Biocatalytic Approaches to Ketodiols and Aminodiols

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Advances in Biocatalysis
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BiCE Programme: Bioconversion Integrated with Chemistry and Engineering: New Routes and Tools for the Synthesis of Chiral Aminoalcohols

- GARY LYE/MARTINA MICHELETTI/NICHOLAS SZITA (MICROSCALE PROCESSING)
- JOHN WARD (METABOLIC ENGINEERING)
- FRANK BAGANZ (METABOLIC MODELLING)
- PAUL DALBY (ENZYME EVOLUTION)
- HELEN HAILES (CHEMISTRY)

Promising drug candidate

Analysis of chemical & biocatalytic options

Directed evolution

Metabolic engineering

Microwell/ Microfluidic studies

Modelling of operations and processes

Verification / refinement at large scale

Process for a new product

GARY LYE/JOHN WOODLEY (PROC. MODELLING)

GARY LYE/MARTINA MICHELETTI/NICHOLAS SZITA (MICROSCALE PROCESSING)
Aim

- To explore approaches to ketodiols and aminodiols

\[
\begin{align*}
\text{RCHO} + \text{HOOC} & \rightarrow \text{OH} \quad \text{TK} \\
\text{OH} & \rightarrow \text{OH} \\
\text{OH} & \rightarrow \text{OH} \\
\text{NH} & \rightarrow \text{NH}
\end{align*}
\]

Azasugars, e.g. mannojirimycin
\(X = \text{OH}\)

Oxazolines

Sphingosines

Antibiotics
\(\text{R} = \text{CHOCH(OH)CH(OH)}\)
\(\text{R} = \text{aryl}\)
\(\text{R} = \text{unsaturated alkyl chain}\)
BiCE Approach

- Directed evolution and metabolic engineering to enhance enzyme activity and performance and widen substrate specificity
- Scale-up studies and modelling
Transketolase (TK) (EC 2.2.1.1)

- Used in stereospecific carbon-carbon bond formation to give $\alpha,\alpha'$-dihydroxy ketones.
- *In vivo* TK catalyses the transfer of the C1-C2 ketol unit from D-xylulose-5-phosphate to D-ribose-5-phosphate.

\[ \text{D-xylulose 5-P} + \text{HCOOH} \rightarrow \text{D-ribose 5-P} + \text{Ketodiol} \]

- When $\beta$-hydroxypyruvic acid (HPA) is used as a donor the loss of CO$_2$, renders the reaction irreversible.

- *E. coli* transformant that over-expresses *E. coli* TK engineered at UCL.
- Propanal had been shown to be a substrate for *E. coli* TK, albeit a poor one.
- **Aims** To establish assays for use with non-$\alpha$-hydroxylated aliphatic substrates, identify TK mutants with improved performance and expand the substrate range.
Approach for Transketolase

- Development of assays to identify active mutants
- Synthesis of ketodiol (and aminodiol) standards for assay validation
Library Construction

- Structural library: residues within 4Å of substrate in TK.
- Discrete library for each residue
- Host strain- XL10( kan') Plasmid-pQR711

- Phylogenetic library (pLib): created to predict common ancestor to E.coli and S.cerevisiae TK

• Model of donor-substrate-TPP adjunct in active site of TK
Ketodiol Standards Needed

- For known TK substrates: use wild-type
- For new TK aldehyde donors: synthesis

\[ HO\rightarrow\text{TK}\rightarrow HO\]

\[ OH\rightarrow\text{TK}\rightarrow OH\]

\[ O\rightarrow\text{TK}\rightarrow O\]

\[ S-\text{isomer Wild-Type} \]

\[ S-\text{isomer Wild-Type} \]

\[ \text{Synthesis} \]

One-pot Synthesis of Ketodiols

During assay development ketodiol product observed with MOPS buffer in control reaction (no TK) and with no TPP/Mg$^{2+}$ present

Good aldehyde acceptor tolerance

Now have access to a range of ketodiols via this one-pot biomimetic reaction

**Colorimetric Assay for TK**

**Assay**

- Tetrazolium Red used to detect $\alpha$-hydroxycarbonyl product
- Hydroxypyruvate must be removed prior to addition of colour reagents - a scavenger resin (quaternary amine resin-Biotage)
- Test with 'wild-type' *E.coli* TK strain - detect >5-10% conversion to ketodiol
- Assay applicable for the screening of non-$\alpha$-hydroxylated aldehydes and used in 96-well format

**Reaction**

1. HPA PA
2. HPA PA
3. HPA PA
4. HPA PA

**No colour**

TK $\rightarrow$ Red Colour with Tetrazolium Red: need to remove HPA

**Red Colour**

Stereoselectivities: propanal

- TK highly enantioselective with $\alpha,\beta$-hydroxylated aldehydes
- Lit: $\text{L}$-erythrulose ($R = \text{CH}_2\text{OH}$) 64% ee ($S$) using spinach TK
- Enantioselectivities using TK from $E. \text{coli}$ and propanal?

