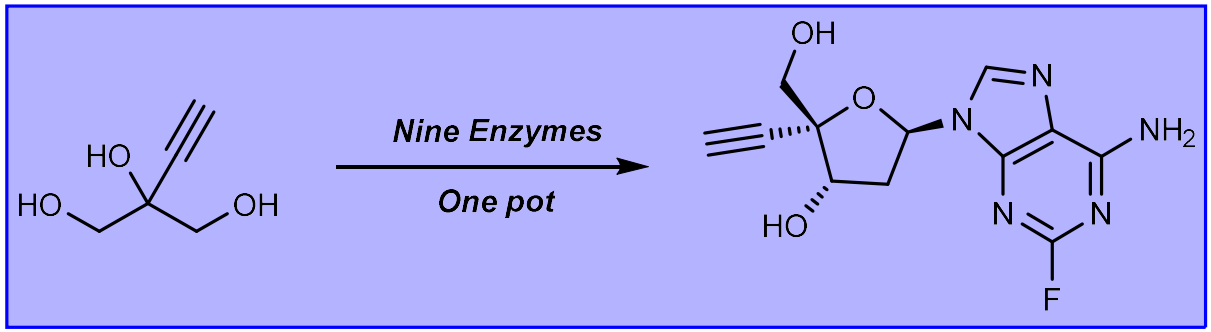
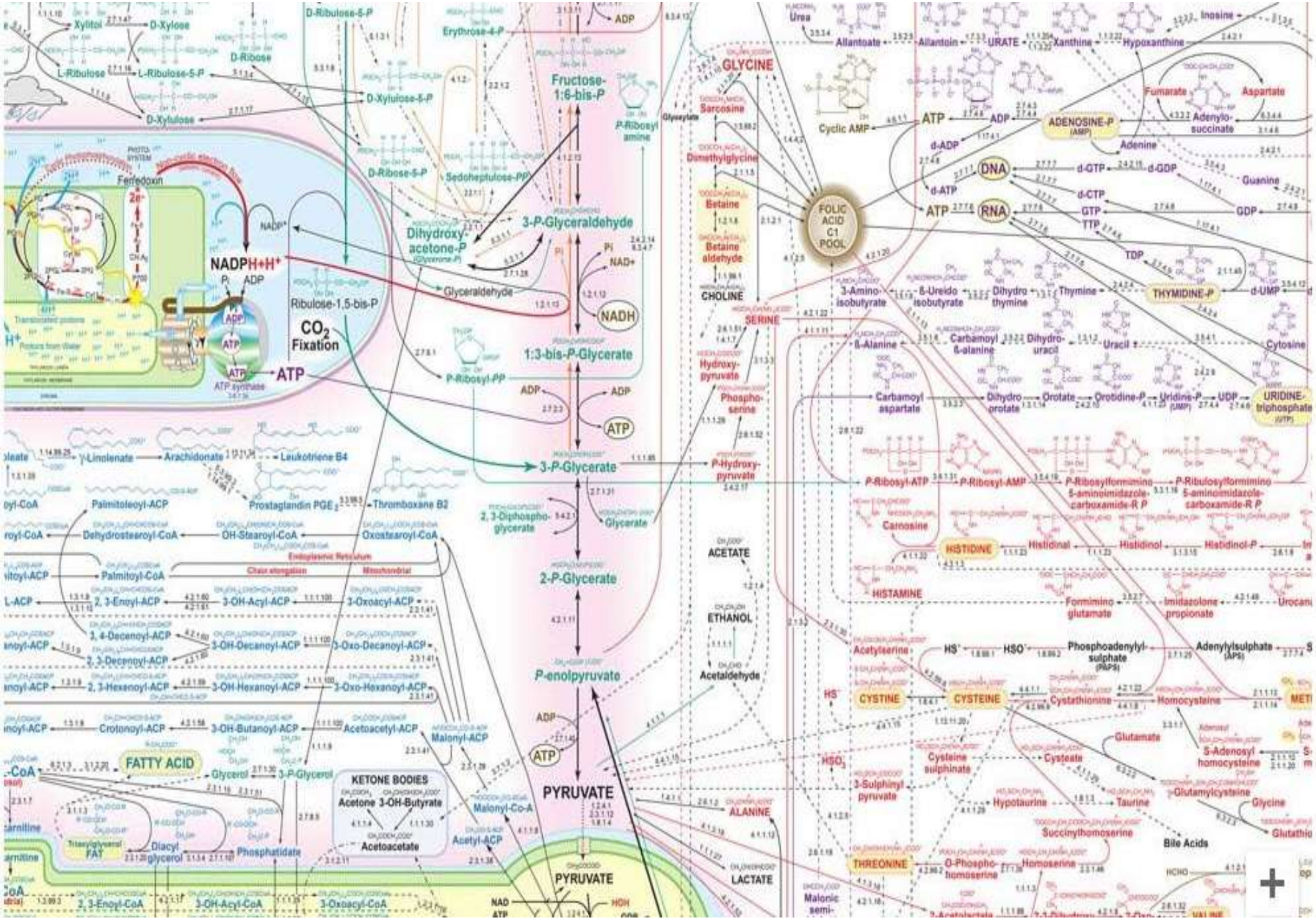
Recent Advances in Biocatalytic Cascades:

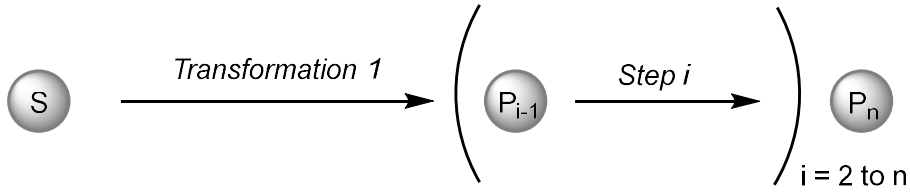
Matt Bock
Denmark Group Meeting
23 November 2021



