“Shortening the Path - Pharmaceutical Materials from Enzymatic Reactions”

Dr. Stefan Mix / ALMAC Group

Organic Process Research & Development
Prague, 17-19 October 2016

www.almacgroup.com
Founded in 1968
Global Headquarters in Craigavon, Northern Ireland
Turnover >$500m
~ 4000 Personnel Globally
Unique ownership – charitable foundation
All Profit Re-invested
Arran Chemical Company - purchased 2015
History of ALMAC

- **1968**: Galen founded by Allen McClay
- **1997**: Galen floated on the stock market
- **2000**: Galen merged with Warner Chilcott
- **2001**: Sir Allen McClay retires
- **2002**: Sir Allen McClay comes out of retirement and acquires Galen service divisions
- **2004**: Almac Group achieves foundation status
- **2006**: Almac successfully rebrand all business units as part of the Almac Group
- **2009**: Galen service divisions are integrated
- **2010**: Passing of Sir Allen McClay
- **2015**: Almac Group acquire Arran Chemicals (non-GMP) facility in Athlone, ROI
- **2015**: Almac Group acquire DP manufacturing facility in Loughborough, England
- **2019**: Opening of regional headquarters in Asia (Singapore)
- **2020**: Opening of regional headquarters in North American (Souderton)
- **2021**: Galen successfully rebrand all business units as part of the Almac Group
Almac provides a range of integrated services to the Pharmaceutical and Biotechnology industries.

Services range across the full spectrum of drug development, from discovery through development to final delivery of commercial drug product.

Almac employs over 4000 people and has evolved into a world-class solution provider of integrated development services and product supply.
Based in Athlone, Co. Roscommon, Ireland

>25 Years experience as a fine chemical provider (building blocks and intermediates)

~60 Staff (chemists, analysts, production)

Current output ~300 ton per annum

Business split of 50% pharma and 50% speciality chemicals provisions

*e.g. flavour and fragrance, monomers, etc.*
Advantages from using enzymes

Why biocatalysis?

- High selectivity
- Conversion of waste to chemicals
- Application from mg to ton
- Renewable feedstocks
- Rapid access to novel biocatalysts
- Low solvent usage
- Tandem biocatalysis
- Avoidance of toxic waste

Our vision
An integrated group of biologists and chemists who:

**Discover.** New enzyme discovery platforms

**Screen.** Screen for and utilize enzymes in processes

**Evolve.** Improve properties of enzymes (process efficiency, economy)

**Supply.** Manufacture (immobilised) enzymes and enzyme-derived products

**Key Expertise:**
- Enzyme discovery (genome mining, metagenomics)
- Building panels of enzymes
- Active site modelling and enzyme design
- Evolution tools (saturation/random mutagenesis)
- Fermentation development & scale-up
- Enzyme supply & immobilisation
- Bioprocess development and manufacture of products
- Metabolite synthesis
What makes a biocatalytic process?

**Natural diversity**
- Characterised organisms
- Sequenced genomes
- Metagenomes

**Protein engineering**
- Directed evolution
- Rational design

**Biocatalysts**
- Recombinant enzymes
- Wild type organisms
- Pathway-engineered organisms

**Key features:**
- Selectivity
- Specificity
- Stability
- TON + TOF

**Synthetic application**
- Process design
- Substrate engineering

**Key features:**
- Cost
- Productivity
- Quality
- Sustainability

"Genomes, Biocatalysts and Pharmaceutical Materials"
07 January 2016, stefan.mix@almacgroup.com
Bioprocess challenges

Development effort and time → off-the-shelf vs bespoke biocatalyst

Catalyst cost contribution → choice of enzyme form

Space-time-yield and CAPEX / manufacturing cost → process design

Competing technologies → watch the market!

Productivity limitations from
  Mass transfer in heterogeneous systems
  Enzyme stability and deactivation
  Substrate/product inhibition
  Thermodynamics

Choice of starting material, route and enzyme class

Product isolation, purification, reduction of bioburden
Biocatalyst libraries off-the-shelf: Speed matters!