**Assay required:**
- GC Supelco $\beta$-Dex 225 chiral column
- Assay scalable down to 96-well format

Absolute stereochemistry: propanal

- Use of Ender’s methodology

Glycolaldehyde Assay

\[
\text{Glycolaldehyde} + \text{Li-HPA} \xrightarrow{\text{TK}} \text{L-Erythrulose}
\]

- To determine ee-derivatisation to the acetate
- \(\text{Ac}_2\text{O/pyr}\) or other amine - polymerised material
- Acid catalysed esterification - racemic material
- Erbium triflate active acylation catalyst
- Chiral HPLC confirmed L-erythrulose ee (WT-TK) 95% (S-isomer)

\[
\text{HO} - \text{H} + \text{O} - \text{CO} - \text{OH} \xrightarrow{\text{Er(OTf)}_3, \text{Ac}_2\text{O}} \text{AcO} - \text{H} - \text{CO} - \text{OAc}
\]

17 h, 30%

Screening of TK Libraries: Propanal

D6
Asp469Ala

D(Asp)469 Library
• (S) -mutants = 69 hits
• Reversal of ee (R) = 14 hits
• Improved ee (S) = 1 hit
• ee knockouts = 13 hits
• Inactives = 0

H(His)26 Library
• (S)- mutants = 29 hits (0 hits >75% e.e.)
• (R)-selective mutants = 42 hits (4 hits > 75% e.e.)
• ee knockout = 12 hits
• Activity knockout = 13 hits

### TK Libraries: Propanal

<table>
<thead>
<tr>
<th>Mutation</th>
<th>e.e.</th>
<th>Activity (vs WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Asp469)</td>
<td>58% (S)</td>
<td>1</td>
</tr>
<tr>
<td>F11 Asp469Thr</td>
<td>64% (S)</td>
<td>4</td>
</tr>
<tr>
<td>E3 Asp469Glu</td>
<td>90% (S)</td>
<td>8</td>
</tr>
<tr>
<td>E8 Asp469Tyr</td>
<td>53% (R)</td>
<td>4</td>
</tr>
<tr>
<td>D6 Asp469Ala</td>
<td>33% (S)</td>
<td>5</td>
</tr>
<tr>
<td>D1 Asp469Ser</td>
<td>12% (S)</td>
<td>-</td>
</tr>
<tr>
<td>His26Tyr</td>
<td>88% (R)</td>
<td>3</td>
</tr>
</tbody>
</table>

Activity of WT TK for production of PKD is 0.05 μmol min\(^{-1}\) mg\(^{-1}\) determined using propanal (50 mM), HPA (50 mM), TPP (2.4 mM), Mg\(^{2+}\) (9 mM) in TRIS buffer (pH 7.0, 50 mM) at 25°C.
TK Libraries and cyclic aldehydes

- Acetaldehyde
- Triethylene glycol
- Cyclopentanone
- Benzaldehyde

TK

OH

OH

D469 Cyclopropanecarboxaldehyde Screen

D469 Cyclopentanecarboxaldehyde Screen
Modified Mosher’s Method: Absolute Stereochemistry

- WT-TK was used to synthesise (S)-PKD
- An NMR chiral assay for screening the absolute stereochemistry of ketodiols has been developed

- The absolute stereochemistry can be predicted from the chemical shift differences $\Delta \delta(\delta_{\text{low}} - \delta_{\text{high}})$ of the major peak for the geminal protons of the methylene attached to ester linkage in the MTPA derivatives.
- The (R)-MTPA ester and the (S)-MTPA ester predict a 58% ee of the (S)-isomer for WT-TK

J. L. Galman, H. C Hailes, in preparation
Transaminase

- Amine donor required e.g. $S-\alpha$-methylbenzylamine (MBA) which generates acetophenone

$$\begin{align*}
\text{Amine donor} & \rightarrow \text{Ketone} \\
\text{Transaminase (TAm)} & \rightarrow \text{Amine}
\end{align*}$$

PLP = Pyridoxal-5-phosphate
New ω-TAmS obtained

• BLAST search using the *V. fluvialis* sequence

- 8 MBA:pyr TAmS (highlighted in yellow)
- 3 β-ala:pyr TAmS (highlighted in green) were tested for ketodiol conversion.
Erythulose

PKD

BKD

Reaction profiles of 3 His-tagged and purified enzymes (CV2025, Sav2612 and PP5182) were measured for conversion of the ketodiols.

• Literature postulates the amination results in a S-product
• Supported by observation that CV2025 only accepts S-(α)-methylbenzylamine
• After TAm step one product by LC-MS, two by HPLC
• Possibly due to a rearrangement in the chemical step or diastereoisomers in TAm step.
• Four isomer mix of products synthesised

Aromatic aminodiols

- 1,2-ketodiol prepared and not bioconverted to aminodiol

- \((1R,2S)\)-Anti-aminodiol prepared in 70% \(de\): (epimer formed 2nd step)

Aromatic aminodiols: Stereoselectivity

- Non-chiral HPLC confirmed that ketodiols give a mixture of \textit{syn} and \textit{anti}-products in 1:1 ratio
- Chiral HPLC established (1\textit{S},2\textit{S}) and (1\textit{R},2\textit{S}) formed in reaction
- \(\omega\)-TAm able to accept both ketodiols-useful in synthesis
- Highly stereoselective in TAm step

\[ \begin{align*}
\text{C. violaceum } \omega\text{-TAm} & \quad \text{PLP, HEPES (pH 7.5)} \\
\end{align*} \]
• TK mutants that convert non-hydroxylated aldehydes into ketodiols with good activities.
• Single point active site TK mutants that generate ketodiols with both (S)- and (R)-enantioselectivities.
• Identification of TK mutants that accept cyclic aldehydes.
• Recruitment of $\omega$-TAms that convert ketodiols to aminodiols in high yields and selectivities ((S)- and (R)-ketodiols accepted).
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