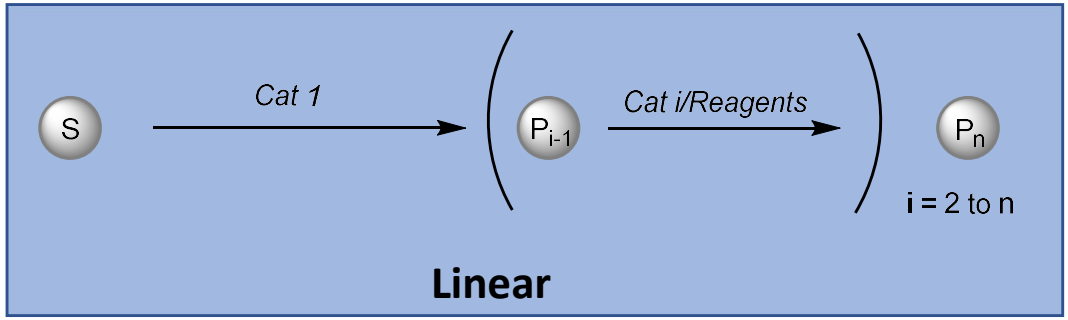

Xylitol
D-Xylose
L-Ribulose
L-Ribulose-5-P
D-Xylulose
D-Xylulose-5-P
D-Ribose
D-Ribulose-5-P
D-Ribose-5-P
Erythrose-4-P
Sedoheptulose-7P
Fructose-1:6-bis-P
P-Ribosyl amine
Ferredoxin
NADPH+H+
NADP+
Pi
ADP
ATP
ATP synthase
Ribulose-1,5-bis-P
CO2 Fixation
Dihydroxy-acetone-P
3-P-Glyceraldehyde
Glyceraldehyde
NAD+
NADH
1:3-bis-P-Glycerate
P-Ribosyl-PP
3-P-Glycerate
2, 3-Diphospho-glycerate
Glycerate
2-P-Glycerate
P-enolpyruvate
PYRUVATE
ACETATE
ETHANOL
Acetaldehyde
ALANINE
LACTATE
GLYCINE
Sarcosine
Dimethylglycine
Betaine
Betaine aldehyde
CHOLINE
SERINE
Hydroxy-pyruvate
Phospho-serine
P-Hydroxy-pyruvate
FOLIC ACID C1 POOL
Urea
Allantoate
Allantoin
URATE
Xanthine
Hypoxanthine
Inosine
Cyclic AMP
ADENOSINE-P (AMP)
Adenylo-succinate
Fumarate
Aspartate
Adenine
d-ADP
d-ATP
DNA
RNA
d-GTP
d-GDP
d-CTP
GTP
GDP
TTP
TDP
Guanine
3-Amino-isobutyrate
β-Ureido isobutyrate
Dihydro thymine
Thymine
THYMIDINE-P
d-UMP
β-Alanine
Carbamoyl β-alanine
Dihydro-uracil
Uracil
Cytosine
Carbamoyl aspartate
Dihydro-orotate
Orotate
Orotidine-P
Uridine-P (UMP)
UDP
URIDINE-triphosphate (UTP)
P-Ribosyl-ATP
P-Ribosyl-AMP
P-Ribosylformimino 5-aminoimidazole-carboxamide-R P
P-Ribulosylformimino 5-aminoimidazole-carboxamide-R P
Carnosine
HISTIDINE
Histidinal
Histidinol
Histidinol-P
HISTAMINE
Formimino glutamate
Imidazolone propionate
Urocani
Acetylserine
HS-
HSO-
Phosphoadenylyl-sulphate (PAPS)
Adenylylsulphate (APS)
CYSTINE
CYSTEINE
Cystathionine
Homocysteine
METI
Glutamate
S-Adenosyl homocysteine
Cysteine sulphinate
Cysteate
3-Sulphinyl pyruvate
Hypotaurine
Taurine
Glutamylcysteine
Glycine
Glutathio
Succinylhomoserine
Bile Acids
THREONINE
O-Phospho-homoserine
Homoserine
HCHO
Malonic semi-
γ-Linolenate
Arachidonate
Leukotriene B4
Prostaglandin PGE2
Thromboxane B2
Palmitoleoyl-ACP
Dehydrostearoyl-CoA
OH-Stearoyl-CoA
Oxostearoyl-CoA
Endoplasmic Reticulum
Chain elongation
Mitochondrial
Palmitoyl-CoA
2, 3-Enoyl-ACP
3-OH-Acyl-ACP
3-Oxoacyl-ACP
2, 4-Decenoyl-ACP
3-OH-Decanoyl-ACP
3-Oxo-Decanoyl-ACP
2, 3-Decenoyl-ACP
2, 3-Hexenoyl-ACP
3-OH-Hexanoyl-ACP
3-Oxo-Hexanoyl-ACP
Crotonoyl-ACP
3-OH-Butanoyl-ACP
Acetoacetyl-ACP
Malonyl-ACP
FATTY ACID
Glycerol
3-P-Glycerol
KETONE BODIES
Acetone 3-OH-Butyrate
Acetoacetate
Malonyl-Co-A
Acetyl-ACP
Triacylglycerol FAT
Diacyl glycerol
Phosphatidate
2, 3-Enoyl-CoA
3-OH-Acyl-CoA
3-Oxoacyl-CoA
NAD
HOH

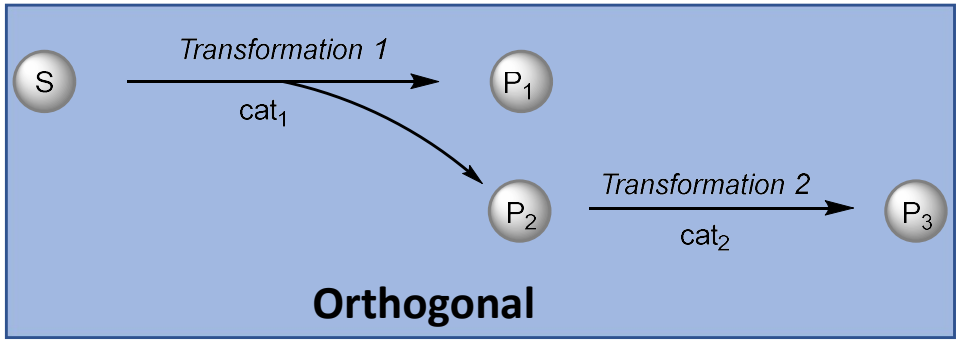
What is a biocatalytic cascade?

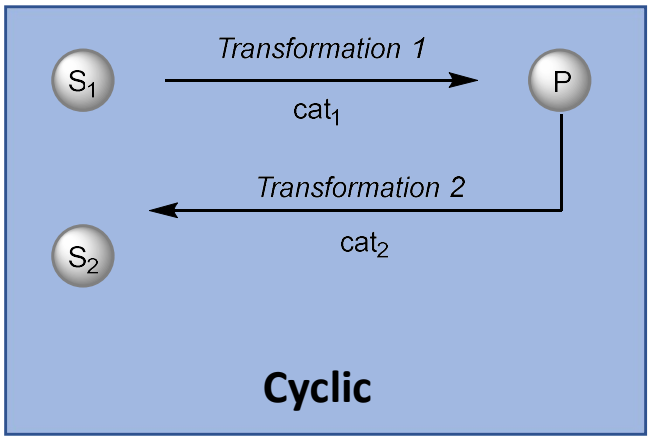
A reaction comprising of two or more transformations in the same pot where one step includes a biocatalyst

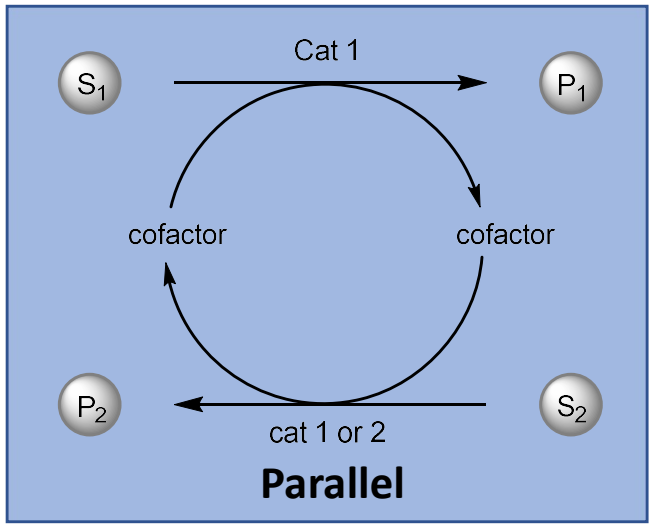


Classifications:





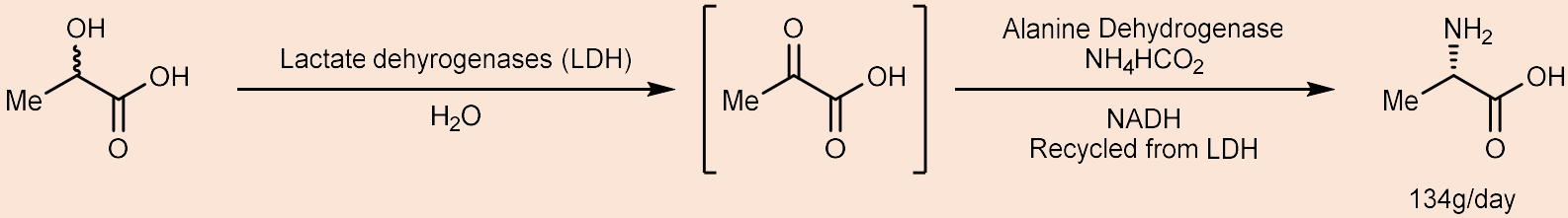


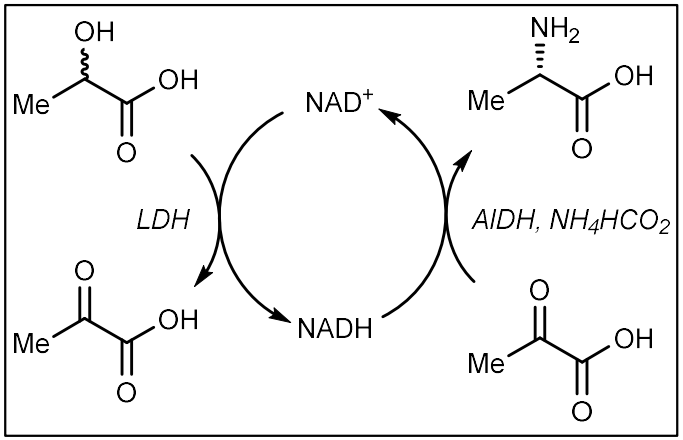


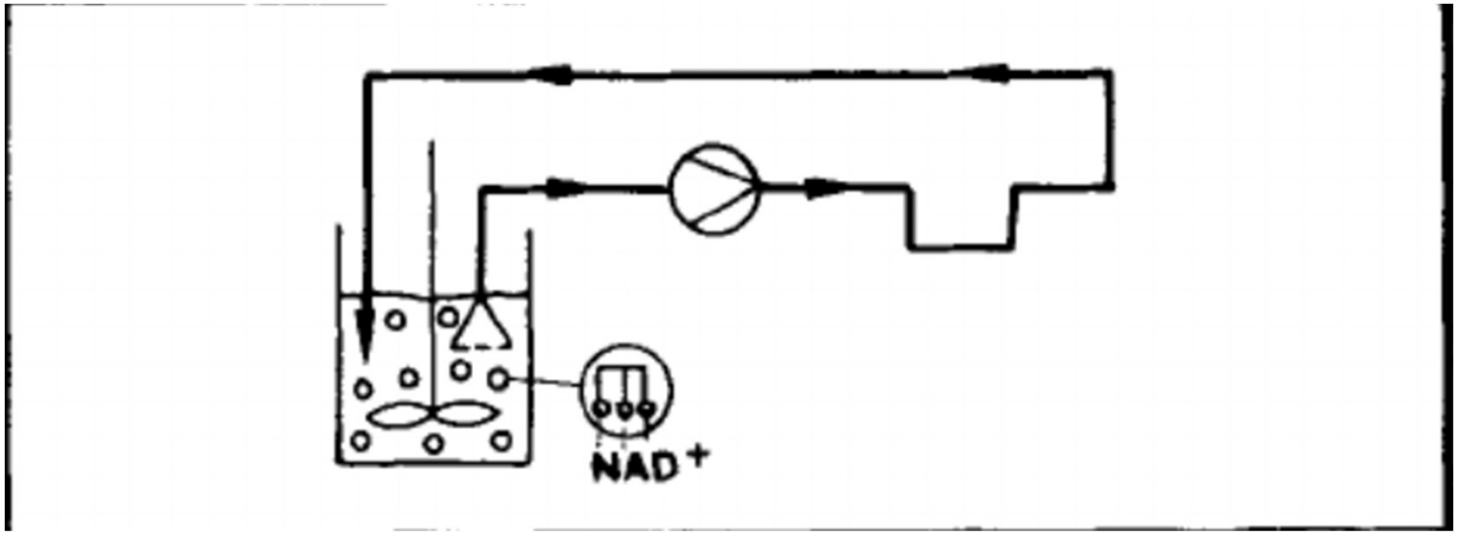
Chem. Rev. **2018**, *118*, 270-348.

The First *in vitro,* Artificial Biocatalytic Cascade

Wandrey and coworkers, **1984**:

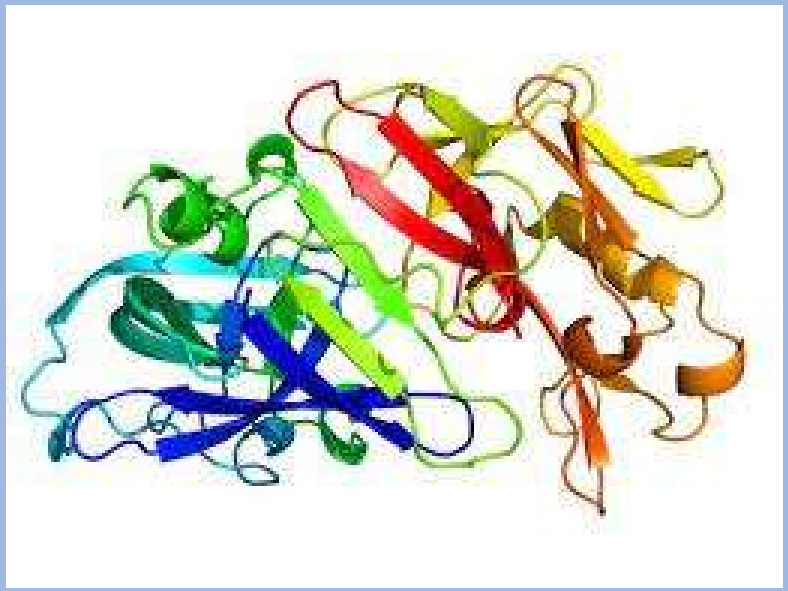




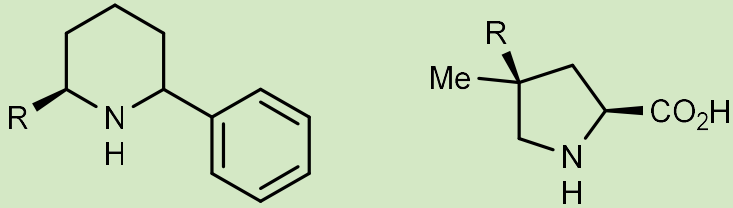


Ann. N. Y. Acad. Sci. **1979**, *326,* 87–95. *Ann. N. Y. Acad. Sci.* **1984**, *434*, 91–94.

