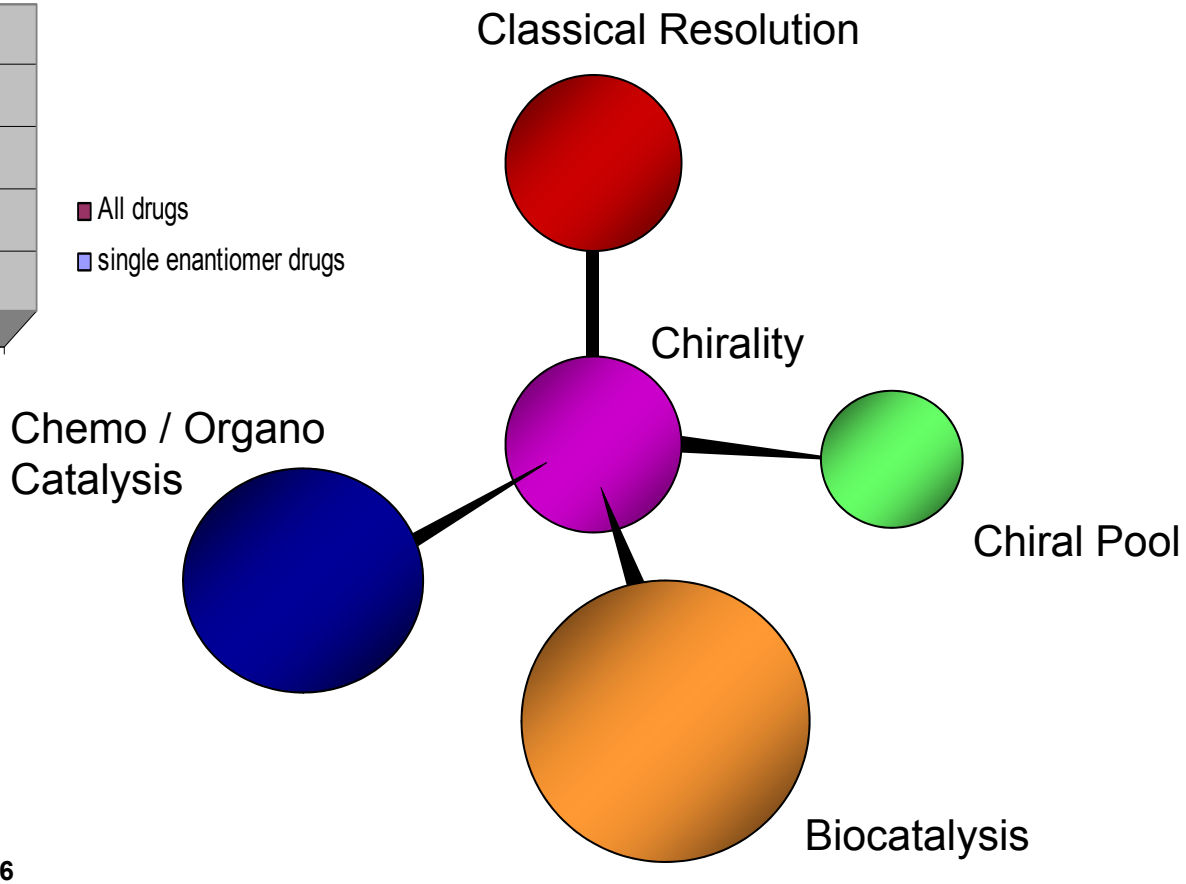
- Dedicated team of chemists, molecular biologists and analysts
- Extensive track record in use of enzymes to produce chiral intermediates / APIs / bulk chemicals

- Enzyme kits: Hydrolase, CRED, TAm, Nitrilase, P450s…..
- Enzyme discovery and evolution
- Scouting of biocatalytic synthesis routes
- Enzyme screening and scale-up (internal and with partners)
- Metabolite isolation, identification and synthesis

Exploring manufacturing routes to chiral building blocks and APIs:



The trend for new chiral pharmaceutical reagents is continuing. In 2000, 35% of intermediates were chiral and this number is expected to increase to 70% by 2010



Woodley et al TRENDS in Biotechnology Vol.25 No.2, **2006**

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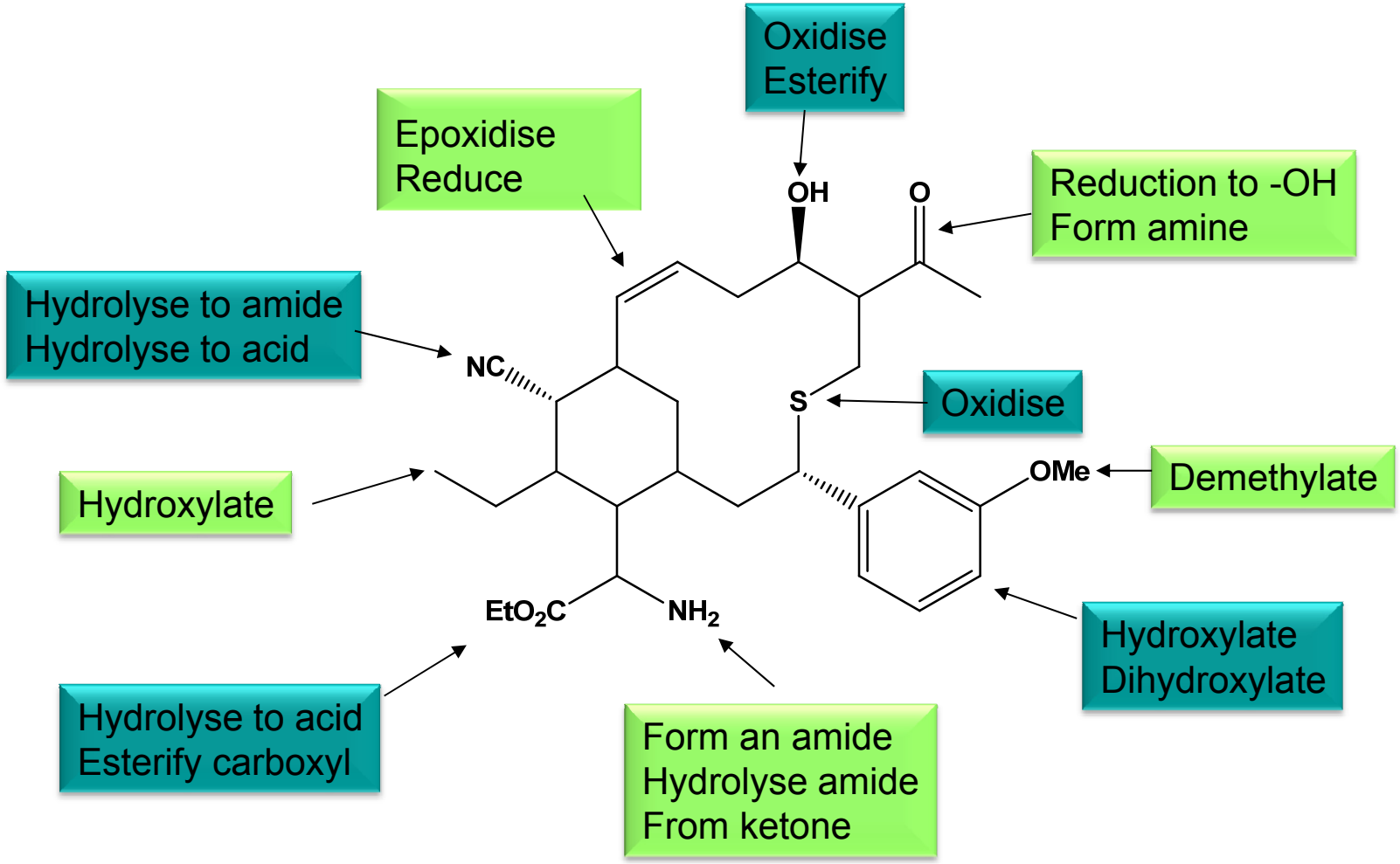
Technology



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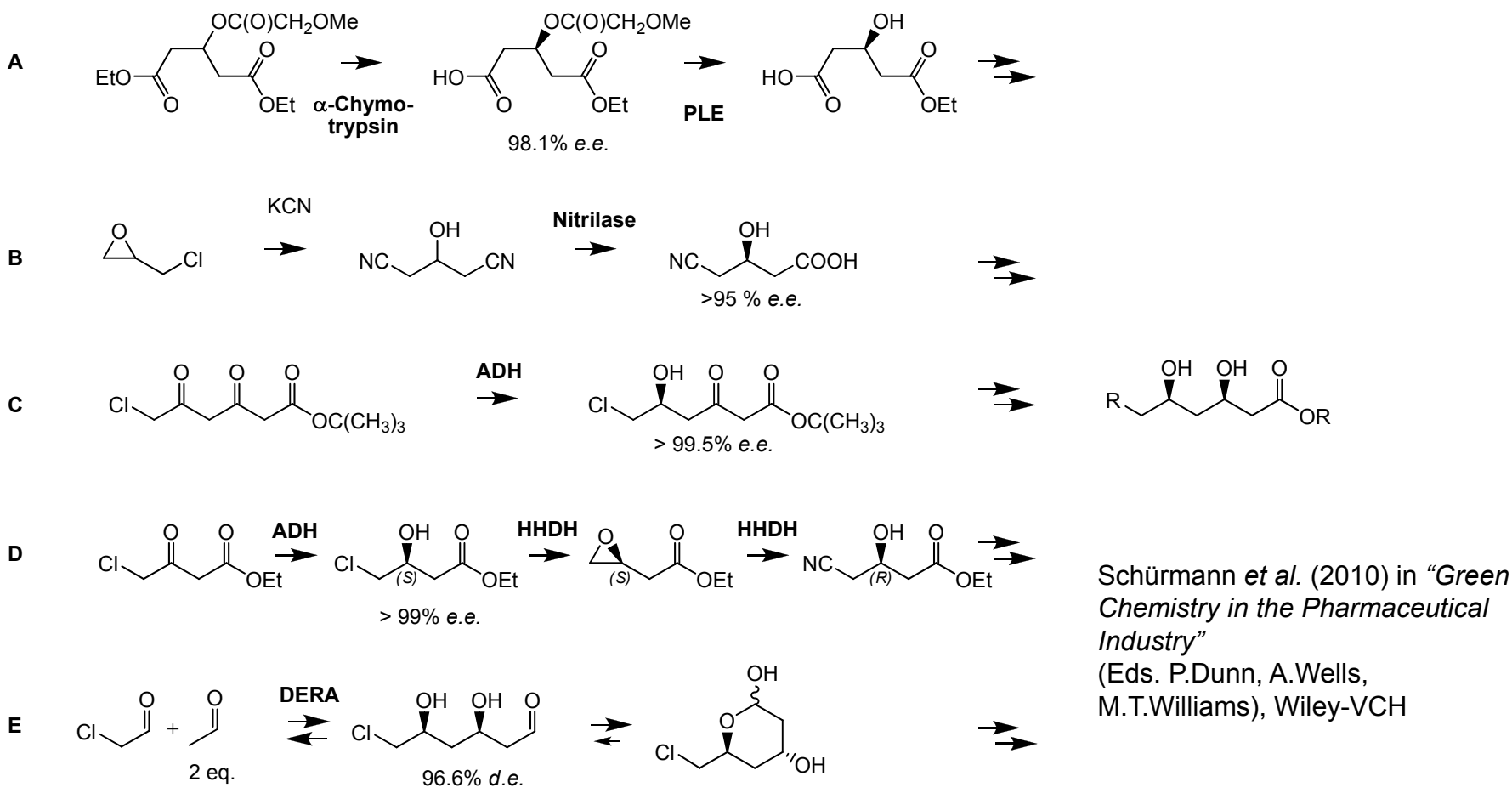
Biocatalytic Approaches to Statin Side Chains



