Biocatalytic Approaches to Ketodiols and Aminodiols

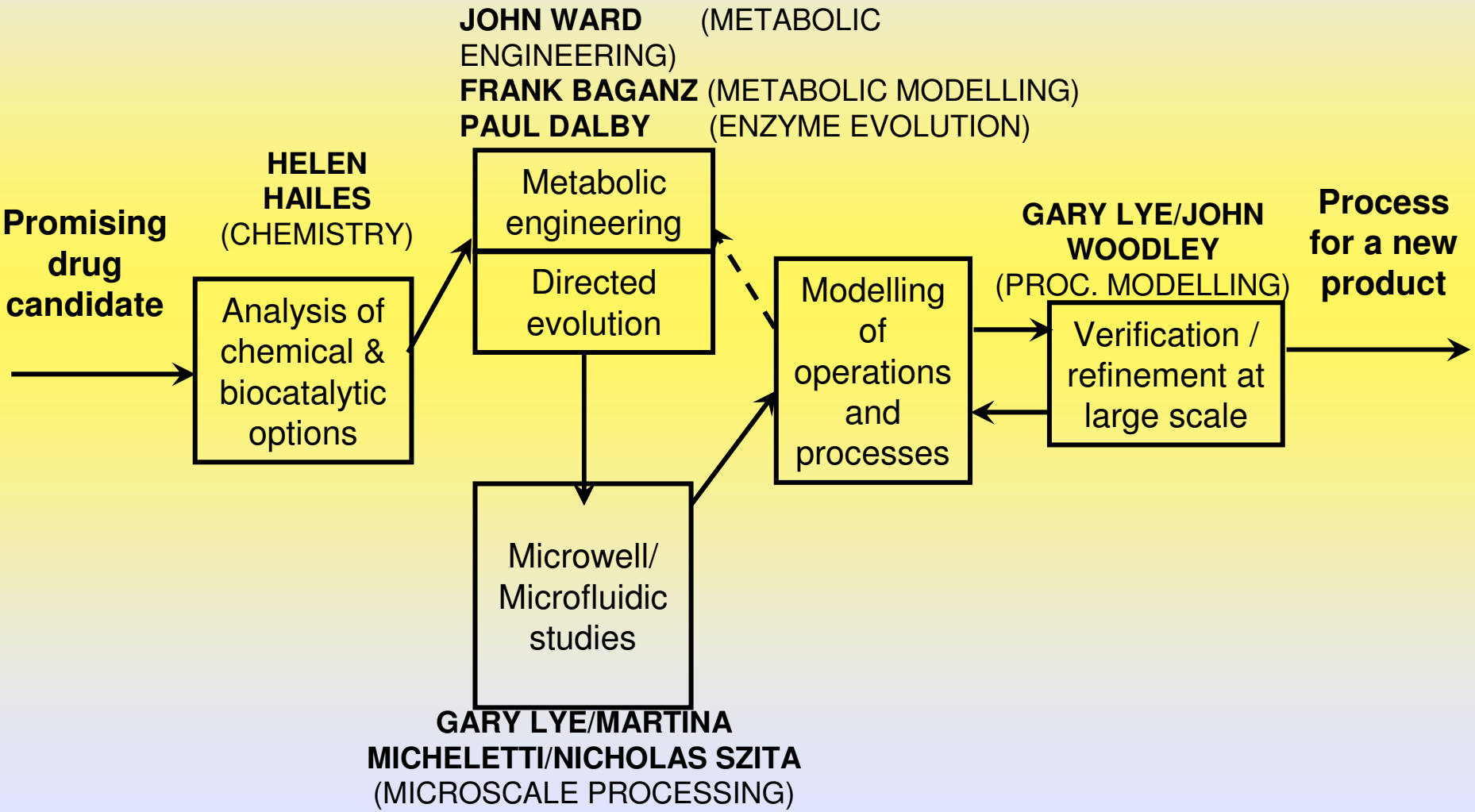
Helen C. Hailes
Department of Chemistry, University College London

Advances in Biocatalysis

UCL 21st April 2009

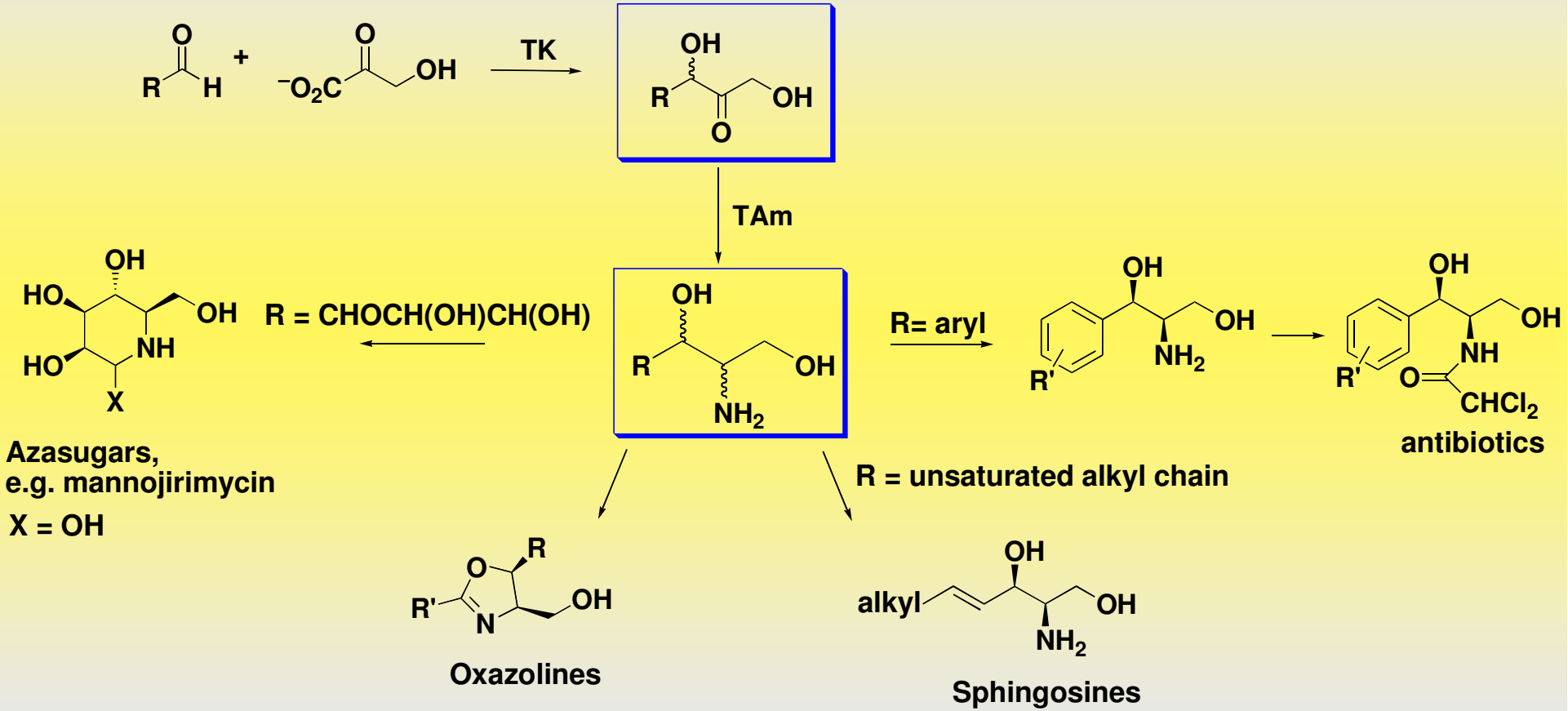
BiCE Programme

Bioconversion **I**ntegrated with **C**hemistry and **E**ngineering:
New Routes and Tools for the Synthesis of Chiral Aminoalcohols

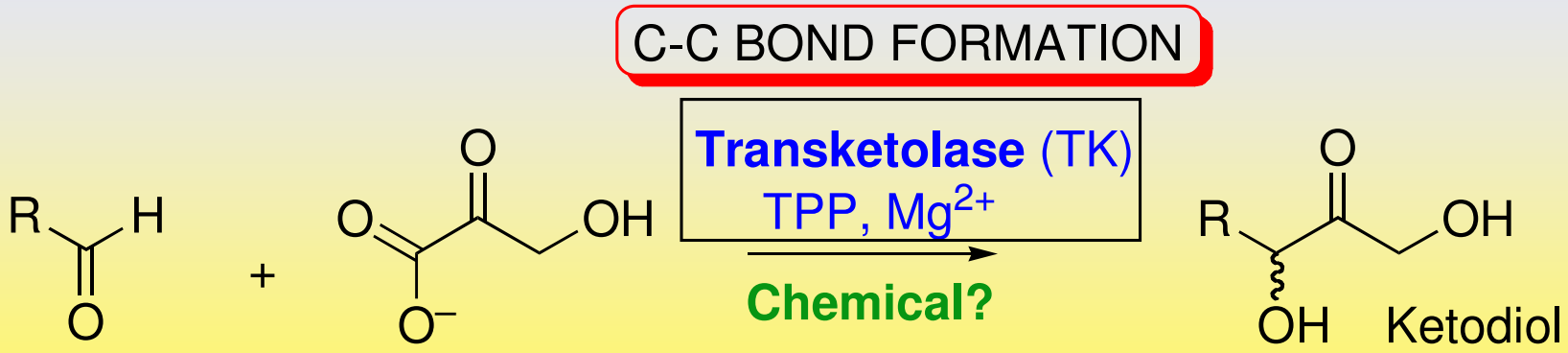


Aim

- To explore approaches to ketodiols and aminodiols



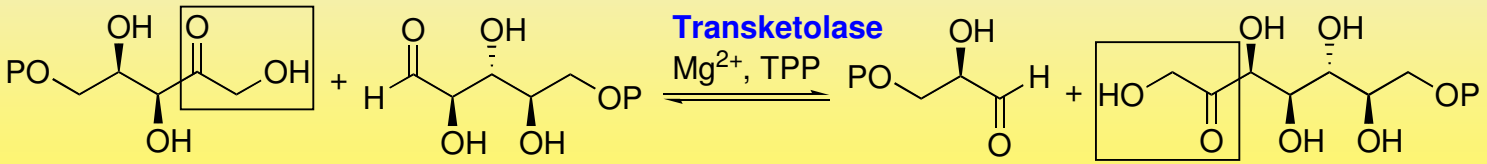
BiCE Approach



- Directed evolution and metabolic engineering to enhance enzyme activity and performance and widen substrate specificity
- Scale-up studies and modelling