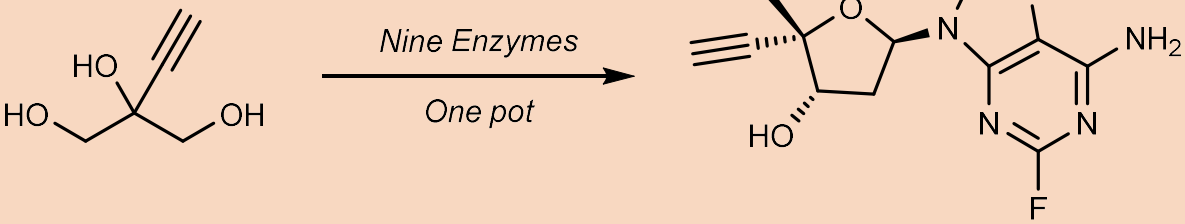
Outline



Enzyme classifications



Biocatalytic cascades for the synthesis of nitrogen heterocycles



MERCK INVENTING FOR LIFE

CODEXIS®

Pharmaceuticals prepared by biocatalytic cascades

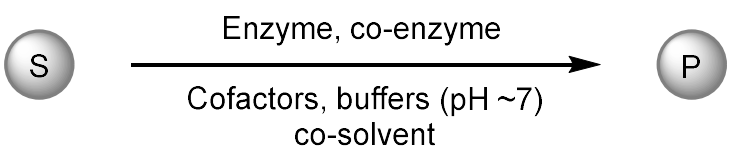
Enzyme Classification

IUPAC's Classification of Enzymes

Class	Reaction Type	Enzyme Examples
Oxidoreductase	Redox Chemistry	Ketoreductases, Iminoreductases, Oxidases
Transferase	FG Addition or transfer	Aminotrasferases, Kinases
Hydrolase	Hydrolytic reactions	Nitrilase, Lipase, Amidase
Lyase	Eliminations	Aldolases, Decarboxylases
Isomerase	Isomerizations	Epimerases
Ligase	Coupling reactions often macromolecules	C-C, C-N, C-O, C-S Ligases

What's in a Biocatalytic reaction?

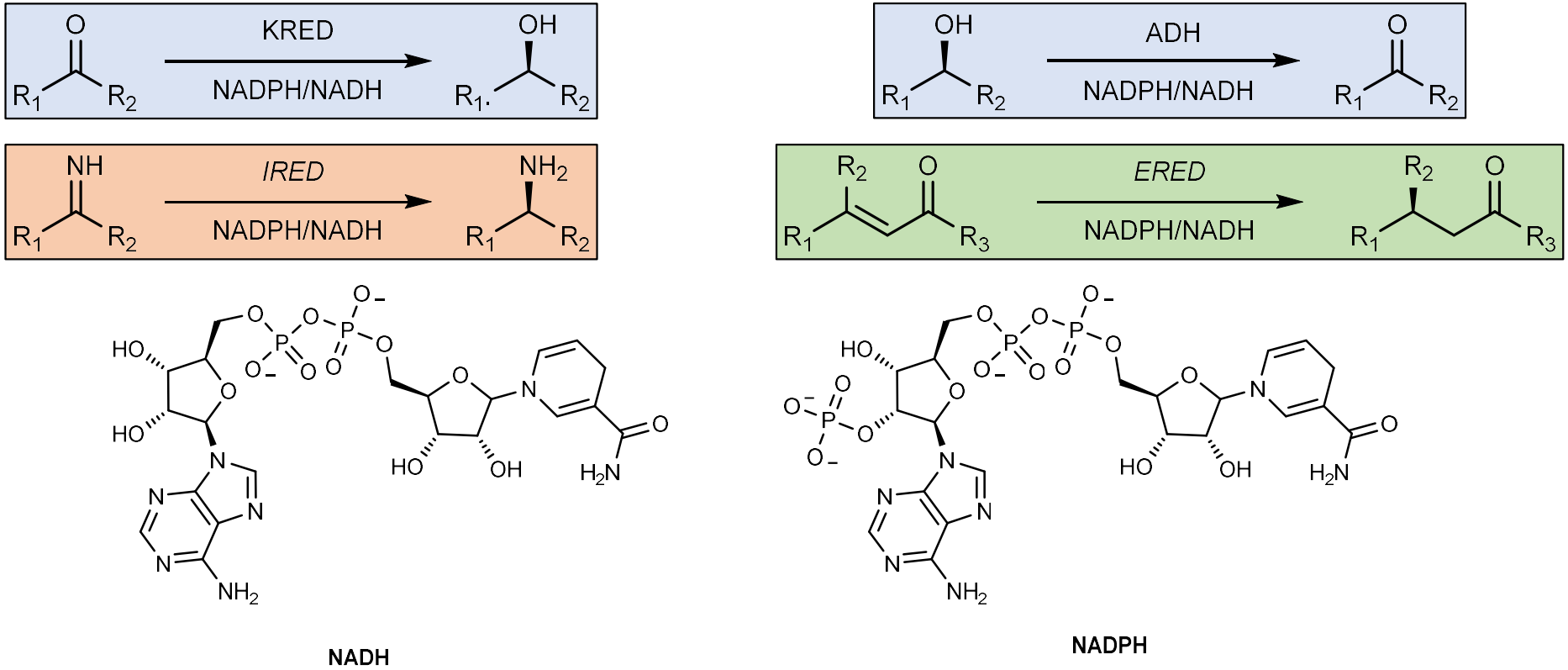
Generalized reaction scheme



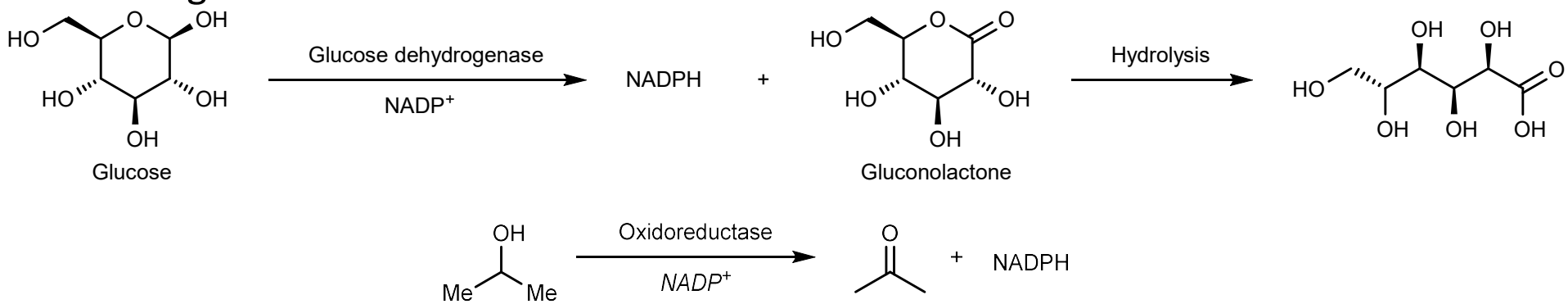
Cofactors: "reagents"
Co-enzyme: Regenerate cofactors or remove byproducts
Buffers: Aqueous. Mixture of salts, system specific
Co-solvent: DMSO, IPA, THF, etc.
Temperatures: room temp or slightly elevated (35 °C)
A significant number of these reactions are reversible!
<u>For the purpose of this talk, specific enzymes are not named, just listed as a general transform</u>

Oxidoreductases

Generalized schemes:



Cofactor regeneration:

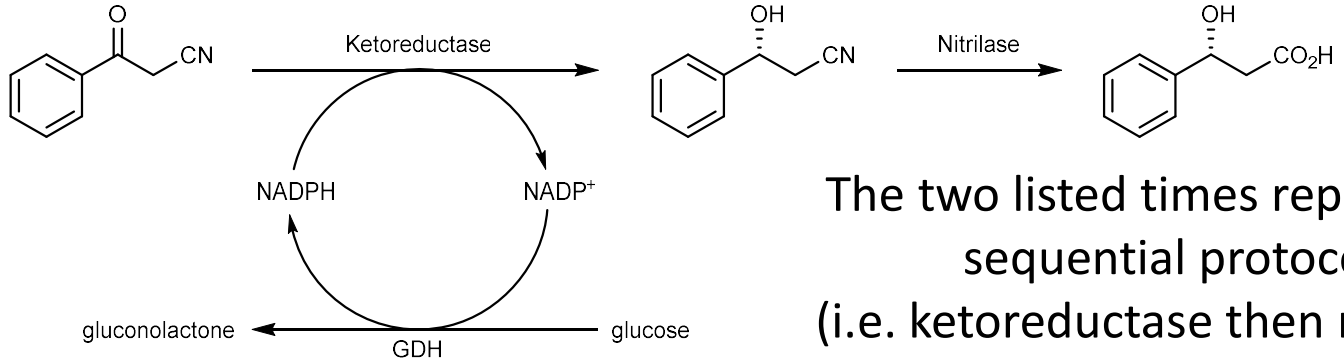


While not as mature, ketoreductases and iminoreductases are starting to see use

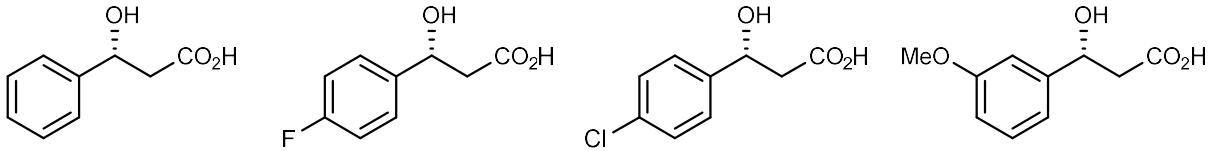
Adv. Synth. Catal. **2019**, *361*, 2421-2432.

Examples of KREDs

Synthesis of β-hydroxy acids

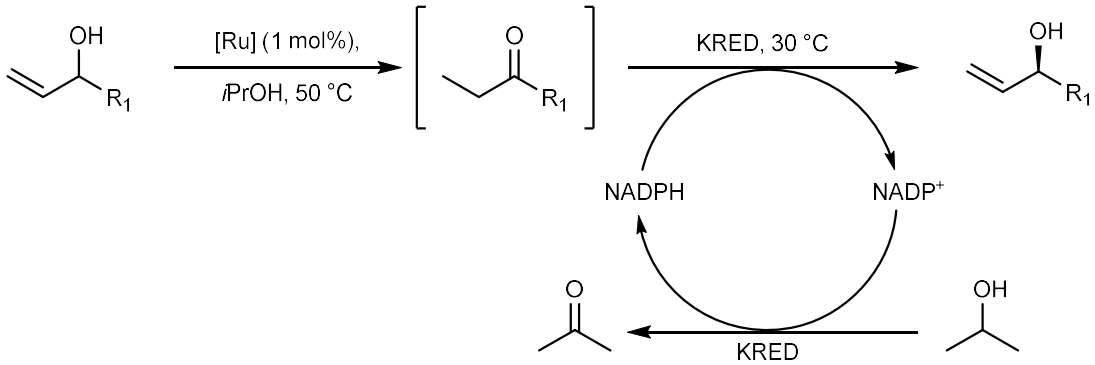


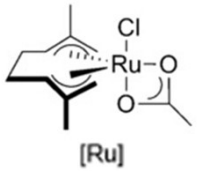
The two listed times represent a sequential protocol (i.e. ketoreductase then nitrilase)

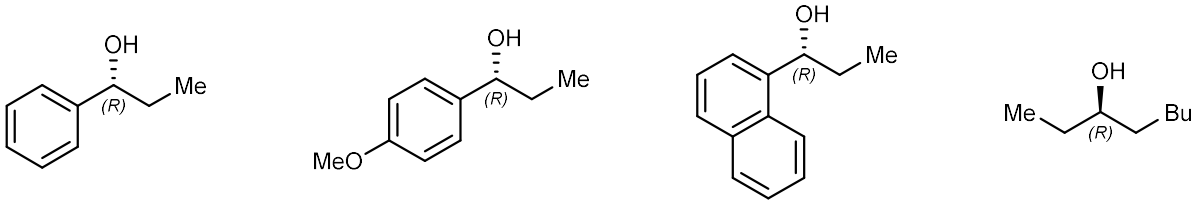


60h/24h	24h/24h	24h/24h	24h/24h
72%, 99% *ee*	77%, 99% *ee*	77%, 99% *ee*	80%, 98% *ee*

Synthesis of benzylic alcohols





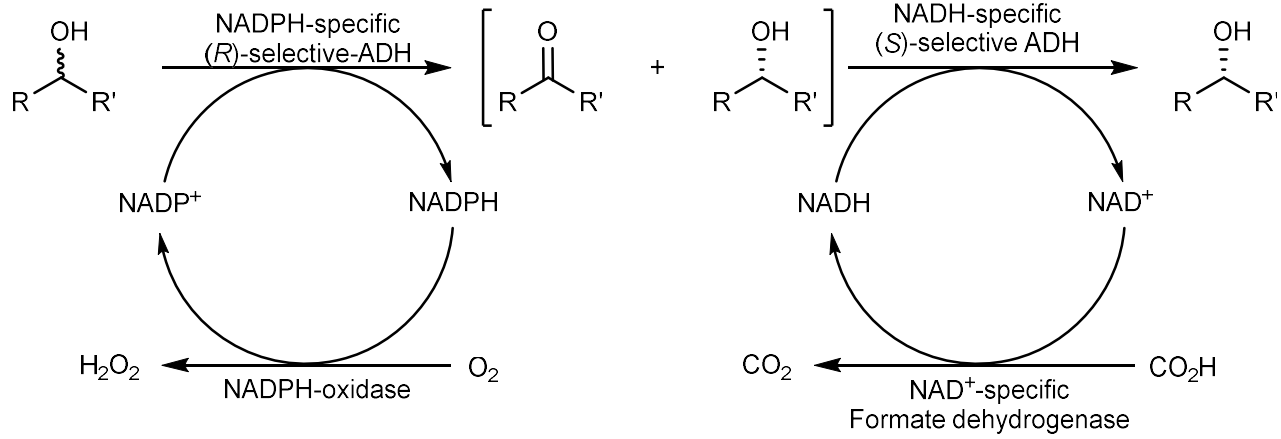


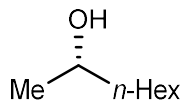
86%	85%	90%	90%
>99% *ee*	>99% *ee*	98% *ee*	>99% *ee*

