One-Flow, kick off meeting 17-1-2017

Enzymatic synthesis of Ursodeoxycholic acid

Prof. Isabel W.C.E. Arends

_in collaboration with Dr. Frank Hollmann, and Prof. Ulf Hanefeld_
Department of Biotechnology
From Industrial to Environmental Biotechnology

**Biocatalysis:** Green chemistry, enzymes as catalysts, novel enzymes, enzyme engineering

**Cell Systems Engineering:** Systems Biology, cell populations, analysis microbial cells

**Bioprocess Engineering:** HT measurements & modelling of biomolecular characteristics, hardware for bioconversion and bioseparation

**Industrial Microbiology:** Microbial performance. Integrating quantitative physiology and molecular biology; Metabolic engineering.

**Environmental Biotechnology:** Waste water treatment; Resource recovery from waste streams; Non-aspectic and inherently stable systems; Biofilms Microbial ecology.

**Biotechnology and Society:** Socially responsible innovation, communication and ethical aspects of biorenewable production.
Biocatalysis Group at Delft

New Enzymes
Tailored Enzymes
Tailored Processes
Integration in Organic Synthesis

Turning an enzyme into a catalyst!
Enzyme reactions not necessarily green
Research Questions

A. Enzymes with novel reactivities
   • Hydrogenases
   • Hydrolyases: C-O bond formation
   • Transketolases: C-C bond formation
   • Oxidoreductases and cofactor regeneration

B: Compatibility of enzymatic and/or chemocatalytic steps
   • Immobilization
   • Compartmentalization
   • Cascades
   • Hybrid enzymes
Ursodeoxycholic Acid

1. Secondary Bile Acid produced by intestinal bacteria

2. Used for treatment of cholestatic liver diseases, solubilizes cholesterol gallstones.

3. Current method multi-step chemical synthesis

4. Chemo-enzymatic synthesis promising alternative

Synthesis UDCA from cholic acid and analogues

CDCA and LCA possible alternatives as starting material (back-ups)

Chemical synthesis UDCA

(According to Hofmann 1963). Current chemical synthesis yield around 30%
Possible route for Chemo-enzymatic synthesis.

- Two-pot reaction in membrane reactors, followed by WK-reduction, in resp. 90 and 88% yld.
- HSDHs from *Clostridium*
- Enzymatic NADP(H) regeneration by ketoglutarate and glucose

Cascades and flow..
a marriage of convenience

- Cascade reactions
  - Linear, orthogonal, coupled (cofactor regeneration) or cyclic

- Flow reactions can assist in compatibility issues
  - Compartmentalization
  - Immobilization

- V. Hessel et al., ChemSusChem, 6, 746-789 (2013)
Enzyme catalysis in flow, benefits

- Expanded process windows
- Higher substrate loadings
- Lower product inhibition
- Shorter reaction times
- Increased enzyme lifetime
- Integrated product recovery
- Adjust reaction times for individual steps in time in order to respond to variation in enzyme activity.

Work package description
- 4yrs PD in Delft. Start apr 2017

**WP1**: Synthesis of UDCA
*Production, and testing of enzymes in separate steps.*
*Choice for best enzyme preparations (stability in solvents etc.)*
*NAD(P) oxidative regeneration step*
*Stabilisation/immobilisation of enzyme*

**WP2**: Shifting equilibrium in one-step epimerization step: One-pot two enzyme reaction. Choice of green spatial solvents

**WP4**: Integrated cascade in flow reactor.
*Choice for concept in flow cascade*
*Coupling with Wolff-Kirschner-reduction (green alternatives ?)*
*In addition, explore fully enzymatic synthesis. This involves dehydroxylation at the 12-position; bioinformatics needed to find the corresponding enzymes*
Fully enzymatic synthesis?