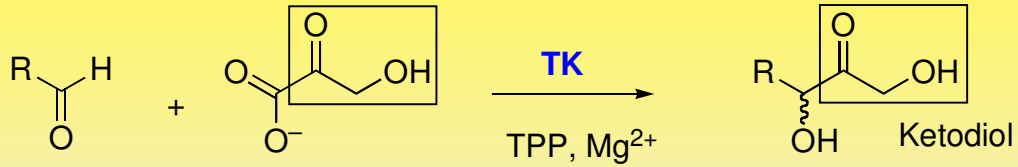
Transketolase (TK) (EC 2.2.1.1)

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



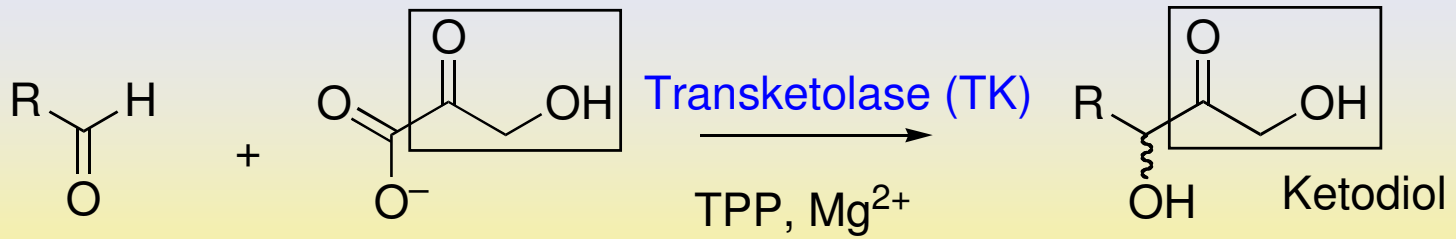
D-xylulose 5-P **D-ribose 5-P**

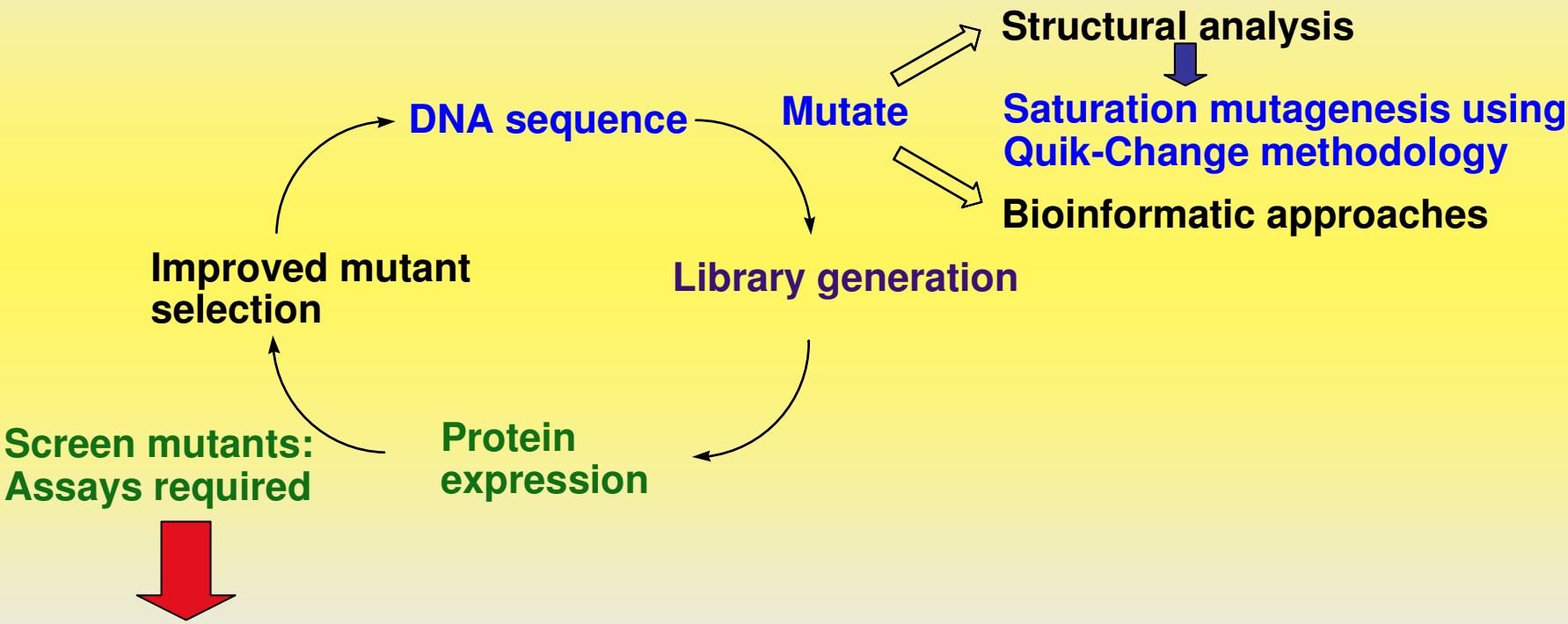
- When β-hydroxypyruvic acid (HPA) is used as a donor the loss of CO_2, renders the reaction irreversible.



- *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-α-hydroxylated aliphatic substrates, identify TK mutants with improved performance and expand the substrate range

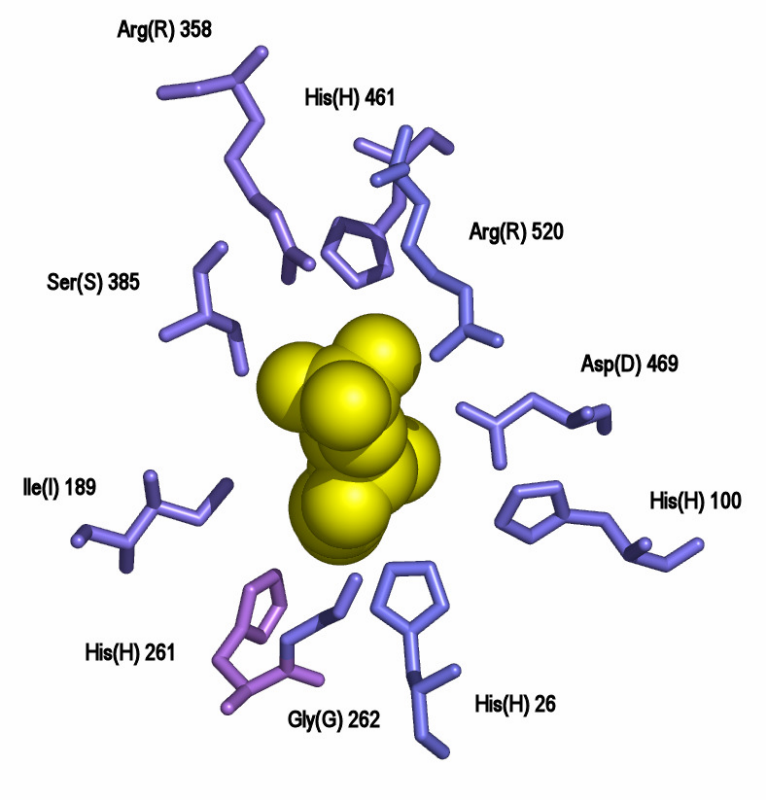
Approach for Transketolase

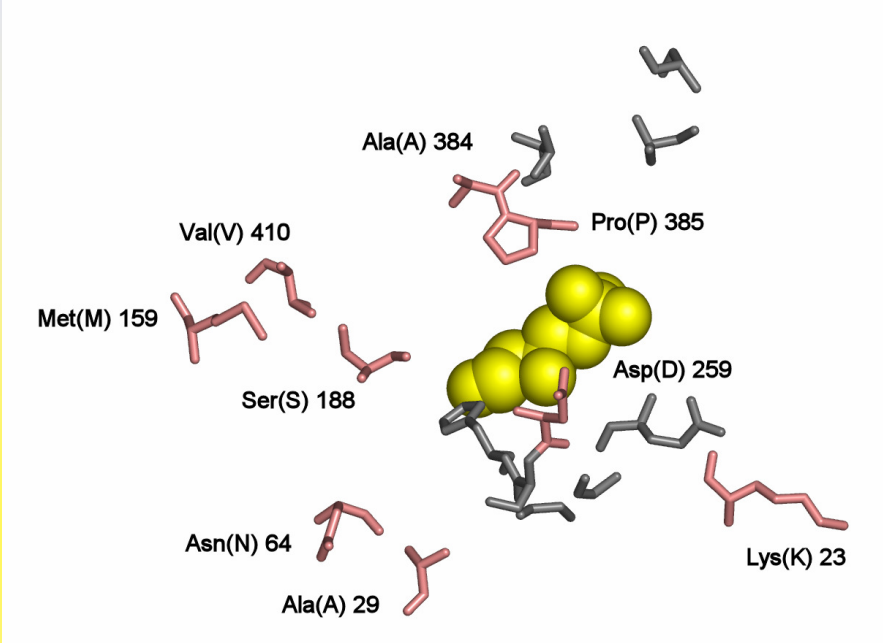




- Development of assays to identify active mutants
- Synthesis of ketodiol (and aminodiol) standards for assay validation