Discovery → Clinical → Launch

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

“Off-the-shelf” biocatalysts

Process Dev

Speed

COST

Enzyme Selection → 100s g → 1-10 kg → 100 kg → Tons

2-3wks → 1-2wks → 3-5wks → 2-3mths → 6-8mths

“Off-the-Self” biocatalysts

Process Development

Genomes, Biocatalysts and Pharmaceutical Materials

07 January 2016, stefan.mix@almacgroup.com
From screening to bioprocess scale up

- **Successful POC**
  - Lab prod. sample

- **Enzyme Screening**

- **Process Development**
  - Reaction design
  - Reaction safety
  - Enzyme form
  - Product isolation

- **Fermentation Development**
  - Reaction design
  - Reaction safety
  - Enzyme form

- **Fermentation Scale-up**
  - Raw material sourcing
  - Quality

- **Scale-up**
  - Bulk Enzyme supply

- **Bioprocess Scale-up**
  - Product isolation+ purification via
    - Solvent extraction
    - Distillation
    - Crystallisation
    - Re-slurry
    - Biopolish
    - Ion exchange
    - Charcoal treatment
    - Ultrafiltration
## Enzyme Platforms

<table>
<thead>
<tr>
<th>Enzyme Platforms</th>
<th>Product Classes</th>
<th>Enzymes</th>
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<tbody>
<tr>
<td>Aldolases</td>
<td>Alcohols, Diols, Amino alcohols</td>
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<tr>
<td>Proteases</td>
<td>Peptides, Amines, Carboxyesters</td>
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<tr>
<td>Lipases and Esterases</td>
<td>Alcohols, Esters, Carboxylic acids</td>
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<td>Ammonia lyases</td>
<td>Amino acids</td>
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<td>Hydantoinases, Carbamoylases, Racemases</td>
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<tr>
<td>Amidases, Acylases</td>
<td>Amino acids, Amides</td>
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<td>Imine reductases</td>
<td>Amines</td>
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<tr>
<td>Hydroxynitrile lyases</td>
<td>Cyanohydrins</td>
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<tr>
<td>Omega-Transaminases</td>
<td>Amines</td>
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<tr>
<td>Carbonyl Reductases</td>
<td>Alcohols</td>
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<td>AA Dehydrogenases</td>
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<tr>
<td>Ene reductases</td>
<td>Ketones, Nitriles, Esters, Amides</td>
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<td>Nitrilases and Nitrile Hydratases</td>
<td>Carboxylic acids, Amides</td>
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<td>Amine oxidases</td>
<td>Amines</td>
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<tr>
<td>Monooxygenases (P450, BVMO)</td>
<td>Alcohols, Sulfoxides</td>
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<td>Epoxide hydrolases</td>
<td>Epoxides, Diols</td>
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<tr>
<td>Halohydrin dehalogenases</td>
<td>Epoxides, Diols</td>
<td>18</td>
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</tbody>
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### 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
How to make chiral amines – spoilt for choice?

- Classical resolution, Auxiliary chemistry
- Transaminase + ketone
- Asymm. hydrogenation, Asymm. PTC
- Aminoacid-DH/ Amine-DH + α-ketoacid/ketone
- Amine oxidase + racemate
- Imine reductase + imine
- Ketoreductase + ketone
- Hydrolase/Nitrilase + ester/nitrile
IRED found by *in-silico* screening

- *In silico* selection of 50 IRED enzyme library
- Gene synthesised, cloned and expressed
- Screened against client substrate - 20% hit rate

<table>
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<tr>
<th>IRED no.</th>
<th>% HPLC peak area @ 260 nm</th>
<th>% HPLC peak area of product enantiomers</th>
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</table>
Enzyme panel building

• Bespoke enzyme panels can be developed within weeks using Almac bioinformatics GIDS platform
• GIDS - gene informed database searching
• Tailored panel of enzymes expressed in 96 well plate format
• 1 enzyme only possible; typical is 25, 50 or 96
• Panels can contain mutants, homologues, or enzymes selected by in-silico screening
IRED process design challenges + solution