Schürmann *et al.* (2010) in *"Green Chemistry in the Pharmaceutical Industry"* (Eds. P.Dunn, A.Wells, M.T.Williams), Wiley-VCH

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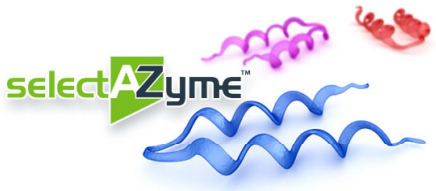

Enzyme Platforms	Product Classes
Aldolases	Alcohols, Diols, Amino alcohols
Proteases	Peptides, Amines, Carboxyesters
Lipases and Esterases	Alcohols, Esters, Carboxylic acids
Ammonia lyases	Amino acids
Hydantoinases, Carbamoylases, Racemases	Amino acids
Amidases	Amino acids, Amides
Acylases	Amino acids, N-Acetyl-Amino acids
Hydroxynitrile lyases	Cyanohydrins
Omega-Transaminases	Amines
Carbonyl Reductases	Alcohols
AA Dehydrogenases	Amino acids
Nitrilases	Carboxylic acids, Nitriles
Nitrile hydratases	Amides, Nitriles
Monooxygenases (P450, Baeyer-Villiger)	Alcohols, Sulfoxides
Epoxide hydrolases	Epoxides, Diols
Haloalcohol dehalogenases	Epoxides, Diols

Extensive portfolio of off-the-shelf enzymes:

- Made in-house
- From partners

On-going enzyme discovery programs:

- In-house
- With industry partners
- With academic partners

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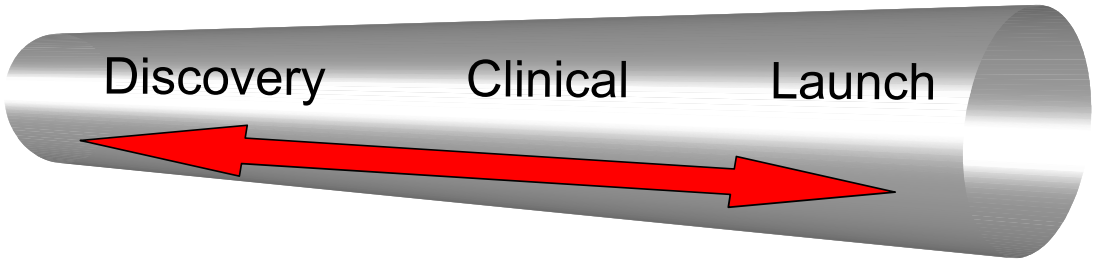
Biocatalyst libraries off-the-shelf: Speed matters!



mg-10g 100g-1Kg 1Kg-100Kg >100Kg

"Off-the-shelf" biocatalysts

Process Dev

Speed

COST

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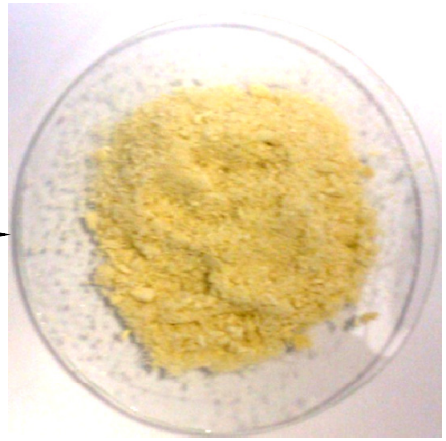
Recombinant enzymes – how they are made:

Cell paste

Homogenisation & Clarification

Liquid CFE

Freeze- or Spray-drying



Solid CFE

- Whole cell biocatalysts are cheapest, but CFE often preferred.
- Further protein purification is possible, but reduces fermentation yield and increases cost.
- Scale up of fermentation, homogenisation and freeze/spray-drying needs PRD!

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Case Studies: Biotransformation route scouting and PRD at Almac, scale up at Almac / other CMO or client

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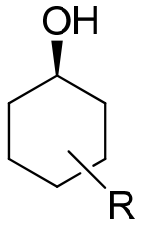
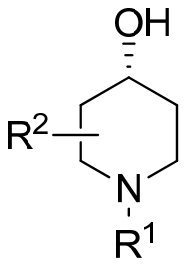
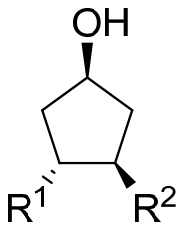
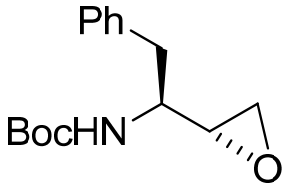
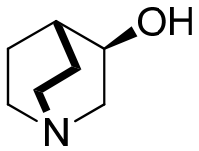
What do these molecules have in common?

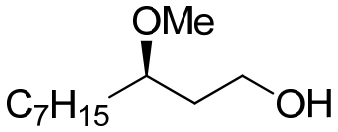


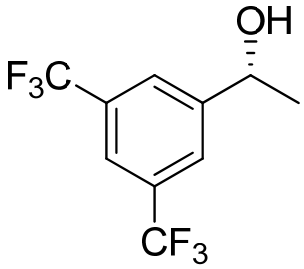


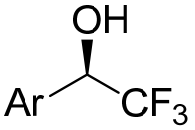






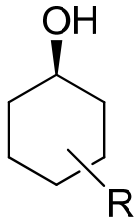


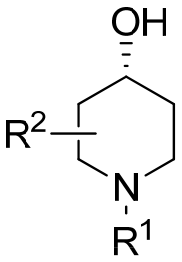


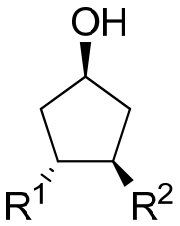


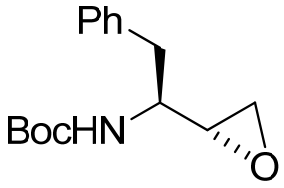
- They are important pharmaceutical intermediates.
- Asymmetric ketone reduction is key step in their synthesis.
- They have been made *via* bioreduction at Almac.

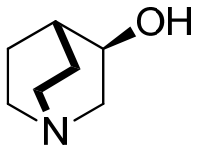
Case study: (R)-3-Methoxydecanol

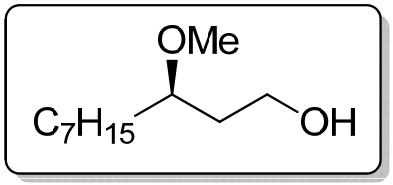


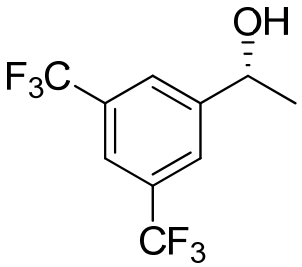


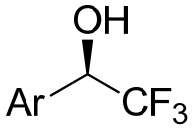












Application of CRED technology enabled large scale manufacture of chiral building block for late phase API.

Success Criteria:

Quality, Quantity, Speed, Cost

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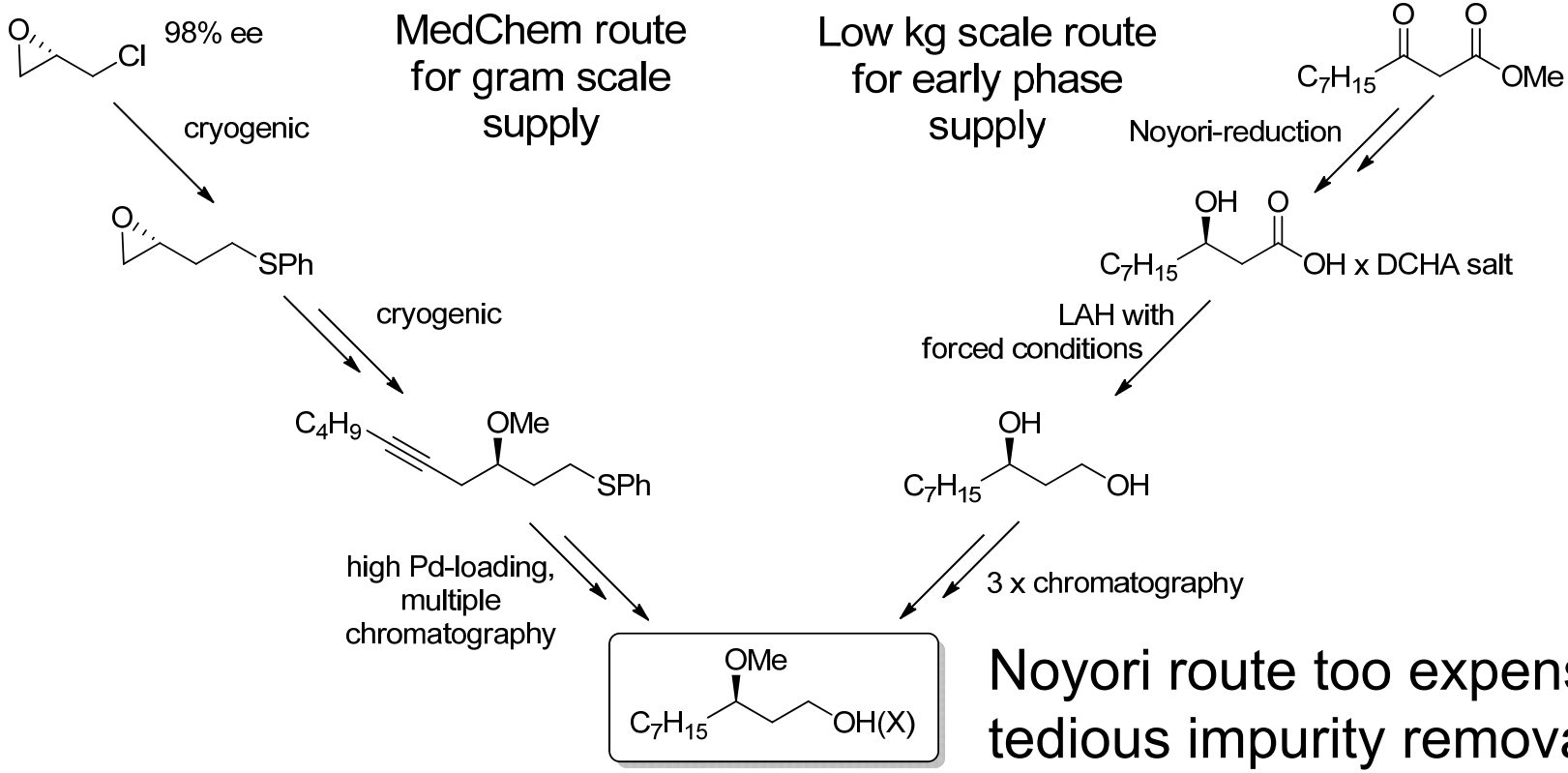
Case study: (R)-3-Methoxydecanol

Why was a new approach needed?



MedChem route very inefficient, hard to scale, and could not deliver >99% ee

Noyori route too expensive, tedious impurity removal, ee borderline – Could not meet late phase requirements

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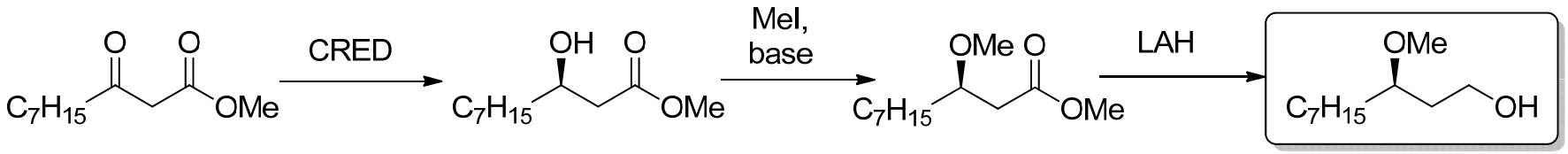
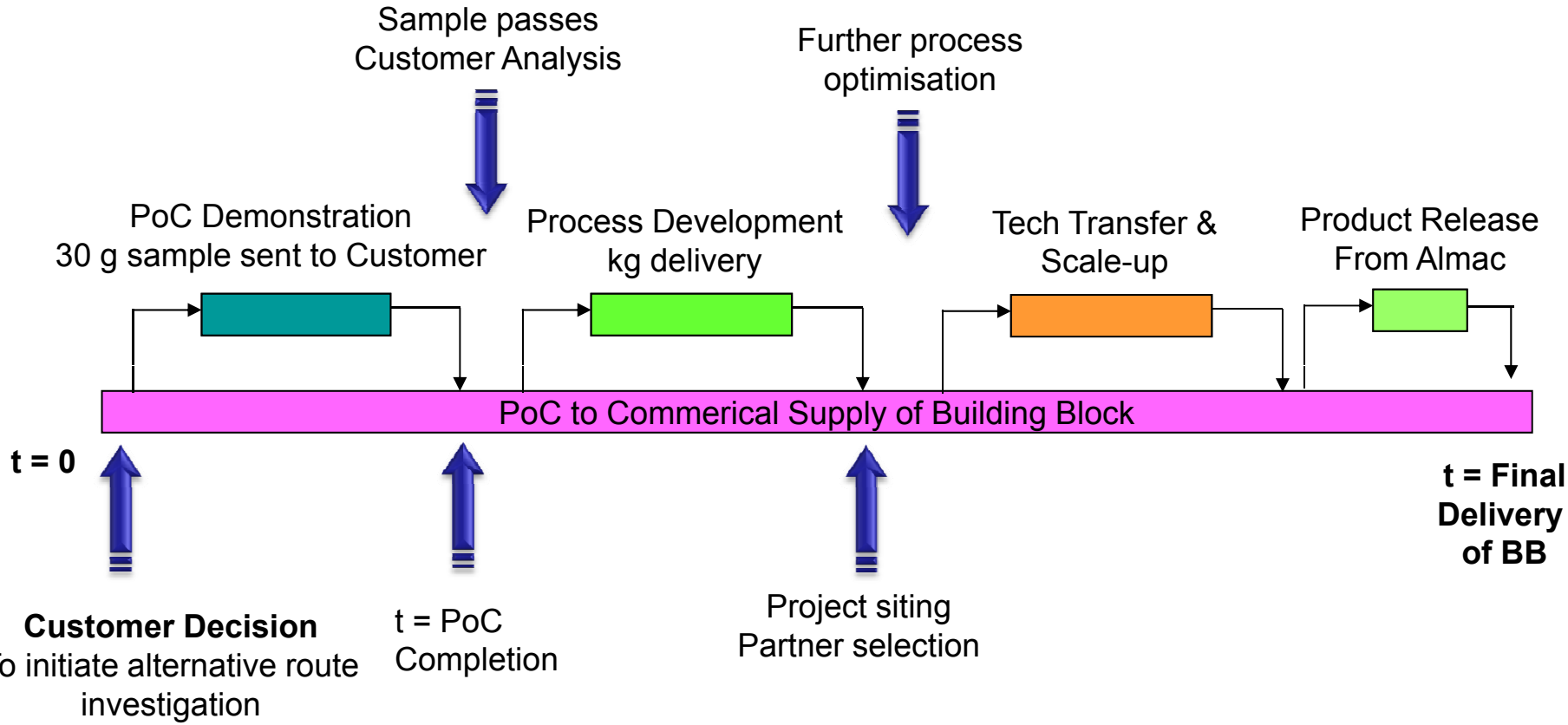
Case study: (R)-3-Methoxydecanol

Proposed bioreduction route





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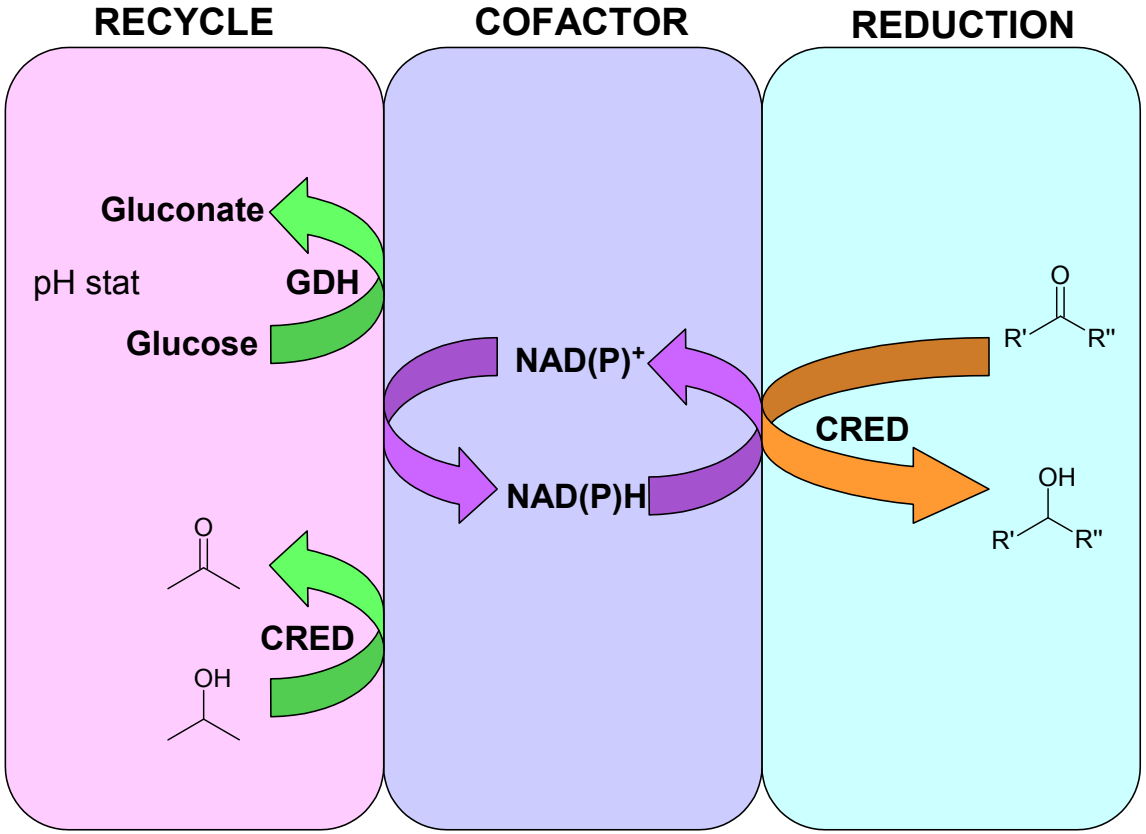
Recombinant CREDs – how they work, and how to work with them:



Screening phase:

- Identify catalyst,
- Identify co-factor,
- Identify recycling system.

ee is what matters!

PRD phase – optimise:

- temperature, pH, co-solvent, enzyme form, catalyst loading, throughput.

Cost is what matters!