Library Construction





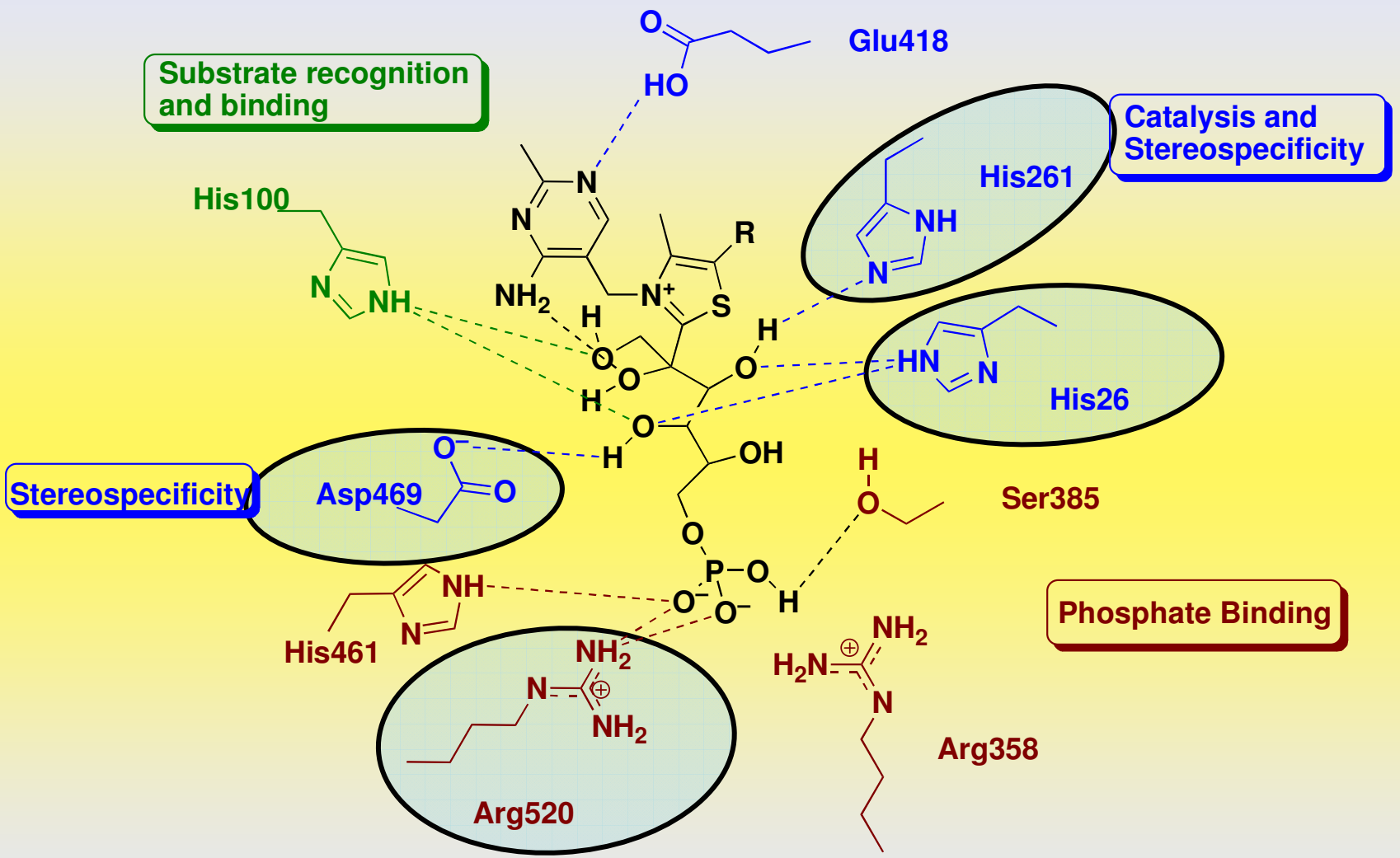
Grey = residues forming 4Å library
Pink = residues forming phylogenetic library

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

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

J. A. Littlechild *et al, Acta Cryst. D*, **1995**, *51*, 1074; Schneider *et al, FEBS Lett.*, **1998**, *424*, 49 and *J. Mol. Biol.*, **1994**, *238*, 387; P. A. Dalby *et al, J. Biotech.*, **2007**.

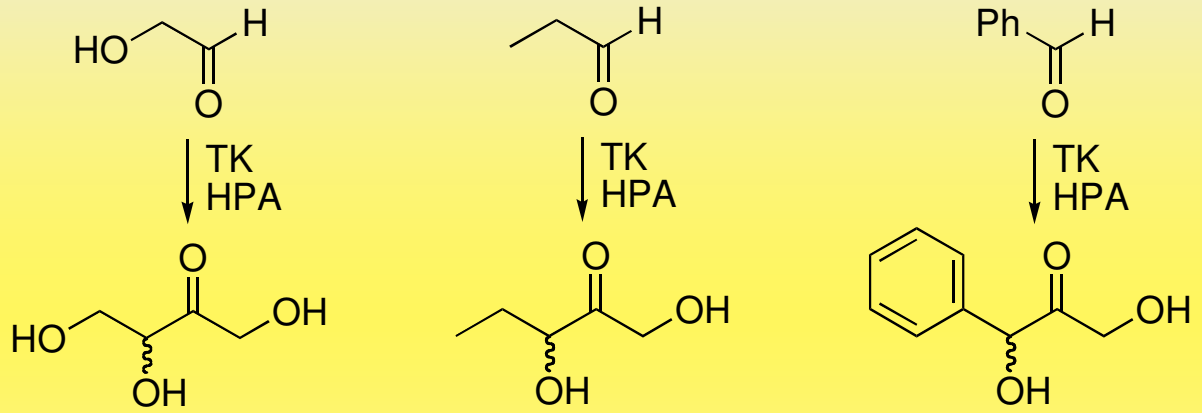
Structural Library Target Residues



- Model of donor-substrate-TPP adjunct in active site of TK

Ketodiol Standards Needed

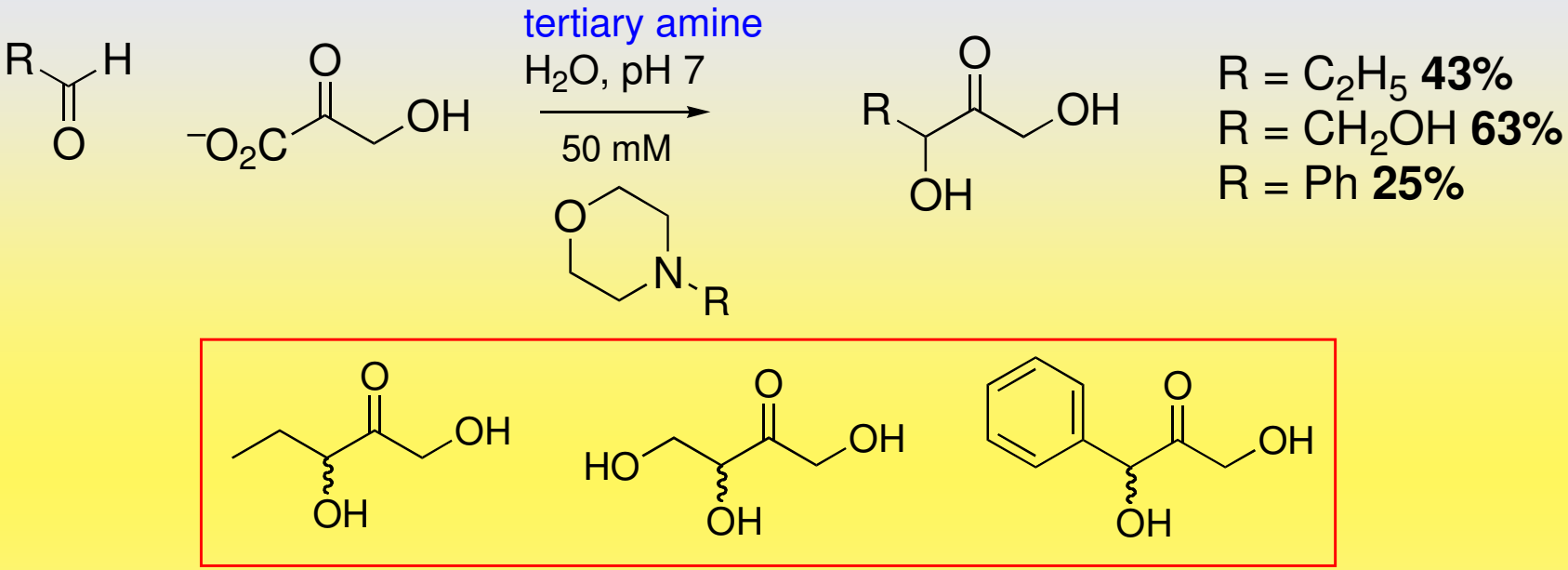
- For known TK substrates: use wild-type
- For new TK aldehyde donors: synthesis



E.coli TK Wild-Type (relative reactivities)	**Yes (100)**	**Yes (20)**	**No**
	***S*-isomer Wild-Type**	***S*-isomer Wild-Type**	**Synthesis**

5 steps: Fetizon *et al*. *Tetrahedron Lett.*, **1985**, *26*, 4925

One-pot Synthesis of Ketodiols



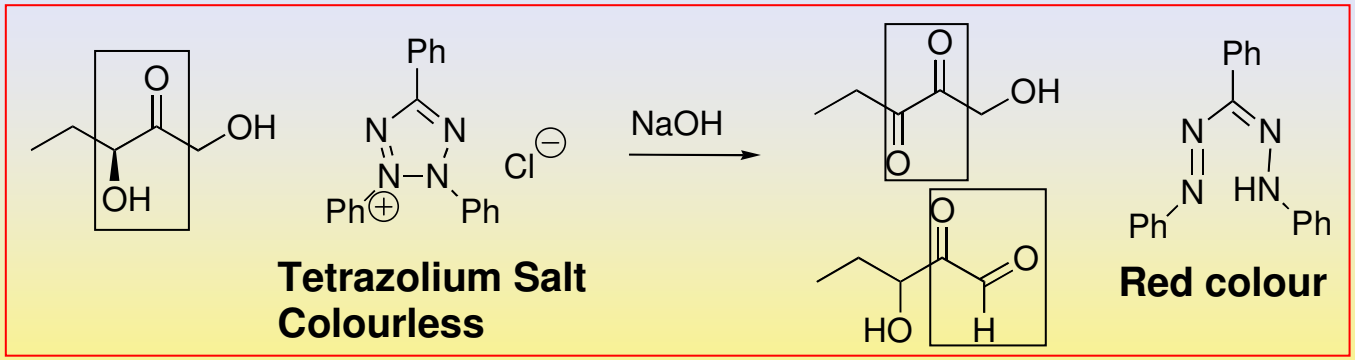
- During assay development ketodiol product observed with MOPS buffer in control reaction (no TK) and with no TPP/Mg^{2+} present
- Good aldehyde acceptor tolerance
- Now have access to a range of ketodiols via this one-pot biomimetic reaction

M. E. B. Smith, K. Smithies, T. Senussi, P. A. Dalby, and H. C. Hailes, *Eur. J. Org. Chem.* **2006**,1121

Colorimetric Assay for TK

UCL

Assay

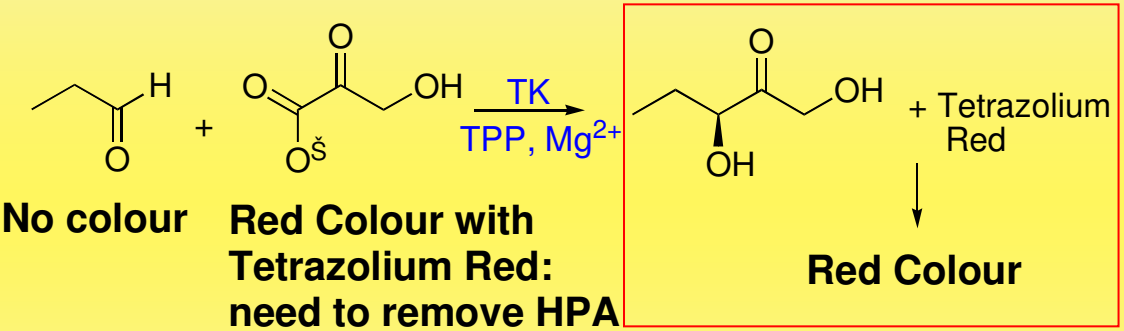


Tetrazolium Salt Colourless

Red colour

Reaction

1	2	3	4
HPA	HPA	HPA	PA
PA	PA	TPP/Mg^{++}	TPP/Mg^{++}
TPP/Mg^{++}	TPP/Mg^{++}	TK	TK
TK			



No colour

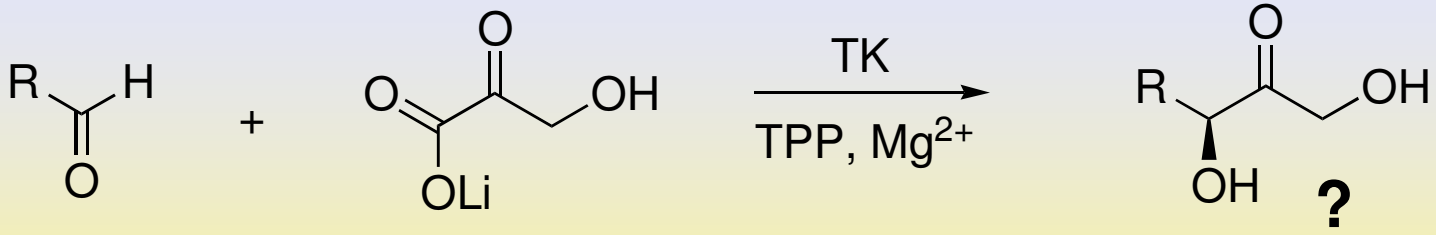
Red Colour with Tetrazolium Red: need to remove HPA