- IRED-20 selected for development
  (100% ee and best activity in screening)

- Enzyme form defined: Spray-dried cells suitable

- Challenges encountered during PRD:
  Inhibition of enzyme limits product titre
  Product highly water soluble \(\rightarrow\) difficult recovery by extraction

- Solution implemented: Ion exchange column in recirculation mode
  \(\rightarrow\) Solves inhibition problem in aqueous reaction medium
  \(\rightarrow\) Enables efficient product recovery with ammonia in methanol
Engineered transaminase for arylethylamine (1)

Rational variant design by protein-substrate interaction modelling

- Bulky substrate - no good for wildtype TAm enzymes
- Smart library approach chosen for enzyme engineering
- Only rationally selected residues were mutated
- Increased chance of success from short engineering program
Rational protein engineering

**Initial Structural analysis**

**Docking/ Mechanism determination**

**Point mutations**

**In silico prediction of mutant Enzymes**

**Wet lab validation**

Typically 20-50 rationally designed mutant enzymes per round

Wet lab validation
Engineered transaminase for arylethylamine (1)

Huang et al. ACS Catalysis 2016, DOI: 10.1021/acscatal.6b02380
Engineered transaminase for arylethylamine (2)

- Large volume product made by Arran via classical resolution
- Cost improvement wanted via implementation of transaminase process using cheap amine donor isopropylamine
- Screening of off-the-shelf R-transamininases gave hit with excellent ee
- However: can’t get to high enough titre AND low enough enzyme loading with screening-hit enzyme
- Challenge by interplay of thermodynamics and enzyme stability
- Enzyme engineering program initiated
Engineered transaminase for arylethylamine (2)

- Limited TAm engineering program conducted
- Best direct amination process with iPr-amine donor gave insufficient improvement of yield, enzyme loading and product titre
- Using ATA-611 in resolution mode resulted in much better overall process (ketone recycling required, but easily accommodated in existing synthetic route)

Are further improvements possible? Sure!
Wanted: asymmetric bioprocess for (R)-CPEA

- R-CPEA is building block of great interest and limited, costly supply
- Very small molecule = challenge for any technology
- Existing process suffers from high cost of resolving agent, manufacturing, and low yield
- Transaminase process investigated, but cost is too high
- CRED + displacement works better than resolution route despite 1 more step
- Long term outlook: Amine dehydrogenase process (still needs some work)

\[
\begin{align*}
\text{ketone} & \xrightarrow{\text{reductive amination}} \text{amine} & \text{classical resolution} & \text{amine} & \xrightarrow{\text{CRED + displacement}} & \text{ketone} \\
\end{align*}
\]

\[
\begin{align*}
\text{long term solution} & \\
0.5\% \text{CRED-A751} & \quad 0.5\% \text{GDH-102} & \quad \text{glucose} & \quad \text{NAD} & \quad 1) \text{Ms}_2\text{O}, \text{Et}_3\text{N} & \quad 2) \text{Phthalimide-K} & \quad 3) \text{N}_2\text{H}_4\cdot\text{H}_2\text{O} & \quad \text{amine-DH, NAD(P) NH}_4\text{Cl GDH/glucose} & \quad \text{ketone} \\
\end{align*}
\]
Desymmetrization route design: D-isovaline

Existing D-isovaline supply: too expensive, too low ee, under IP.
Initial attempts to establish quick Strecker+Resolution process failed.