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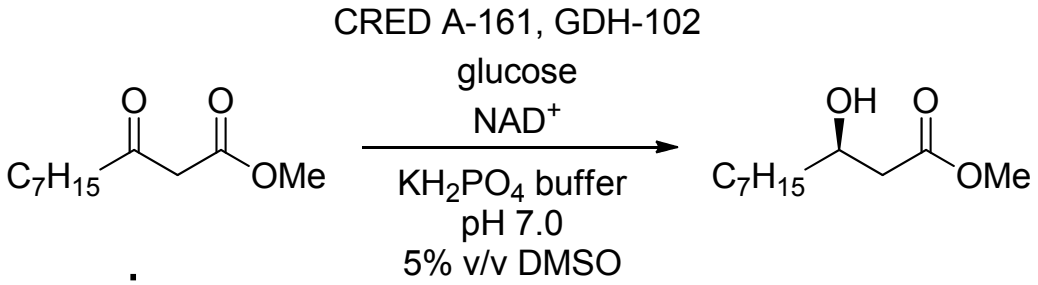
Case study: (R)-3-Methoxydecanol

Key Step Bioreduction



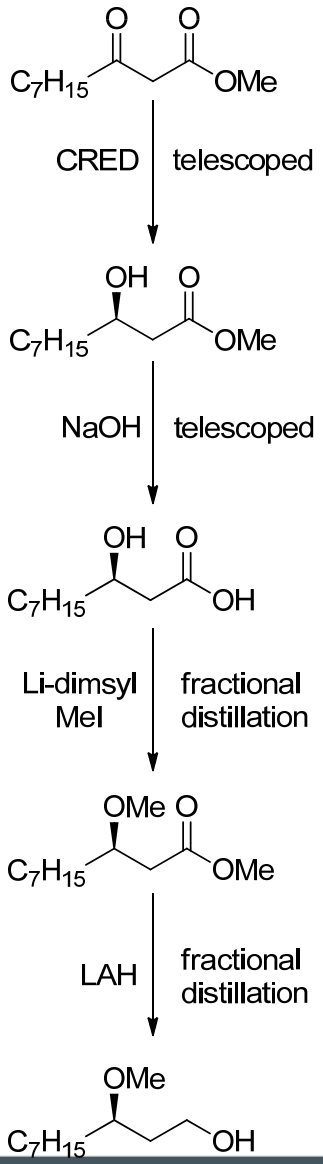
CRED Screening:

- A161 identified as hit with >99.5% ee
- Enzyme is NADH-dependent
- GDH/glucose used for co-factor recycle

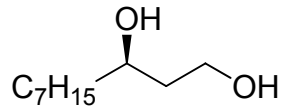
Process Optimisation:

- 5% DMSO co-solvent, pH 7.0, 30 deg C
- 0.1% w/w of lyo cell free extract CRED/GDH sufficient
- 180 g/L substrate >99.8% converted within 12 hours
- Workup by extraction with MtBE; crude product telescoped into next step

Case study: (R)-3-Methoxydecanol – *impurity control*



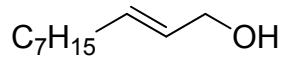
Control of Diol-impurity starts here.
High reaction completion target
of > 99.8% conversion.



Diol-impurity

Control of Dec-3-enol impurity starts here.

High reaction completion targets.
Choice of base for deprotonation.
Unstabilised THF used for methylation.
Fractional distillation in both final steps.



Decenol-impurity

Achieved:
99.3% ee; diol <0.2%; dec-2-enol <0.15%
BHT and Me-BHT <0.2%, all other <0.1%.

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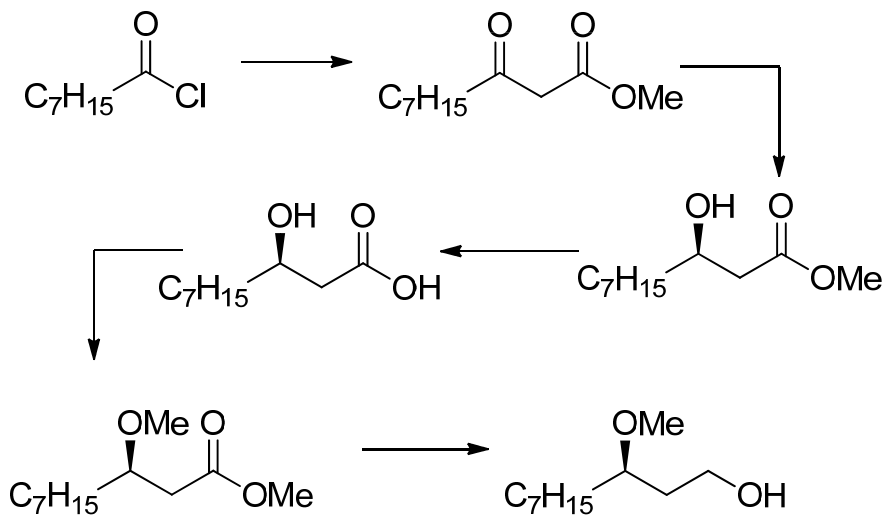
Case study: (R)-3-Methoxydecanol

from POC sample to commercial output

5 Step Process Yields

(%POC / %pilot batch / %final)

Step 1: 75% / 50% / 85%
Step 2: 91% / 90% / 95%
Step 3: 95% / 95% / 98%
Step 4: 80% / 70% / 85%
Step 5: 70% / 70% / 75%



Process modifications after pilot batch experience:

Step 1: switched from Meldrum's acid to methyl potassium malonate SM
Step 4: switched from n-BuLi to n-HexLi, relaxed specification
Step 5: improved hydrolysis protocol

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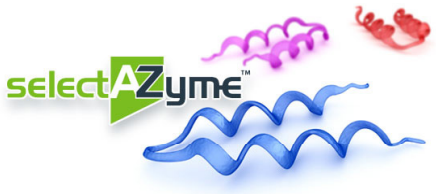
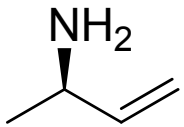
Case study: (R)-3-Methoxydecanol

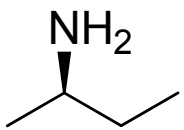
Summary

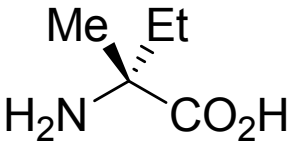


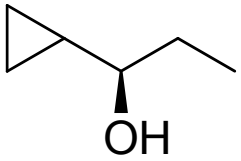
- 5-Step process (one biotransformation)
- Asymmetric chemo-cat route not viable
- Biocatalyst far cheaper and giving superior selectivity
- Biocatalyst was off-the-shelf, facilitating rapid identification and PRD
- Enzyme originally found through genome mining, and then expressed in *E. coli*
- Improved route design and simplified purification strategy to deliver better quality at higher yield
- POC sample followed by 25 kg pilot batch
- Several full scale campaigns split between Almac and two partners with equivalent distillation capability

What do these molecules have in common?









- They are in demand as pharmaceutical intermediates.
- Almac chemists have worked on scaleable syntheses.
- They are very small molecules with even smaller substituent difference at the stereocentre = tricky to make.
- Chiral technology cost contribution is high due to low MW.
- We had some success, but further work is required.

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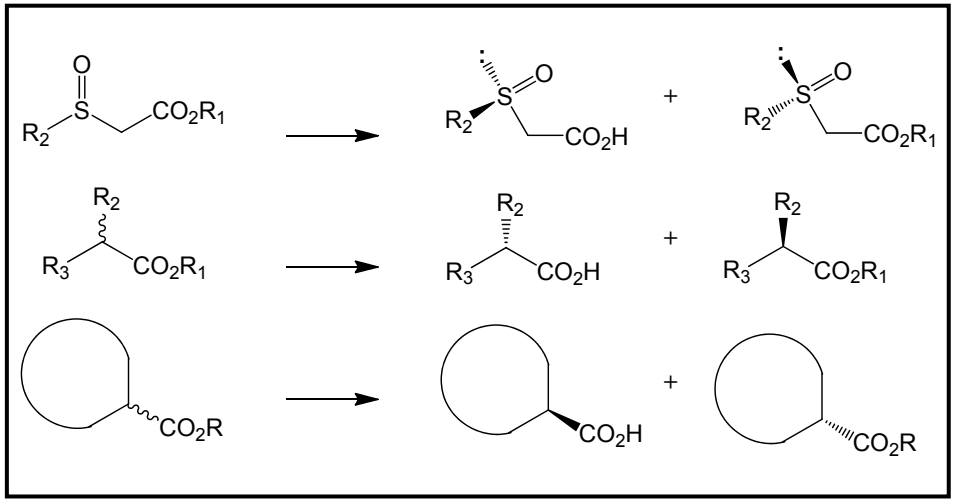
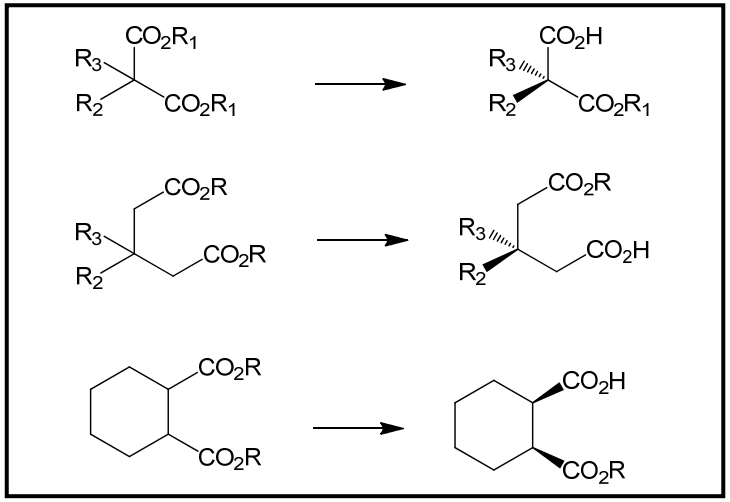
Mature enzyme technologies example: Applications of Hydrolases