Red Colour

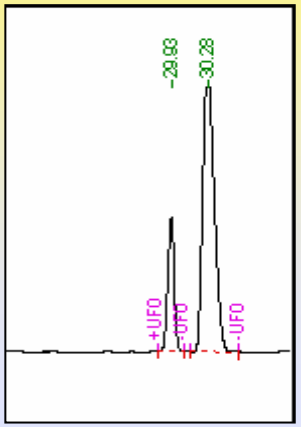
- Tetrazolium Red used to detect α-hydroxycarbonyl product
- Hydroxypyruvate must be to 'removed' prior to addition of colour reagents- a scavenger resin (quaternary amine resin-Biotage)
- Test with 'wild-type' *E.coli* TK strain- detect >5-10% conversion to ketodiol
- Assay applicable for the screening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

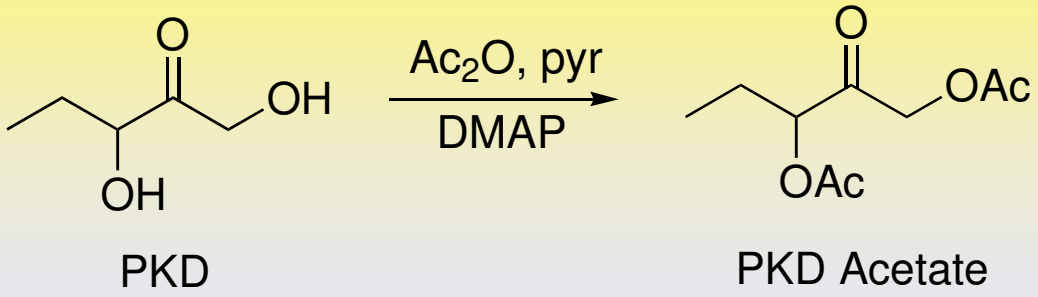
M. E. B. Smith, U. Kaulmann, J. M. Ward, and H. C. Hailes, *Bioorg. Med. Chem.* **2006**, *14*, 7062

Stereoselectivities: propanal



- TK highly enantioselective with α,β-hydroxylated aldehydes
- Lit: L-erythrulose (R = CH_2OH) 64% *ee* (*S*) using spinach TK
- Enantioselectivities using TK from *E. coli* and propanal?
- **Assay required:**
  - GC Supelco β-Dex 225 chiral column
  - Assay scalable down to 96-well format





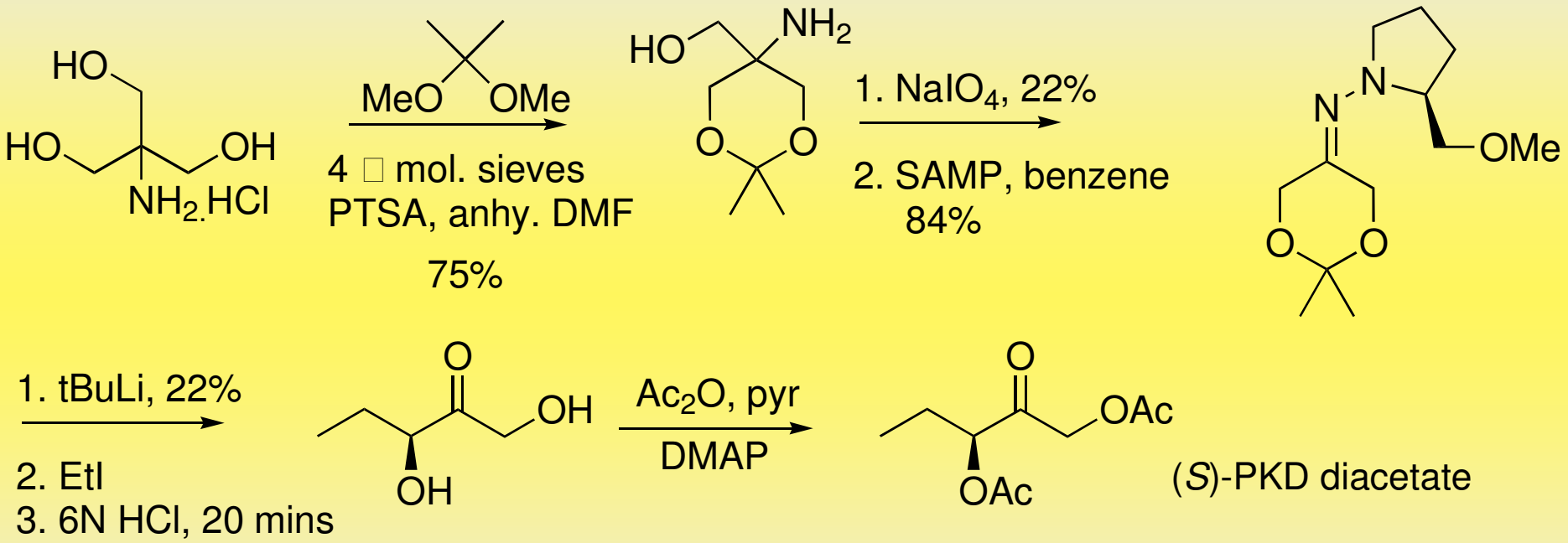
PKD

PKD Acetate

M. E. B. Smith, E. G. Hibbert, A. B. Jones, P. A. Dalby and H. C. Hailes, *Adv. Syn. Cat.*, **2008**, *350*, 2631–2638

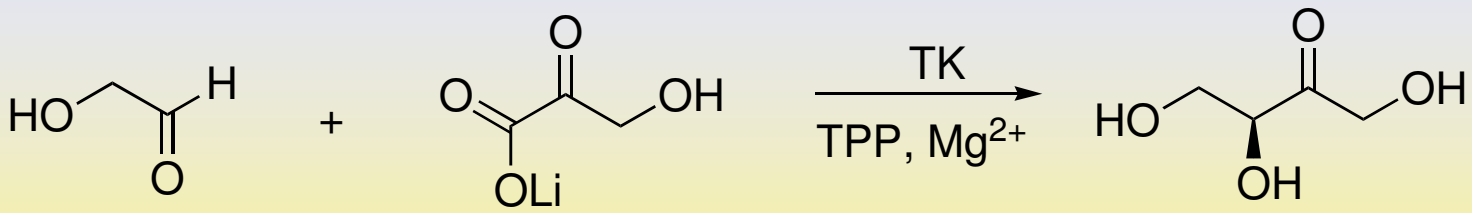
Absolute stereochemistry: propanal

- Use of Ender's methodology



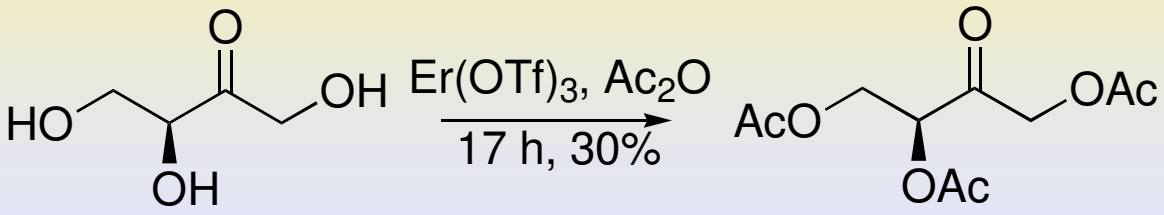
M. E. B. Smith, E. G. Hibbert, A. B. Jones, P. A. Dalby and H. C. Hailes, *Adv. Syn. Cat.*, **2008**, *350*, 2631–2638

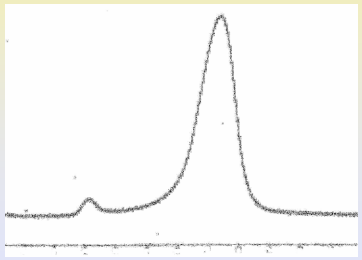
Glycolaldehyde Assay



Glycolaldehyde **Li-HPA** **L-Erythrulose**

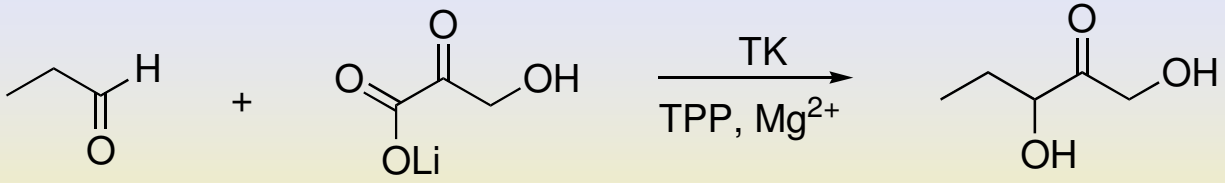
- To determine *ee*-derivatisation to the acetate
- Ac_2O/pyr or other amine - polymerised material
- Acid catalysed esterification - racemic material
- Erbium triflate active acylation catalyst
- Chiral HPLC confirmed L-erythrulose *ee* (WT-TK) 95% (*S*-isomer)