Can we establish a desymmetrization process meeting requirements?
Can the strategy be applied to L-isomer and other amino acids?
Desymmetrization strategy worked - multi kg process implemented
High ee spec of client met via ee upgrade crystallisation

L-isomer accessible via ester switch:

The strategy has been applied to 5 other “exotic” amino acids.
Background:
- Client wanted enzymatic process to replace chiral SMB process for late phase and commercial API

Problems:
- Expensive racemate - yield matters a lot
- Hydrolase-1 had low E-value of 13 → (R)-diester yield < 30%
- Hydrolase-2 had low stability → high enzyme loading required with resulting isolation problems and yield impact

Solutions sought:
- Improve E-value of resolution step via substrate engineering
- Improve stability of desymmetrization enzyme via homologues panel
**Problems solved:**
- Hydrolase-1* has E-value of 60 with dipropyl ester → (R)-diester yield is now 45+%.
- Hydrolase-2 accepts (R)-propyl diester with unchanged selectivity.
- Hydrolase-2* found via homologues search. It was reported to be thermostable, and has a published crystal structure. At maintained selectivity the process now runs at 50 deg C with low enzyme loading.

**Thinking forward:**
- Solutions so far enable maintaining registration schedule but are not all we could wish for.
- Protein engineering of hydrolase-2* to open the way to 3rd generation single enzyme process.
Want single enzyme approach
Substrate docking and rational mutagenesis: 2 rounds of 20 completed
Greatly increased selectivity and catalytic rate
Mutations combined to hit target E value of >100
(R)-Quinuclidinol is key building block for commercial APIs.

Some APIs are becoming generic, increasing need for low cost BB supply.

Historically made by lipase resolution, but racemate is expensive.

ALMAC had investigated Noyori CTH approach, but ee was low, and so was yield after ee upgrade crystallization.
Our first generation CRED process had drawbacks: higher enzyme loading, low titre, tedious workup, high volume recrystallization, limited yield.

Also, our enzyme manufacturing cost was much higher back then.

Our second generation CRED process can now meet <<$1000/kg price target. Cost is dominated by ketone price.

This was enabled by co-expression of improved CRED and GDH, ability run high titre and now uses direct crystallisation.
Addressing inhibition via CRED engineering

- Substrate inhibition: nM affinity
- Molecular modelling, substrate docking, rational mutant selection, gene synthesis, wet screening
- Affinity reduced to mM range with 20 enzyme panel
- Inhibition eased, selectivity of enzyme retained.

Wild type: 88.9 nM
Variant: 12.7 mM
Fluorinated building blocks accessed with CRED technology

All ketones were reduced to both R- and S-alcohols with Almac’s CRED kit at >98% ee.

CREDs are particularly powerful for asymmetric reduction of trifluoromethyl-ketones, when Noyori-catalysis struggles to accept hydrated substrates.

Rowan et al. Tetrahedron: Asymmetry 2013, 24, 1369-1381
Novel access to C-F chirality

Selectively monofluorinating 1,3-dicarbonyl compounds is versatile, efficient and inexpensive.

See e.g. Hutchinson et. al., J. Fluorine Chem. 1998, 92, 45-52; Sandford et. al., Green Chem. 2015, 17, 3000-3009
Accessing C-F chirality – DRKR with model substrate

2-F-acetoacetate was investigated as model substrate.

Literature precedent is slim:

1x CRED published to produce 2R,3S-alcohol at 80% de/98% ee.

1x Pt-cat + quinine/quinidine published giving syn-alcohols at low de/ee and with by-products.
Accessing C-F chirality – DRKR with model substrate

200 off-the-shelf CREDs were screened.

All four stereoisomers can be accessed with high ee/de (not optimised).

Substrate scope investigation is in progress.
- Generic API = tight catalyst cost target
- High SM cost → high yield is critical for product cost
- CRED process offers superior selectivity over chemical reduction
- Co-solvent required for high space-time-yield
- Enzyme deactivation by co-solvent leads to reduced TON
- Enzyme loading reduced by extended co-solvent screen and introduction of improved GDH co-enzyme
- Co-expression of GDH and CRED has lowered enzyme cost per kg
- Low cost, long shelf life enzyme formulation: Spray-dried whole cells
- Going forward: continuous improvement of catalyst and process
A commercially viable biocatalyst is defined by:

**Performance**
- Turnover
- Selectivity
- Volume efficiency

**Supply**
- Cost
- Security
- Scale & Timing

**IP Issues**
- Right to use
- License agreement
- Simple business model
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