Dehydroxylation at C-12 has been observed in the whole cells preps with *Bacteroides* (*Ebenharder 1982*), but never reported afterwards.
Selected examples of research at the Biocatalysis group in Delft
Synthetic Cascades which are enabled by combining biocatalysts with artificial metalloenzymes

Cp*Ir(biot-p-L)Cl] within streptavidin affords an artificial transfer hydrogenase that is compatible with enzymes

Enzyme catalysed oxidations

- Cascades in order activate oxygen
- Enzyme instability issues
- Mass transfer limitations

Alcohol oxidations: oxidases
Oxygen insertion: peroxidases/peroxygenases


Non-conventional cofactor regeneration

Laccase Enzyme

 Activation by oxygen

 messenger molecule: TEMPO

One-electron oxidant

Alcohol Dehydrogenases for chiral oxidations

- Oxidation of racemic profen aldehydes
- Nonsteroidal anti inflammatory drugs
- Shift of equilibrium towards acid by using NADH regeneration
- Cofactor regeneration with oxidase.

Laccase and NADH recycling

Cascading two enzymes:
Laccase from *Myceliophoria thermophila*
ADH from *Thermus sp. ATN1*

Enzymatic cascade for *in situ* H$_2$O$_2$-generation with methanol. Scale-up of peroxygenases from fungi.

AOx alcohol oxidase from *Pichia pastoris* (*PpAOx*, black squares, ■)

AOx alcohol oxidase from *Candida boidinii* (*CbAOx*, red diamonds, ◆)

Methanol oxidation to formic acid: \(2 \text{H}_2\text{O}_2\)

**FDM:** formaldehyde dismutase from *Pseudomonas putida*

**Diagram:**
- The reaction pathway shows the conversion of methanol (MeOH) to formic acid (HCO\(_2\)H) using formaldehyde dismutase (FDM) and alcohol oxidase (AOx).
- The reaction involves the use of dioxygen (\(\text{O}_2\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)).

**Graphs:**
- The graphs illustrate the concentration of 1-phenylethanol (1-Phe) and product formation over time (h).
- The x-axis represents time in hours, ranging from 0 to 2.
- The y-axis represents concentration in mM.
- Data points show the concentration increase over time for AOx + FDM and AOx alone.

**Equation:**

\[2 \text{MeOH} + 2 \text{H}_2\text{O}_2 \rightarrow \text{HCO}_2\text{H} + 2 \text{H}_2\text{O} + \text{C}_{\text{1-phenylethanol}}\]
Methanol oxidation to CO$_2$: 3 H$_2$O$_2$

From methanol to formic acid: 2 H$_2$O$_2$

From formic acid to CO$_2$: 1 H$_2$O$_2$

Two-liquid-phase system

Higher substrate concentration and product stability for enzymatic oxidation

Selectivity = [1-phenylethanol] (mol) / [total products] (mol).

Selectivity 97%; ee>98%

(R)-1-phenylethanol (■,■)
acetophenone (△,△)
Photocatalytic NAD\(^+\) regeneration

- Fast kinetics
- Flavin true catalyst

\[ \text{TF(FMN)} = 237 \text{h}^{-1} \]

Photoenzymatic lactonization of diols

- Non-optimized conditions:
  - $\text{TTN(NAD)}=170$, $\text{TTN(FMN)}=340$
  - Full conversion

Carbonitrides active photocatalysts, even for hydroxylation.

W. Zhang, F. Hollmann
Cascade with water splitting catalysts
Taking electrons from sunlight

- OYE from *Thermus scotoductus*
- 323 K
- 10 g/l photocatalyst

Sunlight promotes yield in chloroperoxidase catalyzed sulfoxidation

Reaction on 25mL scale: (buffer pH5/BuOH)=3:1; T=25°C; [Thioanisol] = 50 mM; [CPO]=3.9 μM; [EDTA]=50 mM.

Chloroperoxidase loaded in polymersomes

Hans-Peter de Hoog
Collaboration Nijmegen-Delft (Nolte, Rowan, Cornelissen, van Hest)

Three-enzyme cascade in polymersome-loaded hydrogel reactor