M. E. B. Smith, E. G. Hibbert, A. B. Jones, P. A. Dalby and H. C. Hailes, *Adv. Syn. Cat.*, **2008**, *350*, 2631–2638

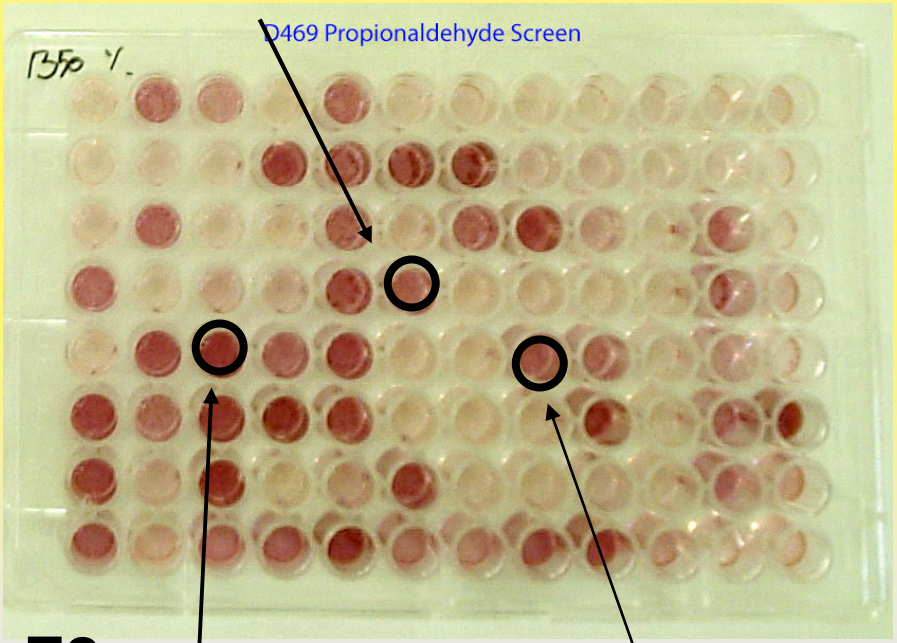
Screening of TK Libraries: Propanal



Li-HPA

Propanal ketodiol (PKD)

D6
Asp469Ala



E3
Asp469Glu

E8, Asp469Tyr

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

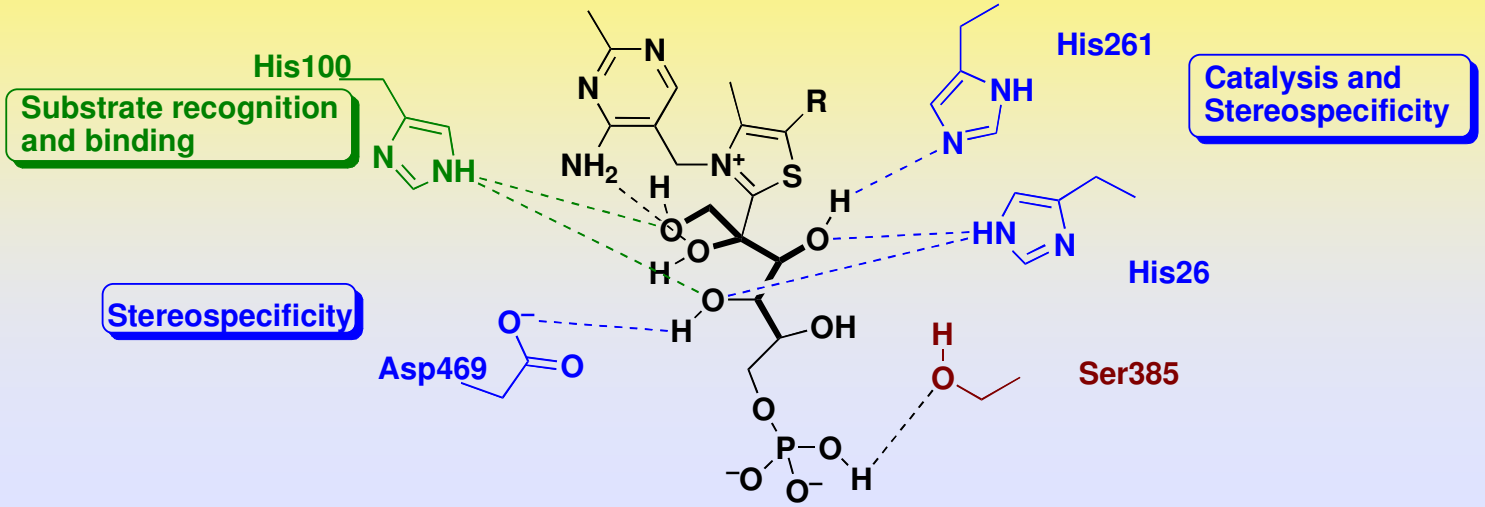
- (*S*)- mutants = 29 hits (0 hits >75% e.e.)
- (*R*)-selective mutants = 42 hits (4 hits > 75% e.e.)
- *ee* knockout = 12 hits
- Activity knockout = 13 hits

E. G. Hibbert, et al., *J. Biotechnology,* **2008**, *134,* 240–245; M. E. B. Smith, et al., *Adv. Syn. Cat.*, **2008**, *350*, 2631–2638

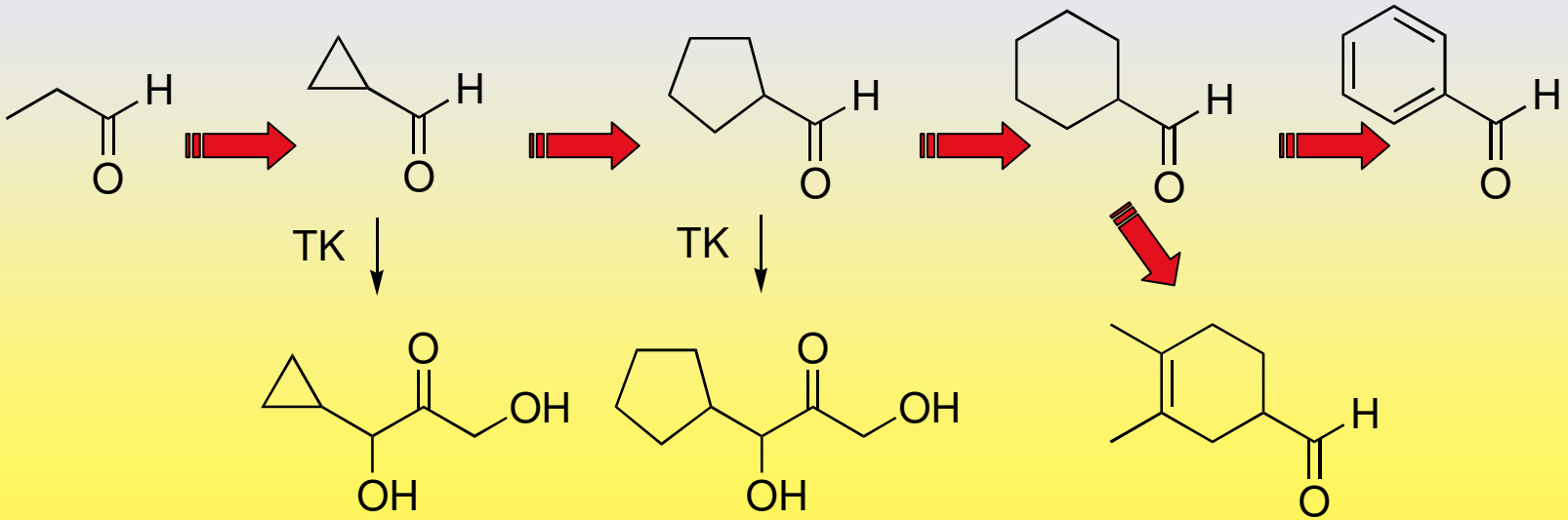
TK Libraries: Propanal

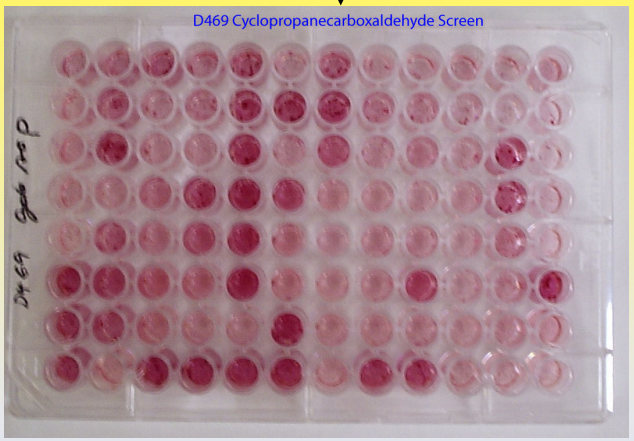
Mutation	e.e.	Activity (vs WT)
WT (Asp469)	**58% (*S*)**	1
F11 Asp469Thr	64% (*S*)	4
E3 Asp469Glu	**90% (*S*)**	8
E8 Asp469Tyr	53% (*R*)	4
D6 Asp469Ala	33% (*S*)	5
D1 Asp469Ser	12% (*S*)	-
His26Tyr	**88% (*R*)**	3

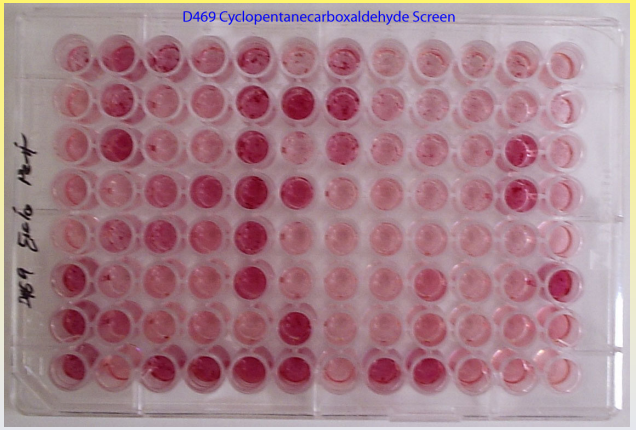
Activity of WT TK for production of PKD is 0.05 μmol min^{-1} mg^{-1} determined using propanal (50 mM), HPA (50 mM), TPP (2.4 mM), Mg^{2+} (9 mM) in TRIS buffer (pH 7.0, 50 mM) at 25 °C.