Kinetic resolution



50% yield

Desymmetrization



100% yield

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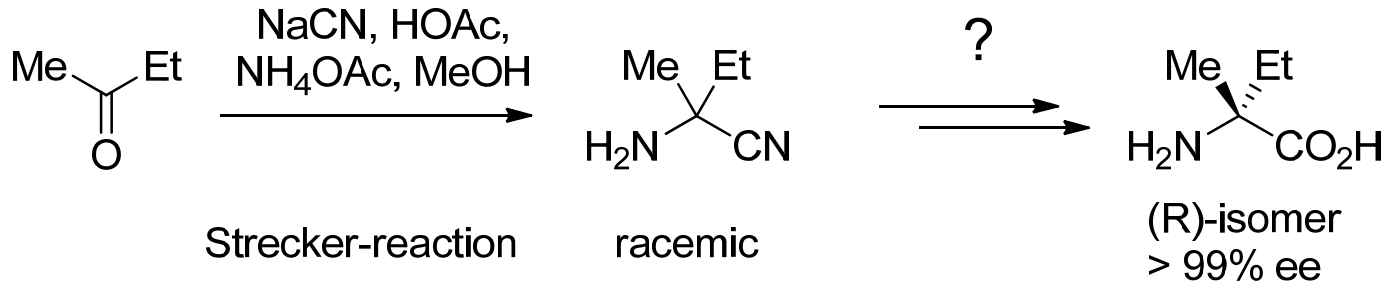
Case study: D-Isovaline

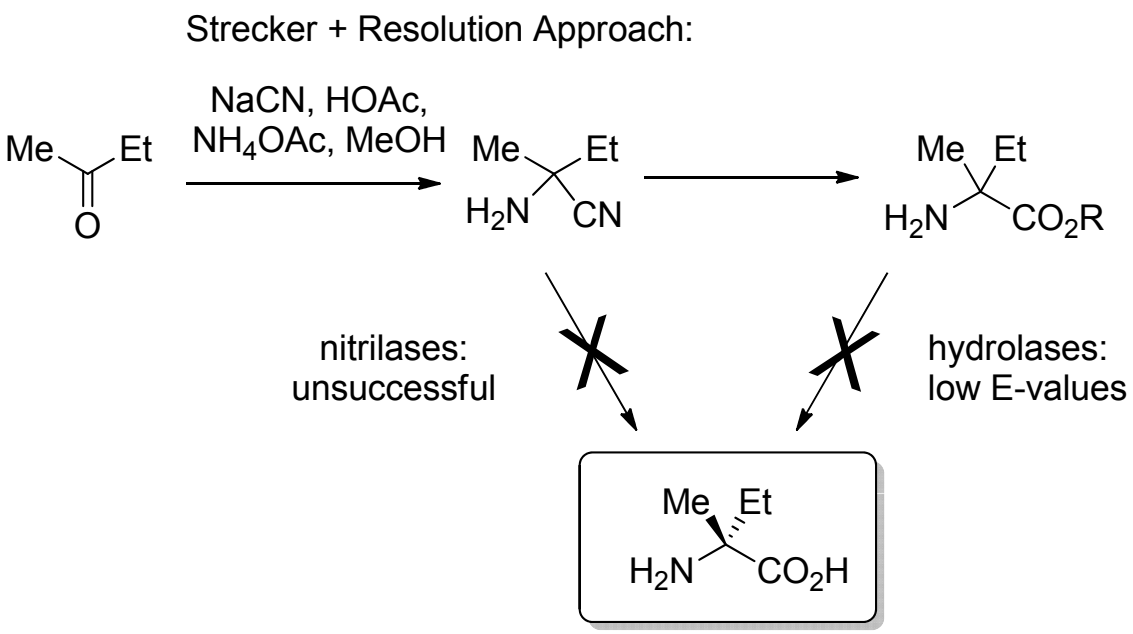


Background:

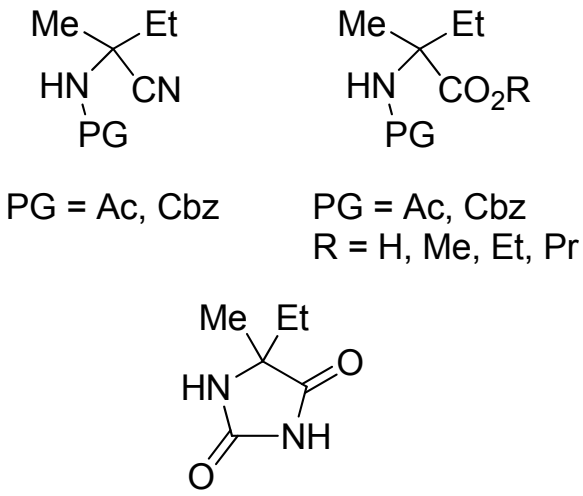
- A number of synthetic methods are known for this type of structure, and a number of suppliers list it as stock item.
- But price is very high (>$500/g) and quality insufficient.
- Strecker-reaction provides easy access to racemate.
- Classical resolution on basic amine intermediate un-successful after screening >30 commercial resolving agents.

Is there a biocatalytic alternative to enable viable multi kg supply?

Case study: D-Isovaline



other substrates made and tried:



Strecker + bioresolution approach envisaged to give shortest route and quickest success from cheap commercial starting material.

However, this did not work due to low E-values and / or lack of reactivity.

10 different substrates screened against >200 enzymes – no luck.

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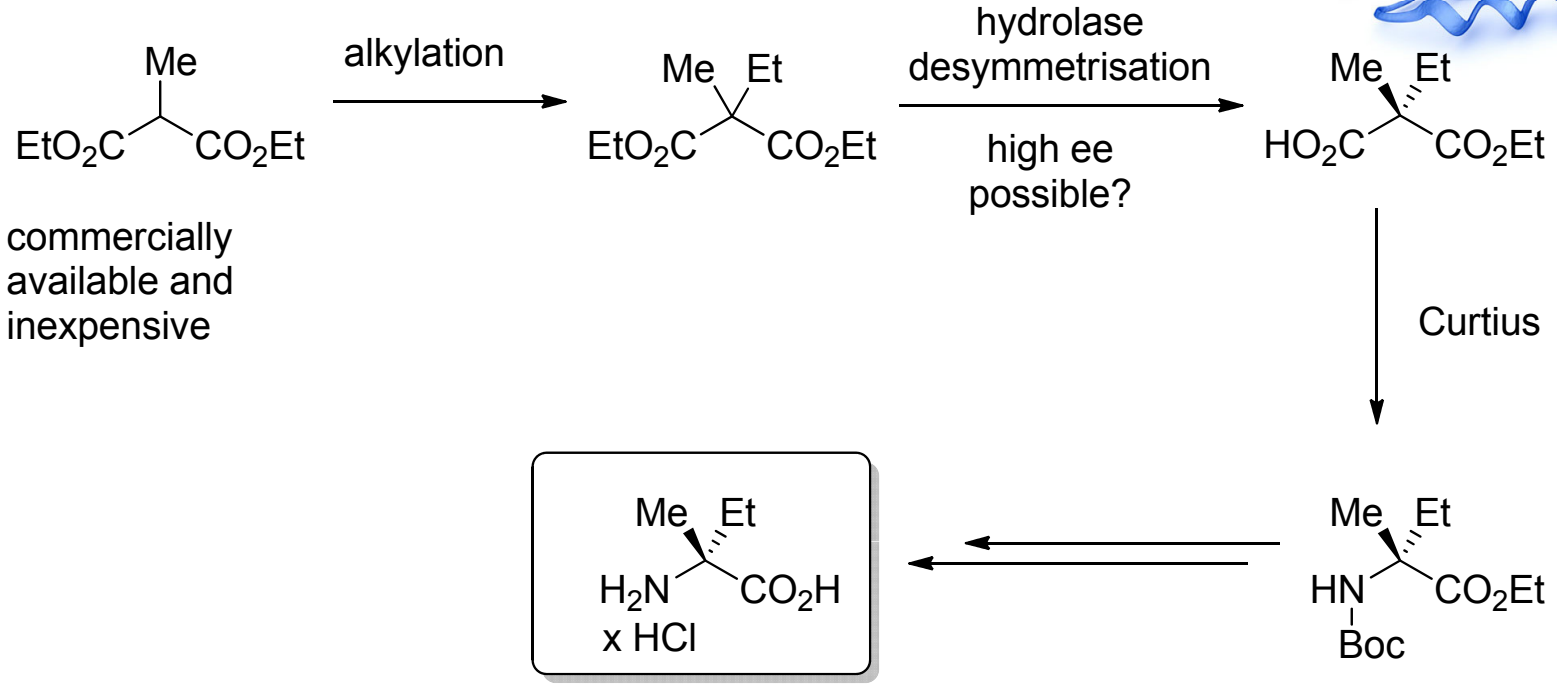
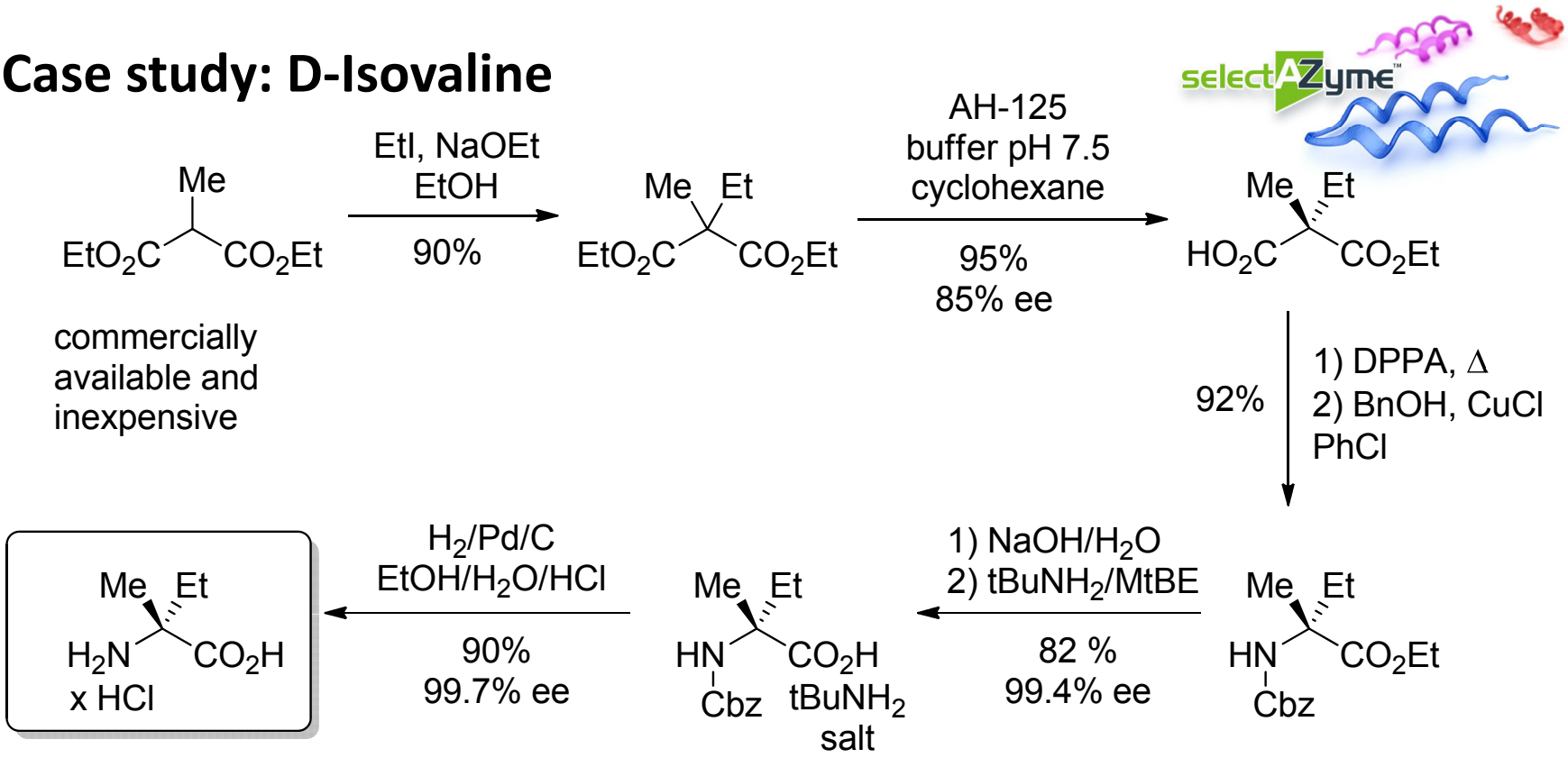
Case study: D-Isovaline