J. Org. Chem. **2009**, *74*, 1685-1662. *ACIE* **2016**, *55*, 8691-8695.

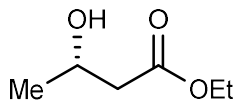
Examples of ADHs

Deracemization with two ADHs

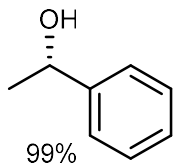




99%
>99% *ee*

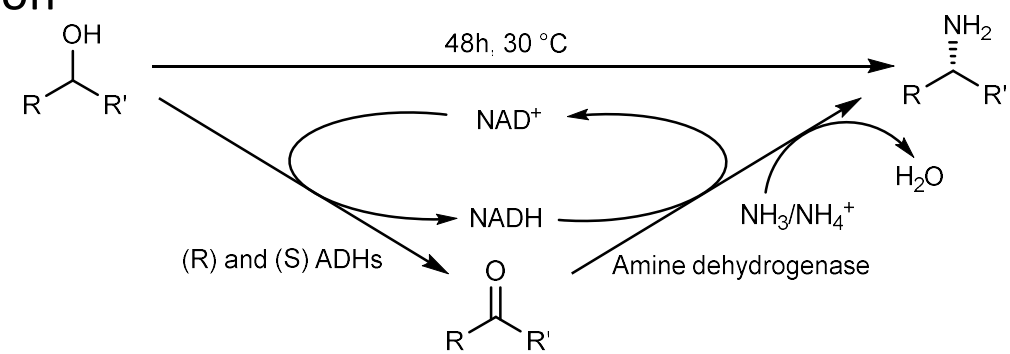


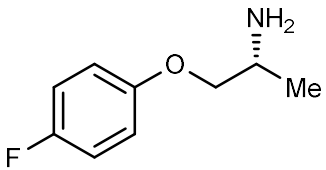
99%
>99% *ee*



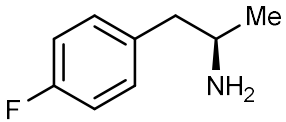
99%
>99% *ee*

Another deracemization

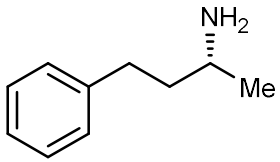




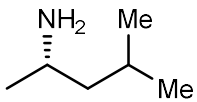
84%
>99% *ee*



66%
>99% *ee*



92%
83% *ee*

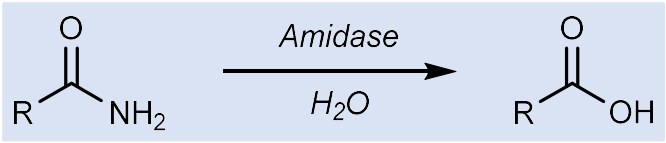


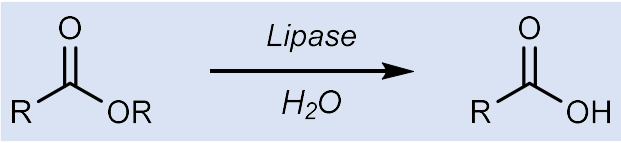
80%
99% *ee*

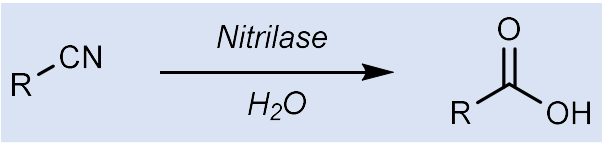
J. Am. Chem. Soc. **2008**, *130*, 13969-13972.; *Science* **2015**, *349*, 1525-1529.

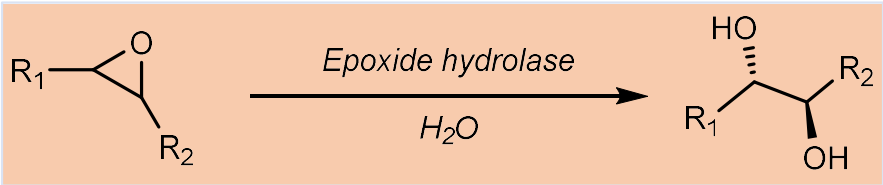
Hydrolases

Generalized schemes:

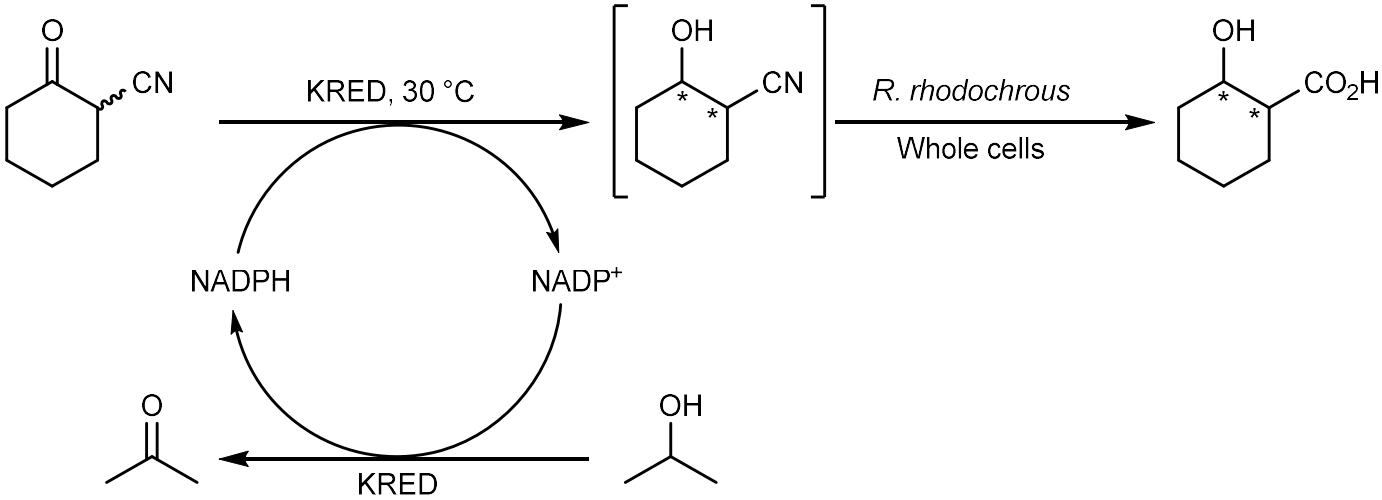


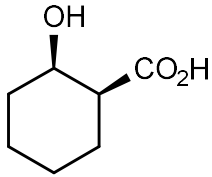




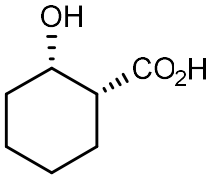


Synthesis of cyclic acids

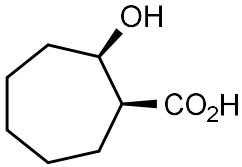




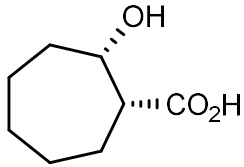
83% yield
>99:1 *d.r.*, >99% *ee*



90% yield
>99:1 *d.r.*, >99% *ee*



91% yield
>99:1 *d.r.*, >99% *ee*

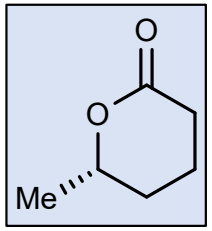


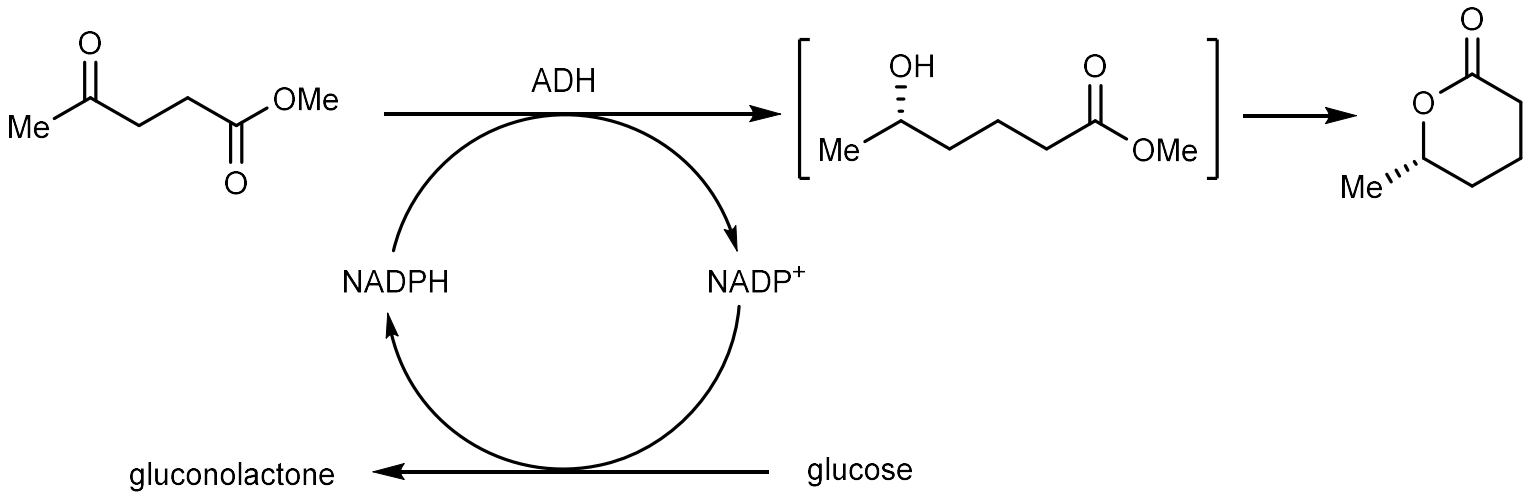
97% yield
>99:1 *d.r.*, >99% *ee*

Adv. Synth. Catal. **2019**, *361*, 2421-2432.; *Org Lett.* **2016**, *18*, 3366-3369.

Group Problem #1

Using a biocatalytic cascade, prepare the following compound

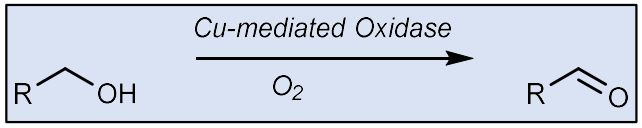


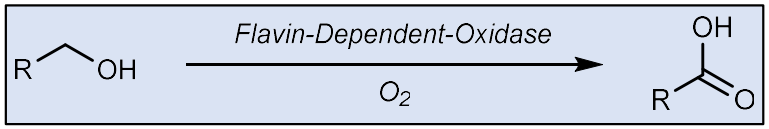


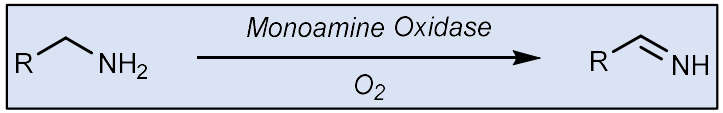
Chem. Rev. **2018**, *118*, 270-348.

Oxidases

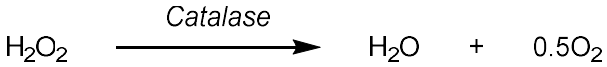
Generalized schemes:



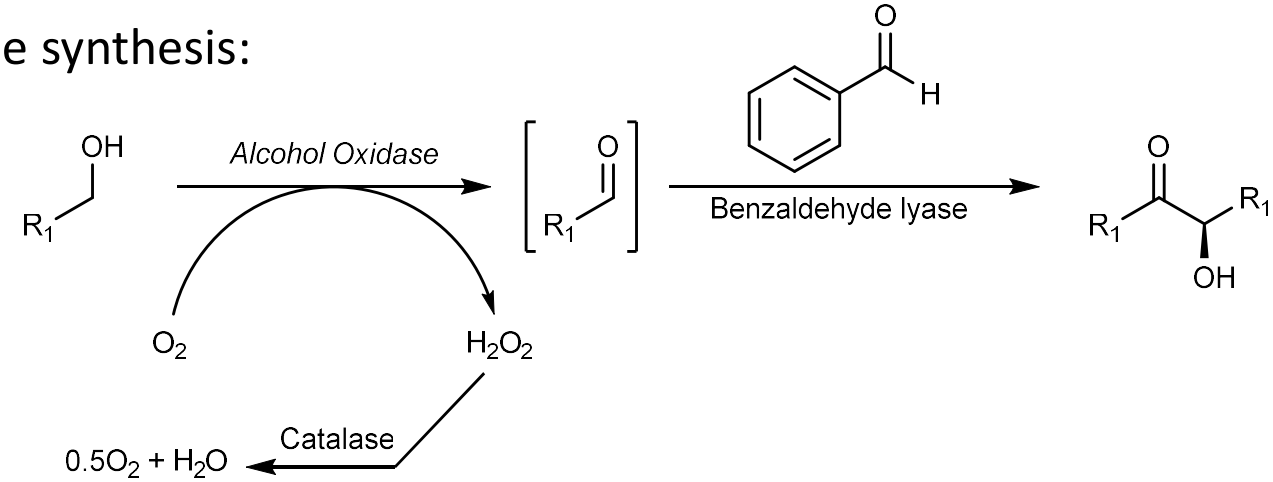


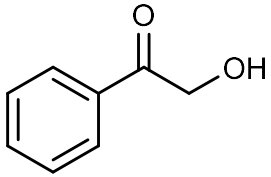


- Often very substrate specific.
- Normally paired with one or two co-enzymes
  - Catalase for breakdown of H_2O_2
  - A peroxidase to maintain Cu oxidation state

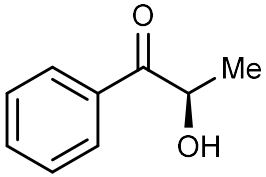


α-hydroxy ketone synthesis:

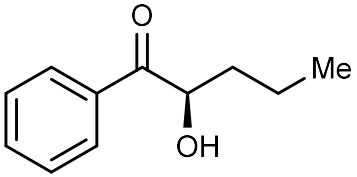




> 99% Conv.



> 99% Conv.
99% *ee*

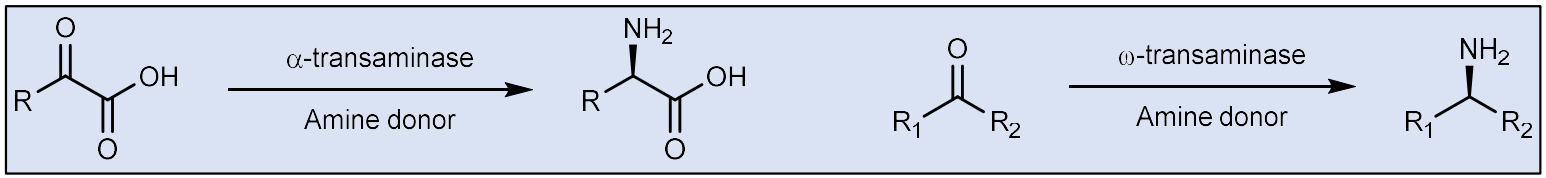


> 91% Conv.
98% *ee*

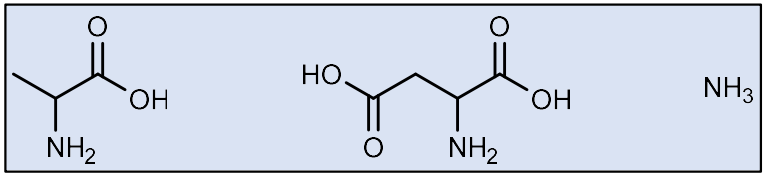
ChemSusChem **2019**, *12*, 2859-2881.; *ChemCatChem* **2013**, *5*, 2512-2516.