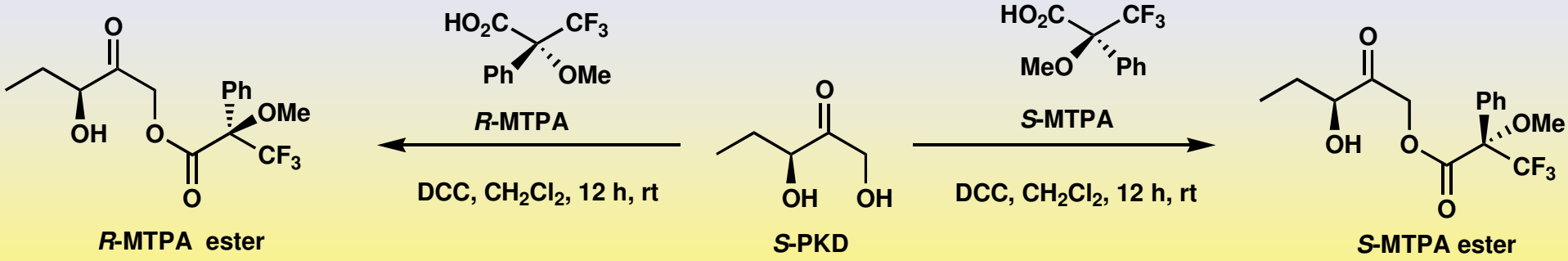
TK Libraries and cyclic aldehydes

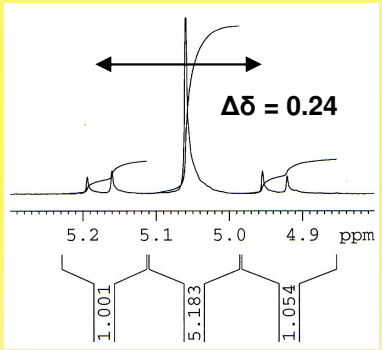




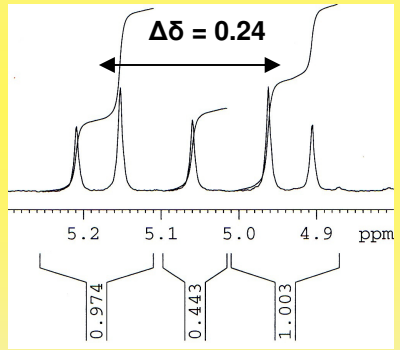


Modified Mosher's Method: Absolute Stereochemistry





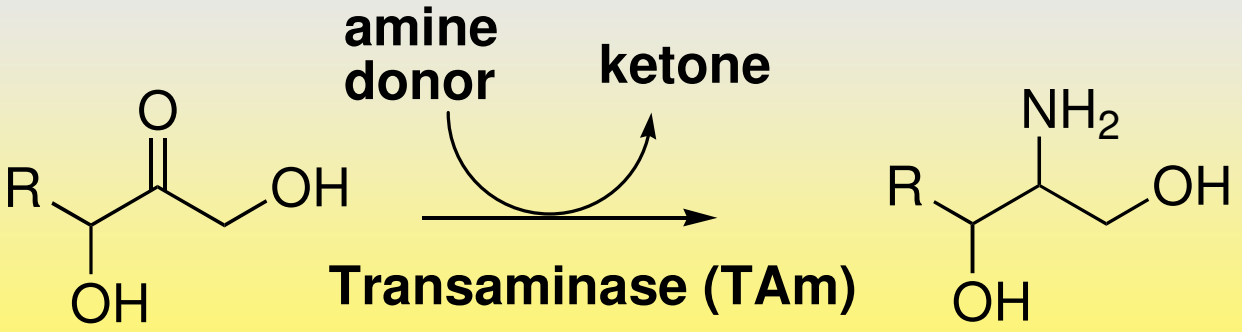
- WT-TK was used to synthesise (*S*)-PKD
- An NMR chiral assay for screening the absolute stereochemistry of ketodiols has been developed

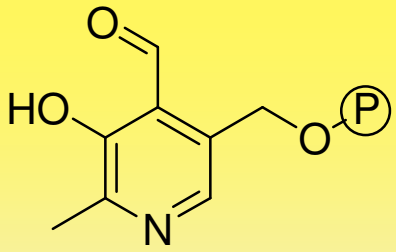


- The absolute stereochemistry can be predicted from the chemical shift differences $\Delta\delta(\delta_{low} - \delta_{high})$ of the major peak for the geminal protons of the methylene attached to ester linkage in the MTPA derivatives.
- The (*R*)-MTPA ester and the (*S*)-MTPA ester predict a 58%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

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

Transaminase



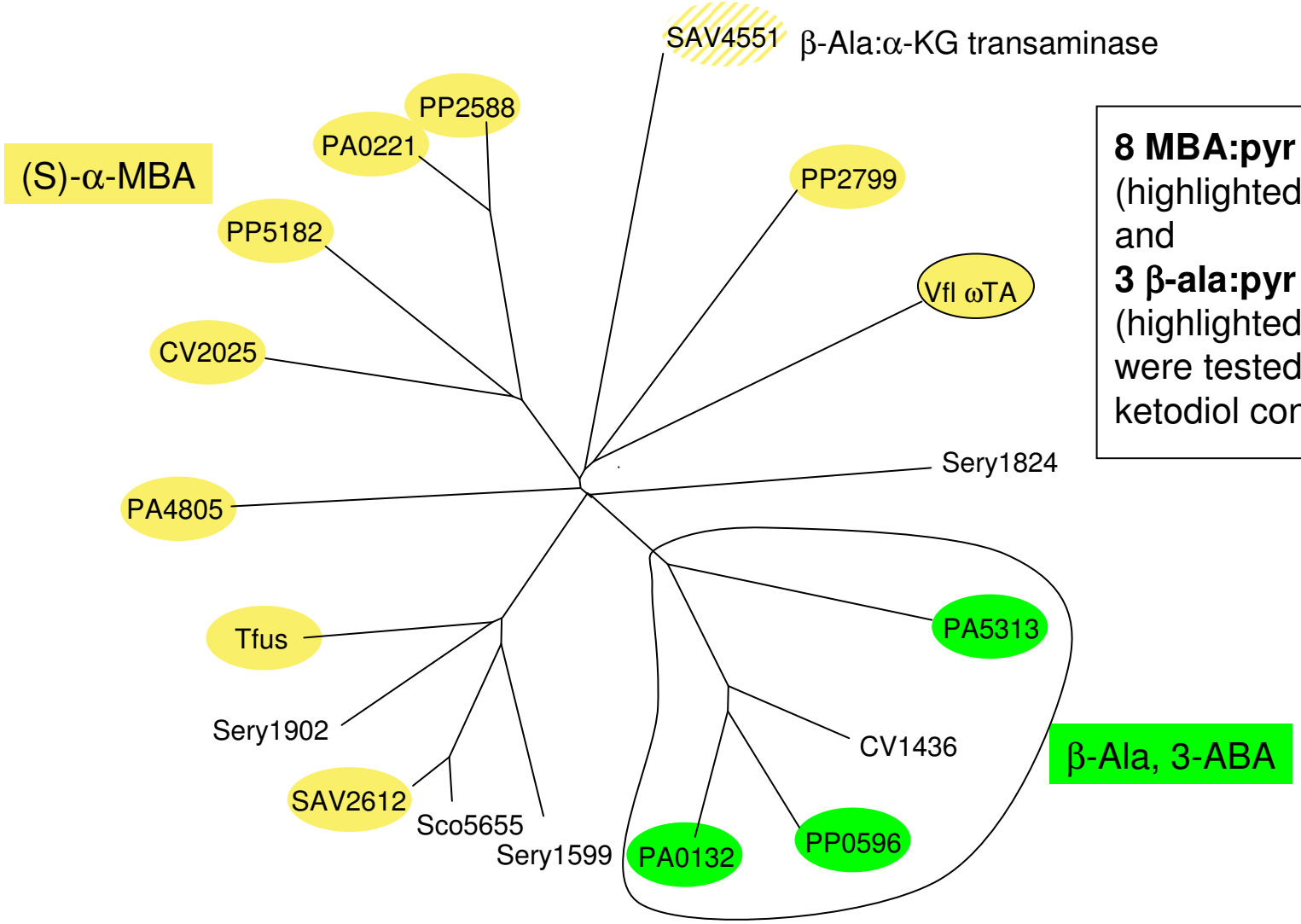


PLP = Pyridoxal-5-phosphate

- Amine donor required e.g. *S*-α-methylbenzylamine (MBA) which generates acetophenone