Envisaged alternative approach with hydrolase mediated desymmetrisation as key step – this did work. *Finally!*

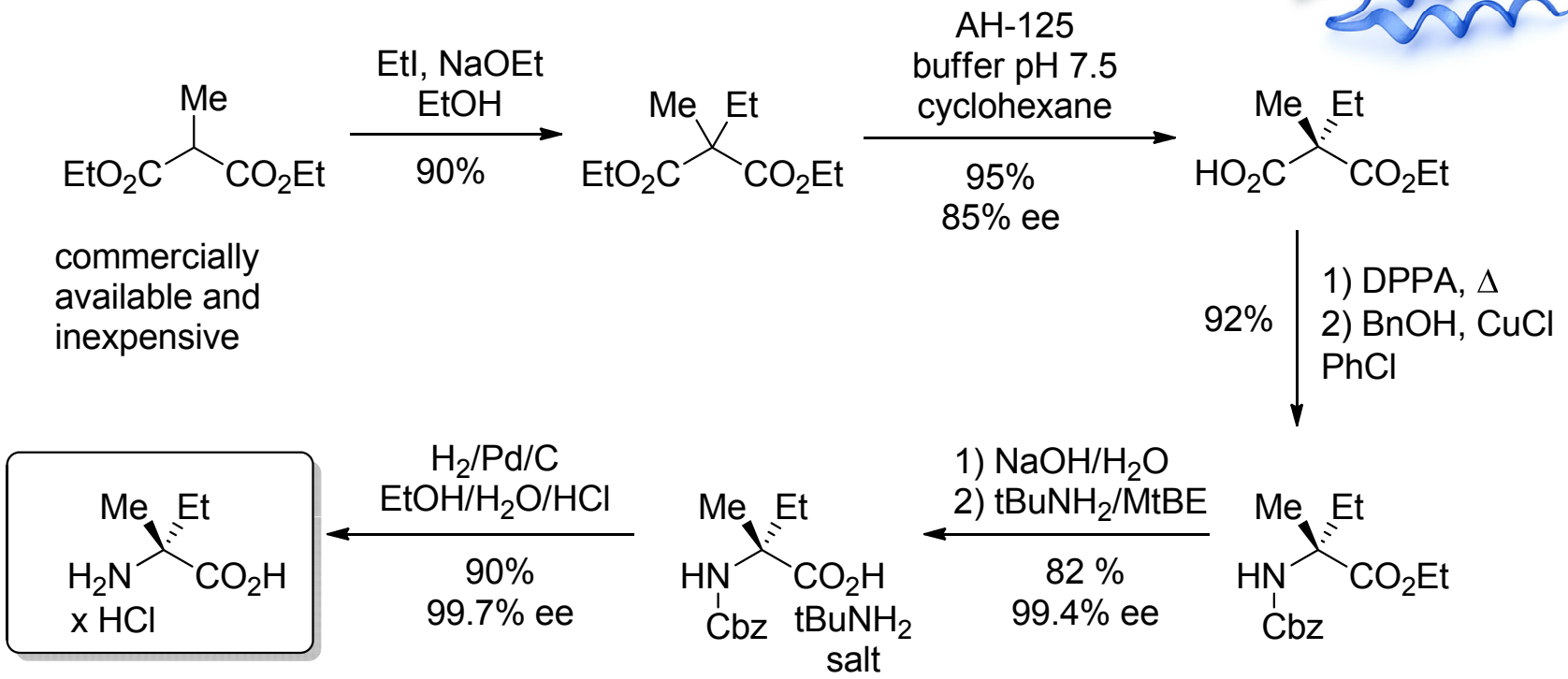
Screening of 180 off-the-shelf hydrolases gave a hit, BUT ee was only 85%, AND Curtius gave low yield.

Case study: D-Isovaline



Enzyme step PRD was straightforward. 0.5% w/w recombinant enzyme were sufficient, used as cell free extract.
Curtius problem was solved by switching to Cbz-product.
ee-Upgrade was achieved *via* tBu-amine salt.
Final step workup yielded highly pure product.

Case study: D-Isovaline – *Summary & Outlook*

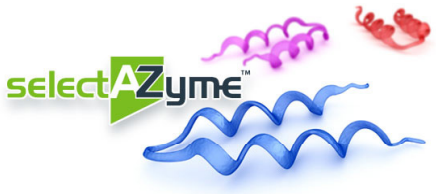


10 kg of pilot batch material were made and delivered.

Room for future improvement:

Remove need for ee-upgrade by substrate engineering or protein engineering. Use cheaper enzyme preparation for larger scale. Alternative enzyme with reversed selectivity would enable replacing Curtius with Hofmann-rearrangement.

What if no recombinant off-the-shelf biocatalyst is good enough?



- **Option Zero**: Substrate and reaction engineering

- **Option A**: Check for wild type microorganisms to do the job
  - Traditional approach – does traditional mean outdated?
  - Often - but not always! - narrow substrate scope and low productivity
  - Still preferred if complex multi-enzyme pathways are involved
  - Engineering of metabolic pathways possible *via* strain mutation

- **Option B**: Check for enzyme homologues in nature, clone and express (£)
  - Modern approach, enabled by advances in bioinformatics and genome mapping
  - Related: Meta-genomics approach
  - Can replace or merge with option C

- **Option C**: Start a directed evolution program (£££)
  - Modern approach, enabled by advances in gene synthesis, protein engineering and understanding of protein structure-activity-relationship
  - Impressive results but high up-front investment
  - Expanded off-the-shelf catalyst libraries as by-product useful to others

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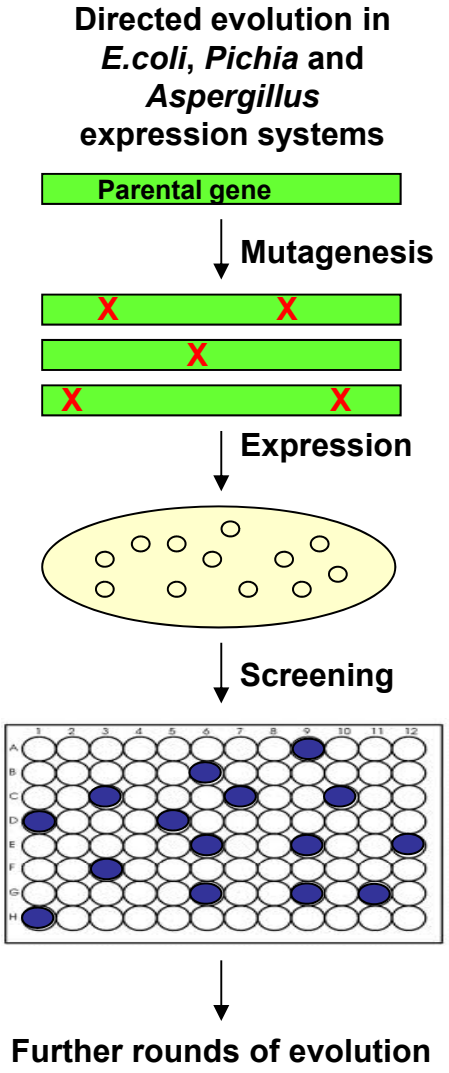
Enzyme Evolution



- Almac perform directed evolution by both random and site-specific mutagenesis.
- Expertise in a number of different expression systems such as *E.coli, Pichia* and *Aspergillus.*
- Bioinformatic expertise allowing saturation mutagenesis
- Novel system that allows glycosylation of proteins in *E.coli*