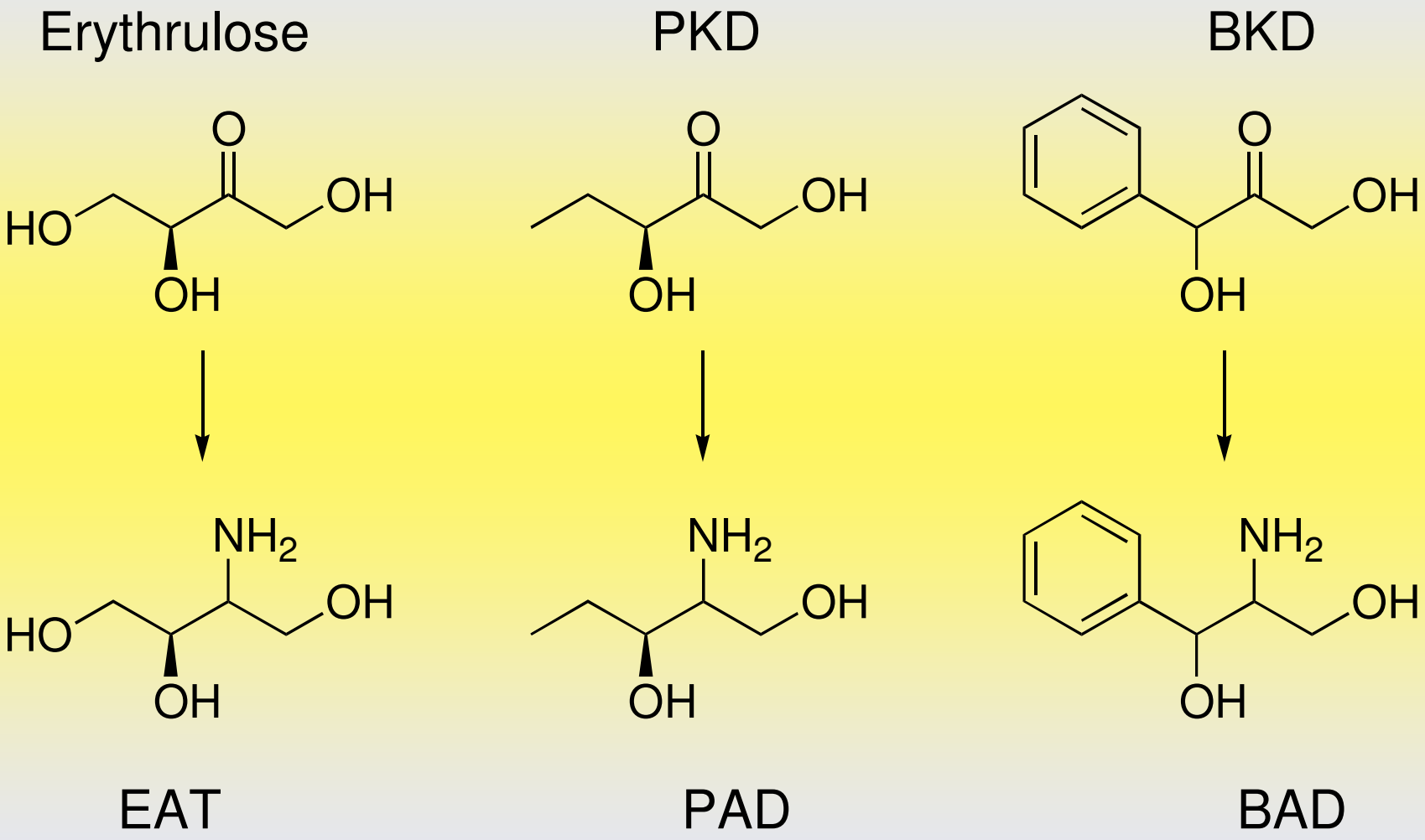
New ω-TAms obtained

- **BLAST search using the *V. fluvialis* sequence**



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

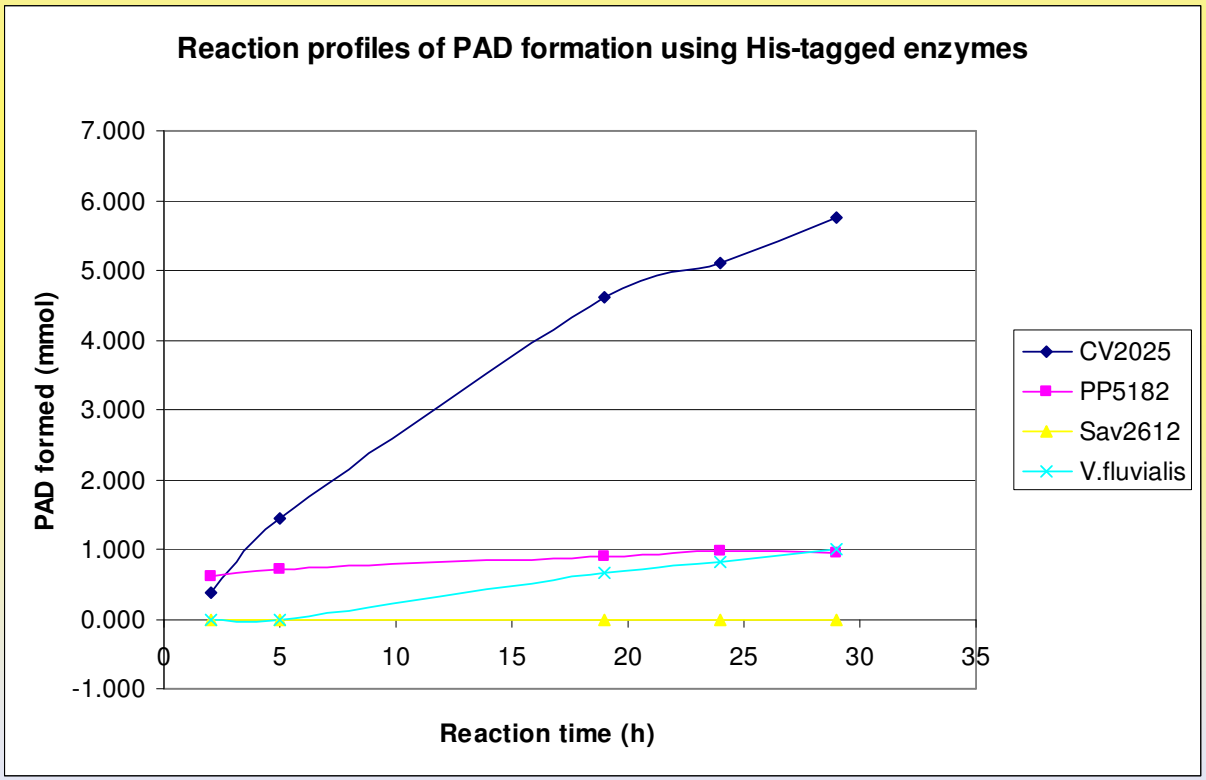
Key Transaminase Reactions

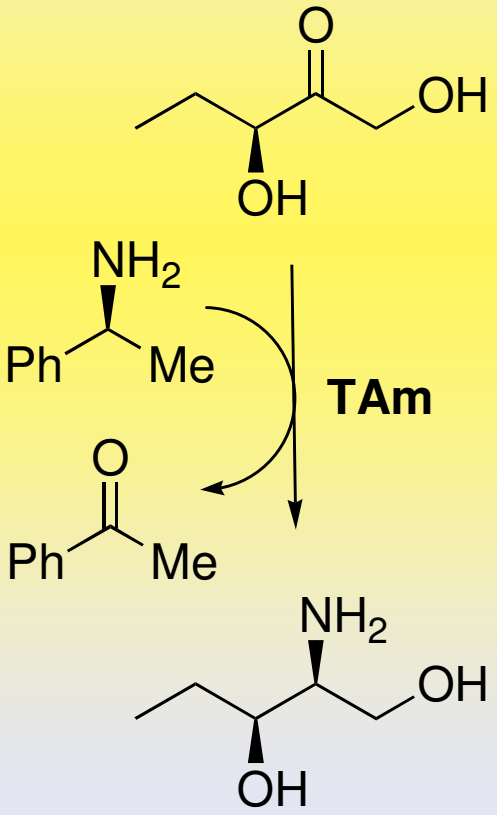


EAT: C. U.Ingram, M. Bommer, M. E. B. Smith, P. A. Dalby, J. M. Ward, H. C. Hailes, and G. J. Lye, *Biotech. Bioeng.* **2007**, *96,* 559–569.

Aliphatic Ketodiol

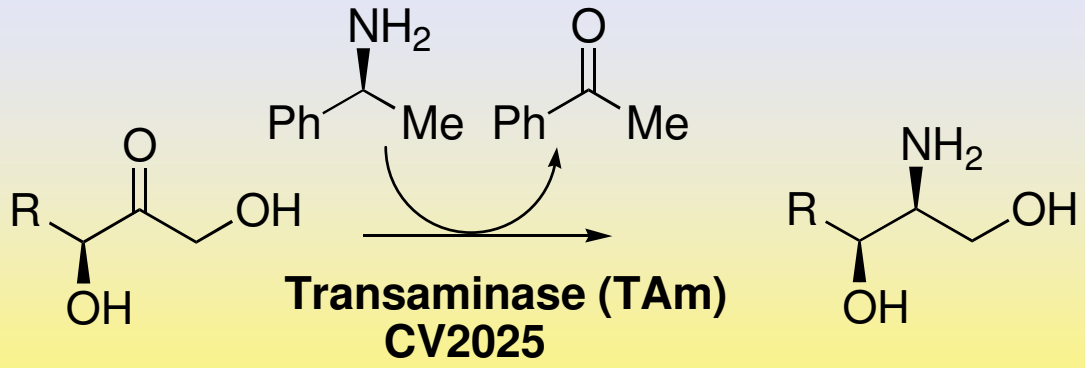
- Reaction profiles of 3 His-tagged and purified enzymes (CV2025, Sav2612 and PP5182) were measured for conversion of the ketodiols



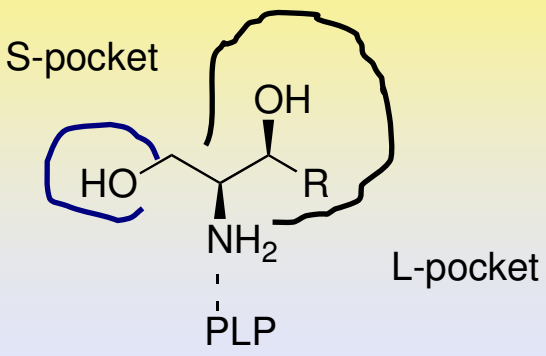


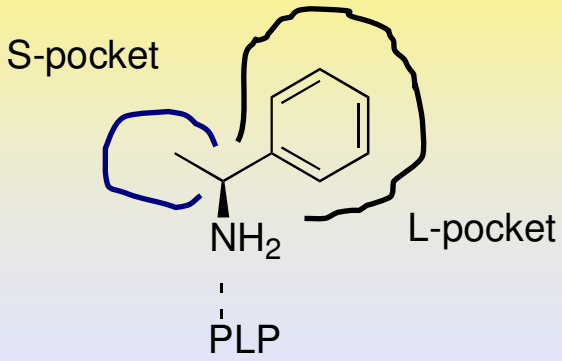
U. Kaulmann, K. Smithies, M. E. B. Smith, **H. C. Hailes**, and J. M. Ward, *Enz. Microb. Tech.,* **2007***, 41,* 628

TAm selectivity



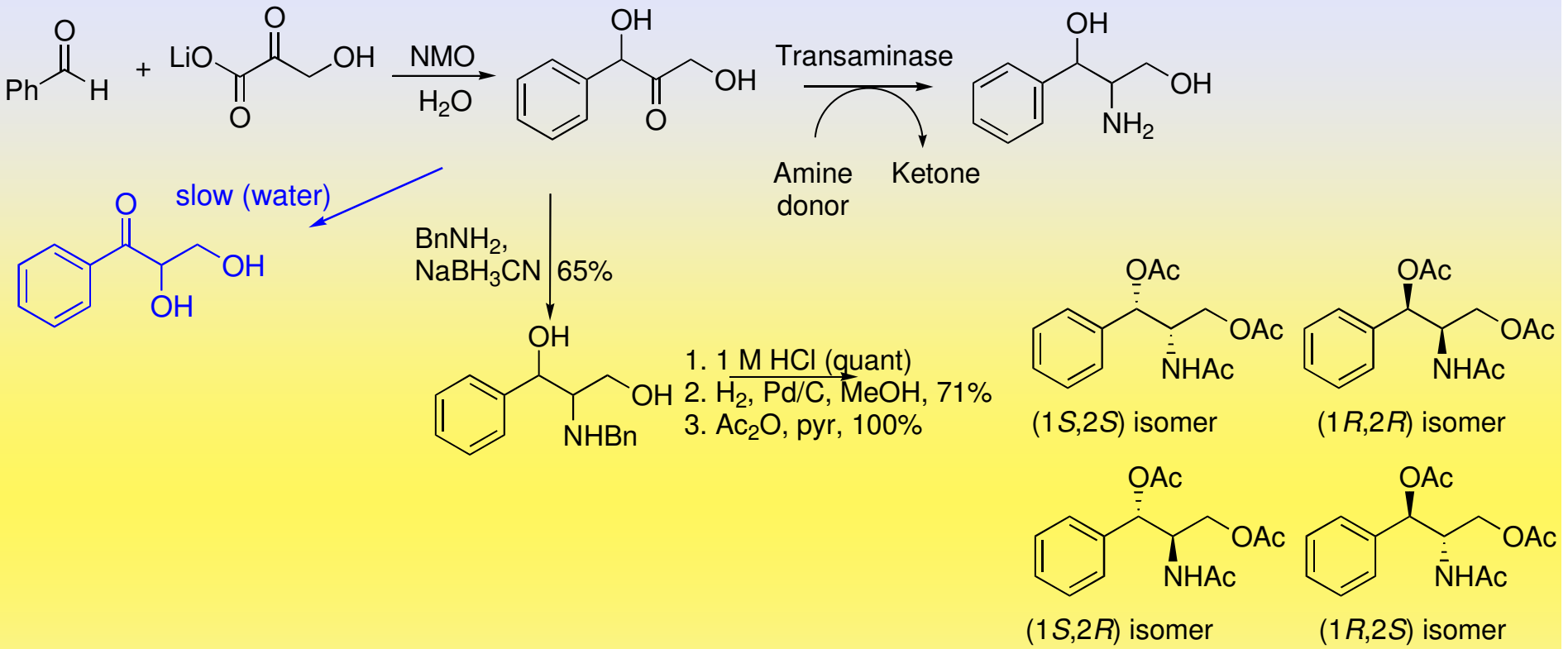
- Literature postulates the amination results in a *S*-product
- Supported by observation that CV2025 only accepts *S*-(α)-methylbenzylamine