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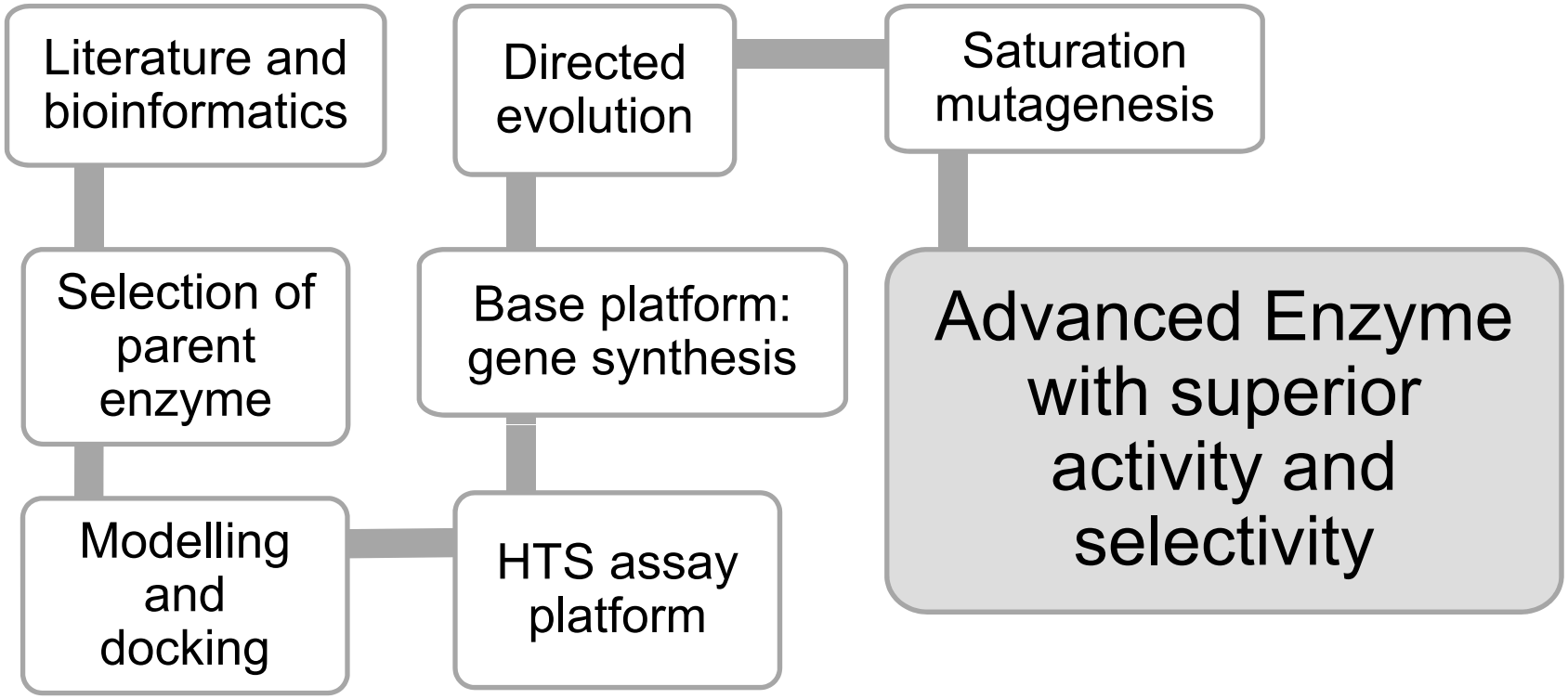
Evolution Strategy



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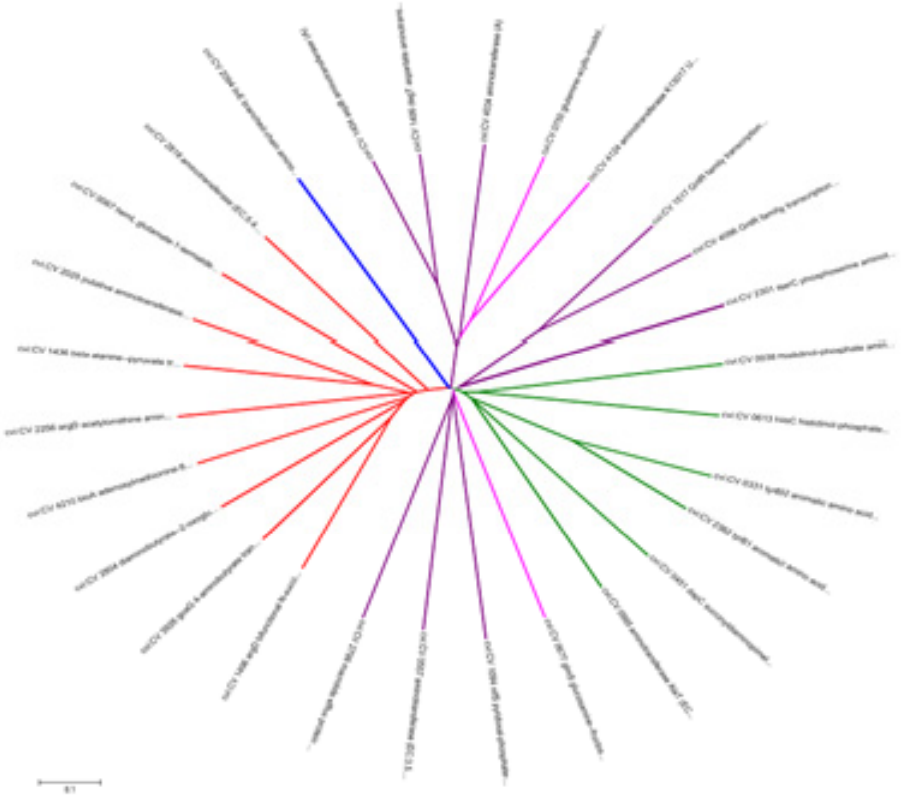
Biocatalyst improvement *via* homologues



Homologues can be identified *via* sequence and structural alignment.

These have a similar core motif, but are derived from a range of organisms.

Priority will be given to homologues with similar docking characteristics but originating from extremophile organisms.

Involves bioinformatic studies, gene synthesis, host transformation and reaction screening.

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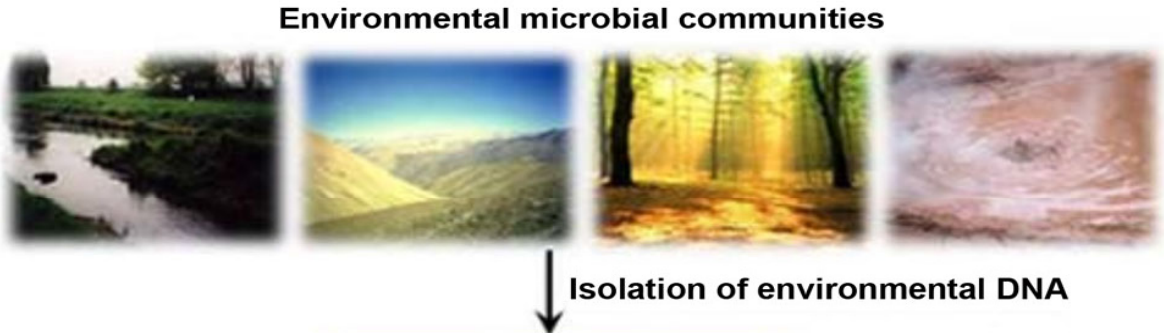
Metagenomics

Metagenomics: application of genomics to uncultured microorganisms

1st STEP: Isolation of environmental DNA (eDNA)



- the *ex situ* method: cells are isolated and concentrated from environment prior to their lysis
- the *in situ* methods: cells are lysed directly within the soil material
- the enrichment method: microorganisms are enriched for a desired enzyme activity prior to the isolation of eDNA

Extraction of novel natural biocatalysts

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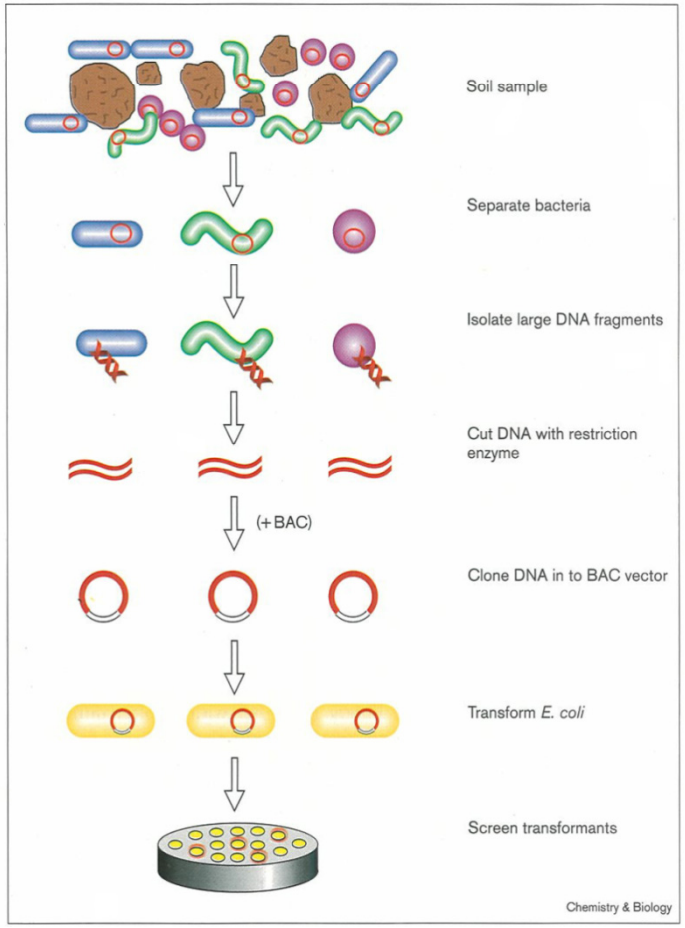
Metagenomics



Handelsman et al. 1998 – CHEM BIOL. 5:R245-R249

Classical

Cloning eDNA into vectors

•Gene identification by DNA sequencing (optional)

•Expression cloning into appropriate vectors

metagenome libraries

Almac & UCL

Isolation of DNA

•2nd Generation sequencing

•Bioinformatic libraries

• Expression and cloning

metagenome libraries

Extraction of novel biocatalysts

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Metagenomics

- **Novel salt mine enzyme discovery with QUB**
- Grown under both aerobic and anaerobic conditions, range of temperatures
- Novel microbes identified
- **Novel enzymes identified**
- **Salt mines in Northern Ireland**

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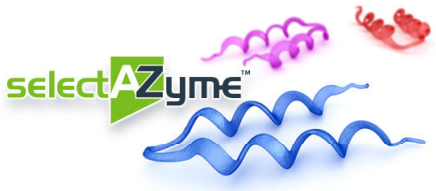
How to use whole cell biocatalysts



Phase 1: Screening — Selection from panel of 50-100 organisms

Phase 2: PRD — Optimisation of growth + reaction conditions for maximised yield and productivity

Phase 3: Full scale manufacture — *via* either of two modes:

Fermentation mode	**vs**	***Re-suspension mode***
one-pot microbial growth and desired reaction		**off-line biomass growth, reaction with re-suspended cells**

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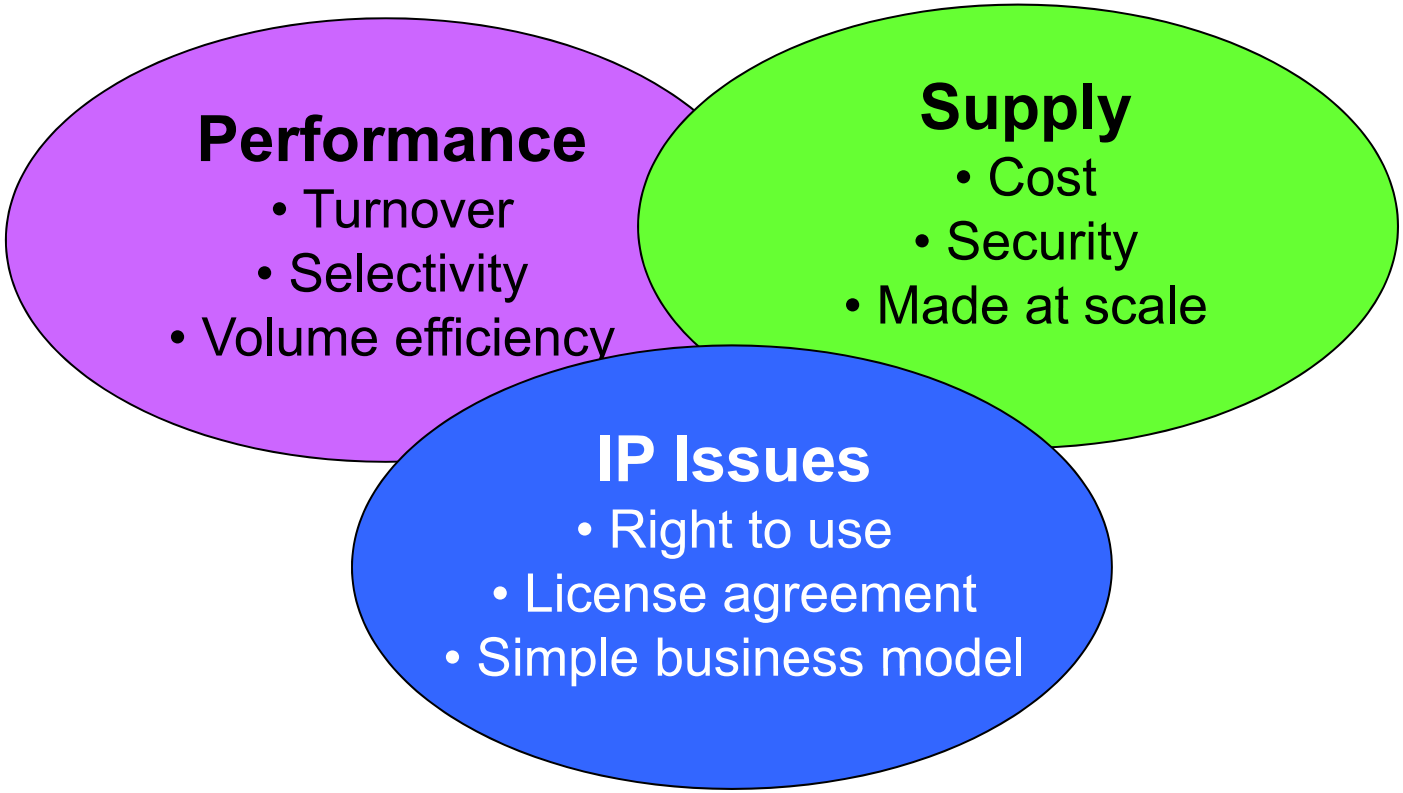
A ***commercially viable*** biocatalyst is defined by…..



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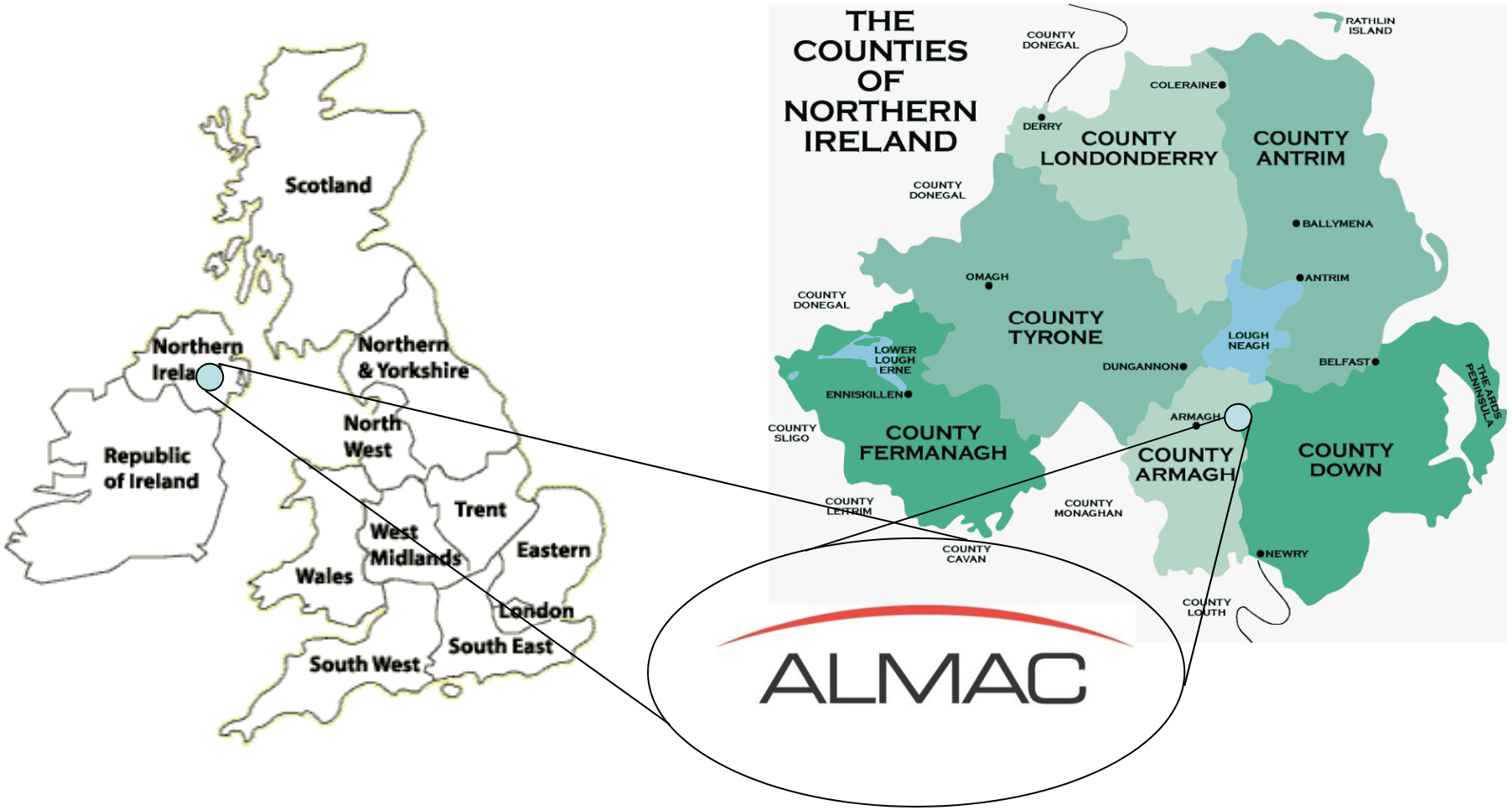
Where we are:



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Where we are:

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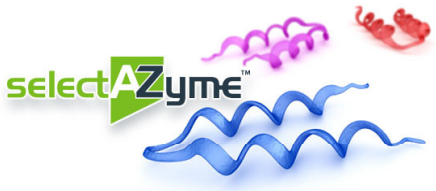
Contacts



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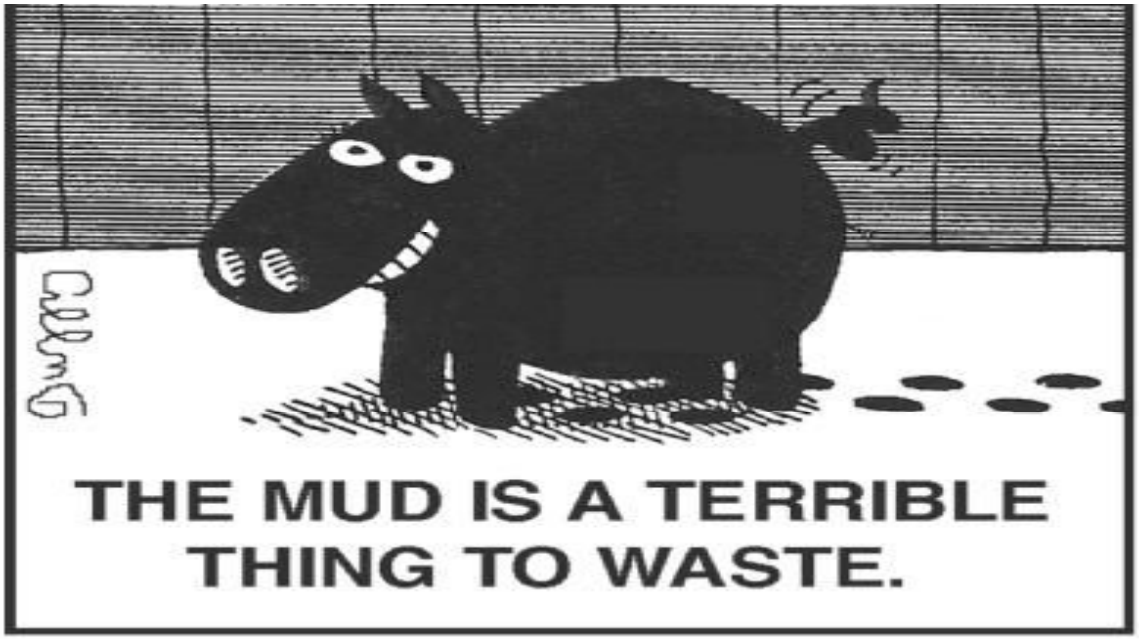

European Union

European Regional Development Fund
Investing in your future

Thank you for listening!

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