L (large)-pocket: accepts carboxy, aromatic, larger aliphatic groups
S (small)-pocket: sterically constrained

Aromatic aminodiols-stereoselectivity

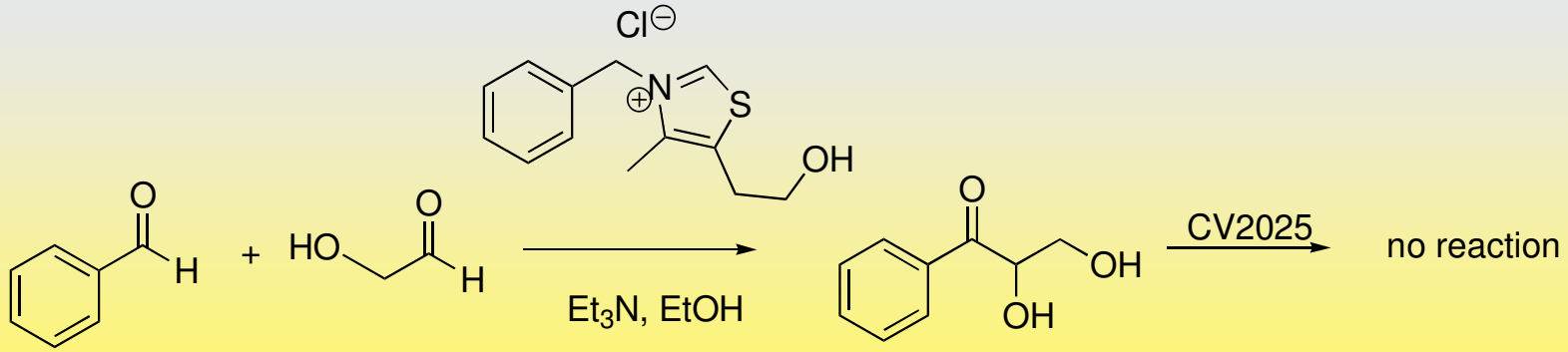


- After TAm step one product by LC-MS, two by HPLC
- Possibly due to a rearrangement in the chemical step **or** diastereoisomers in TAm step.
- Four isomer mix of products synthesised

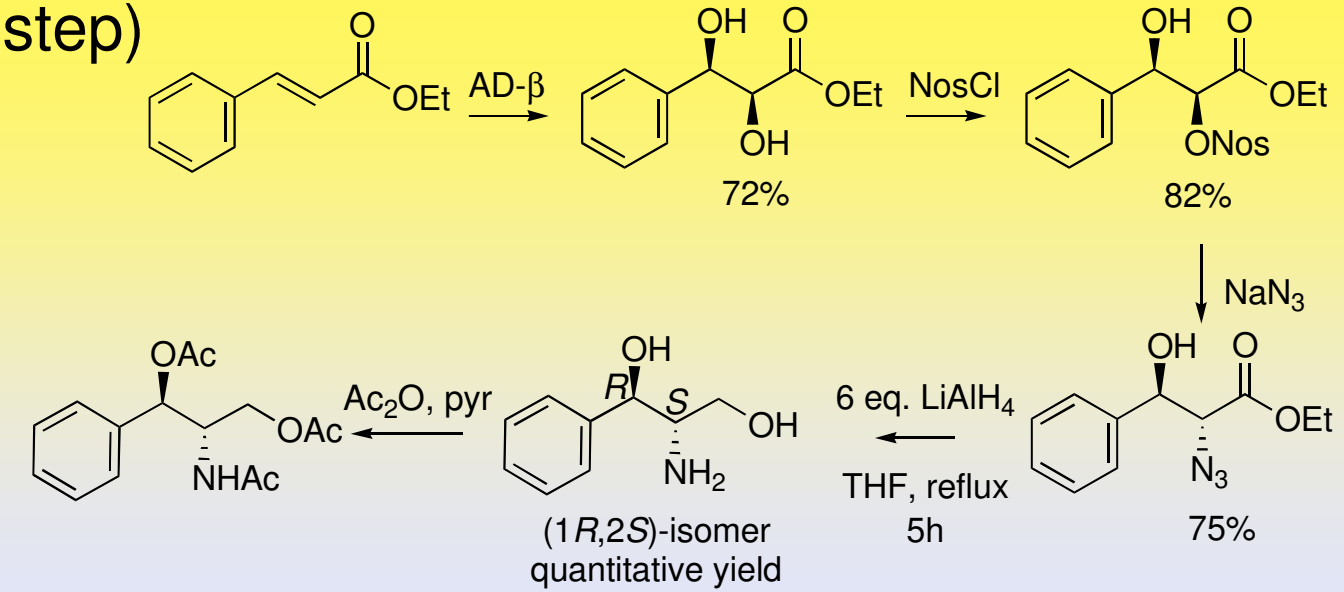
K. Smithies, M. E. B. Smith, U. Kaulmann, James L. Galman, J. M. Ward, H. C. Hailes, *Tetrahedron: Asymm.* **2009**, doi:10.1016/j.tetasy.2009.03.012

Aromatic aminodiols

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



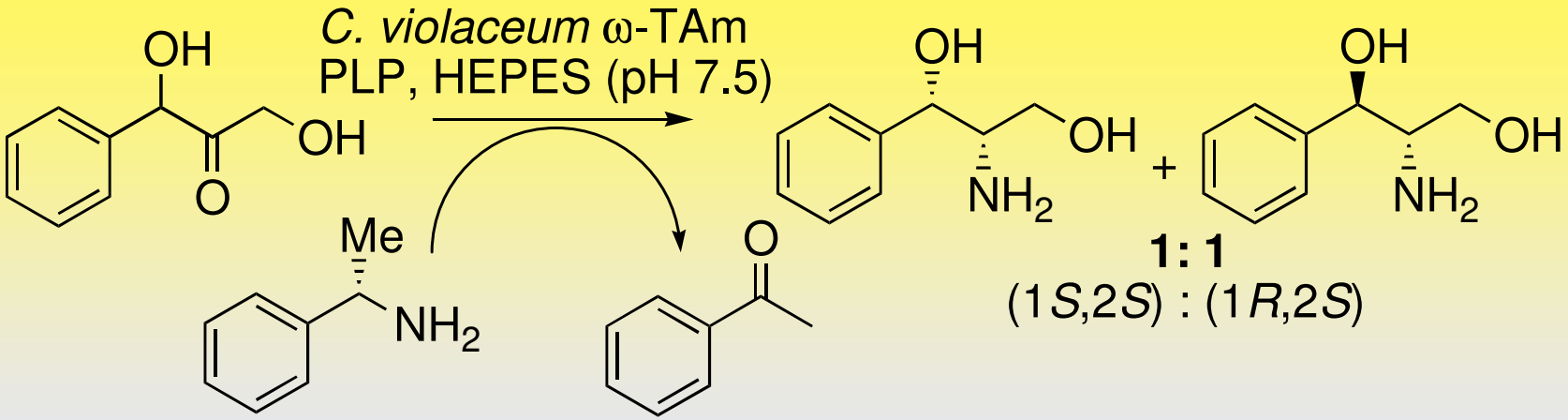
- (1*R*,2*S*)-Anti-aminodiol prepared in 70% *de:* (epimer formed 2nd step)



K. Smithies, M. E. B. Smith, U. Kaulmann, James L. Galman, J. M. Ward, H. C. Hailes, *Tetrahedron: Asymm.* **2009**, doi:10.1016/j.tetasy.2009.03.012

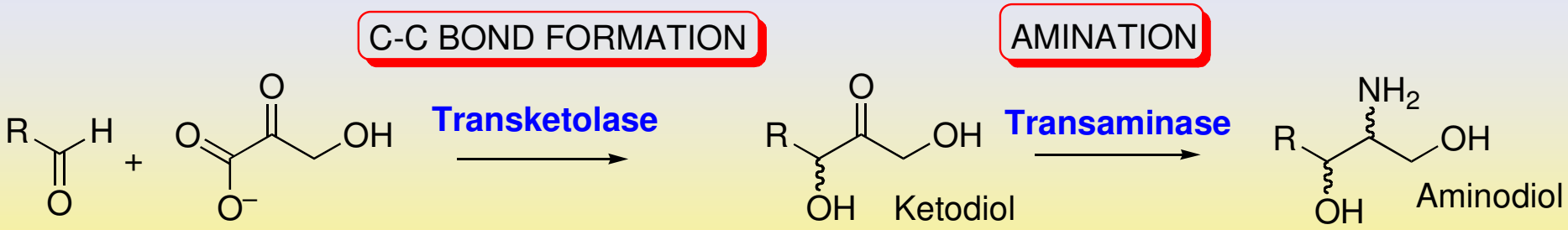
Aromatic aminodiols: Stereoselectivity

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



K. Smithies, M. E. B. Smith, U. Kaulmann, James L. Galman, J. M. Ward, H. C. Hailes, *Tetrahedron: Asymm.* **2009**, doi:10.1016/j.tetasy.2009.03.012

Summary



- 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 ω-TAms that convert ketodiols to aminodiols in high yields and selectivities ((*S*)- and (*R*)-ketodiols accepted)

Acknowledgements BiCE

- Chemistry
  - **Mark Smith**
  - **Kirsty Smithies**
  - **Alexander Jones**
  - **James Galman**
  - **Armando Cazares**
- Biochemical Engineering
  - **John Woodley**
  - **Paul Dalby**
  - **Frank Baganz**
  - **Gary Lye**
  - **Nicolas Szita**
  - **Ed Hibbert**
  - Tarik Senussi
  - Christine Ingram
  - Bing Chen
  - **Martina Micheletti**
  - **Lydia Coward**
- Molecular Biology
  - **John Ward**
  - **Ursula Kaulmann**
- Financial Support
  - EPSRC
  - **BiCE companies**
    - Merck
    - Lonza
    - DSM
    - AstraZeneca
    - Novacta
    - Degussa
    - Fluka
    - Sirius
    - Radleys
    - Hel
    - Almac
    - Tecan
    - Alphamerix