Transaminases

Generalized scheme:



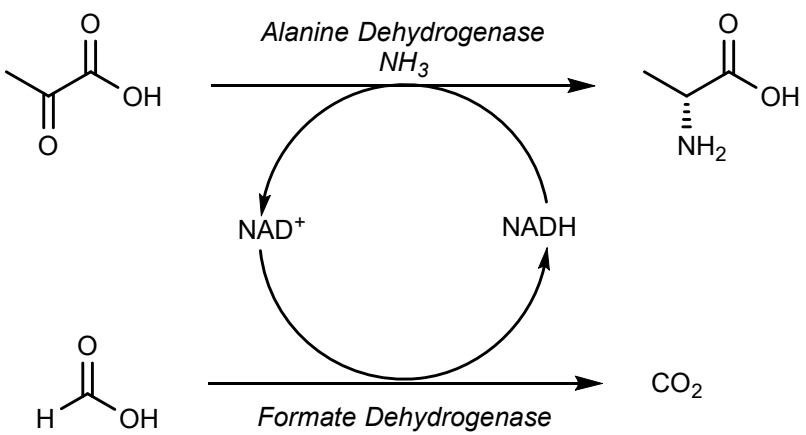
Common Amine donors



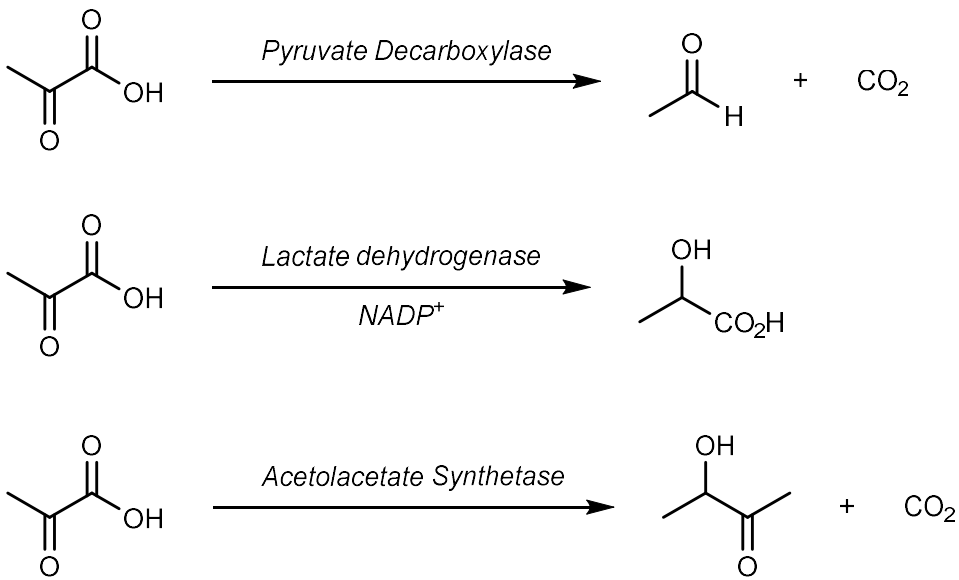
While a powerful reaction, equilibrium is favored for starting material.

Strategies to Shift Equilibrium:

Regeneration of amine donor



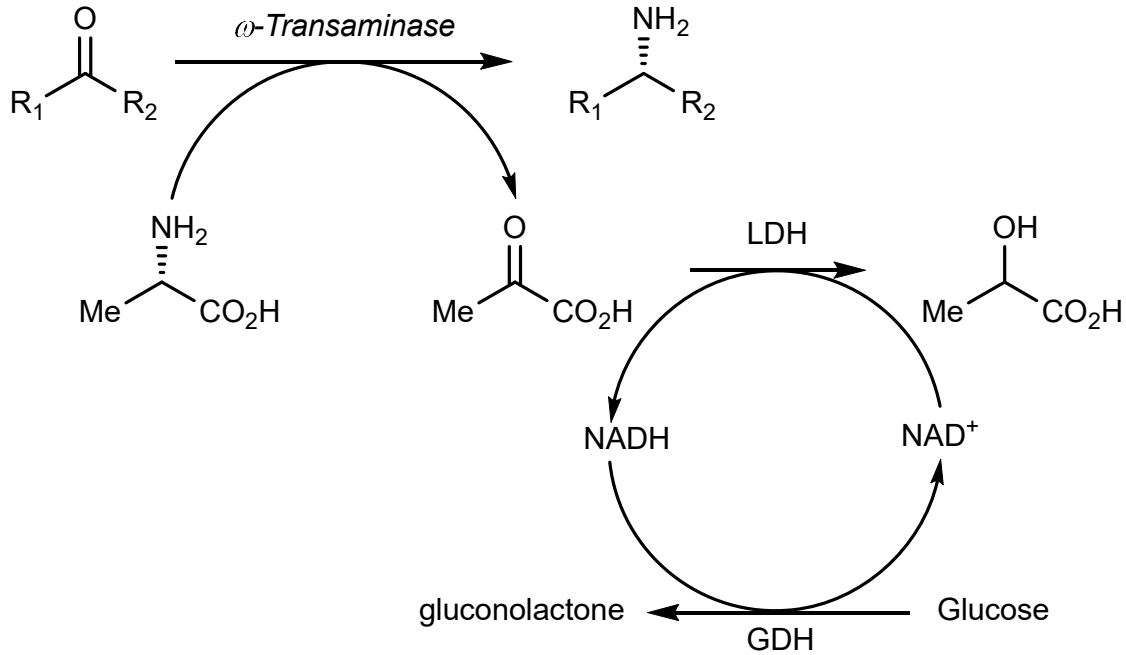
Removal of keto-acid byproduct

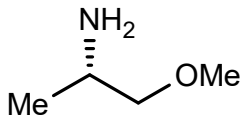


Adv. Synth. Catal. **2011**, *353*, 2239-2262. *ACS Catal.* **2014**, *4*, 129-143.

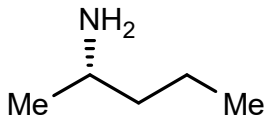
Transaminases

Asymmetric reductive amination

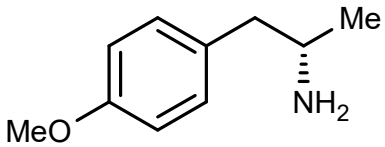




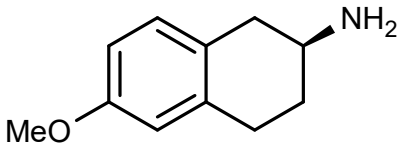
97% Conv.
>99% *ee*



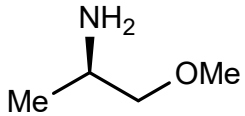
97% Conv.
>99% *ee*



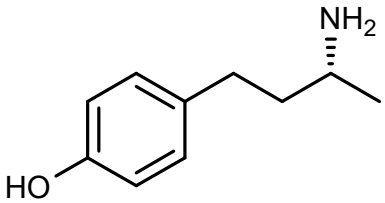
94% Conv.
>99% *ee*



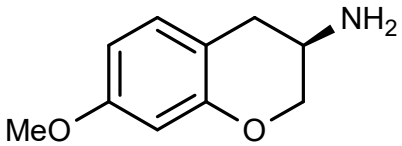
99% Conv.
>99% *ee*



99% Conv.
>99% *ee*



99% Conv.
>99% *ee*



99% Conv.
>99% *ee*

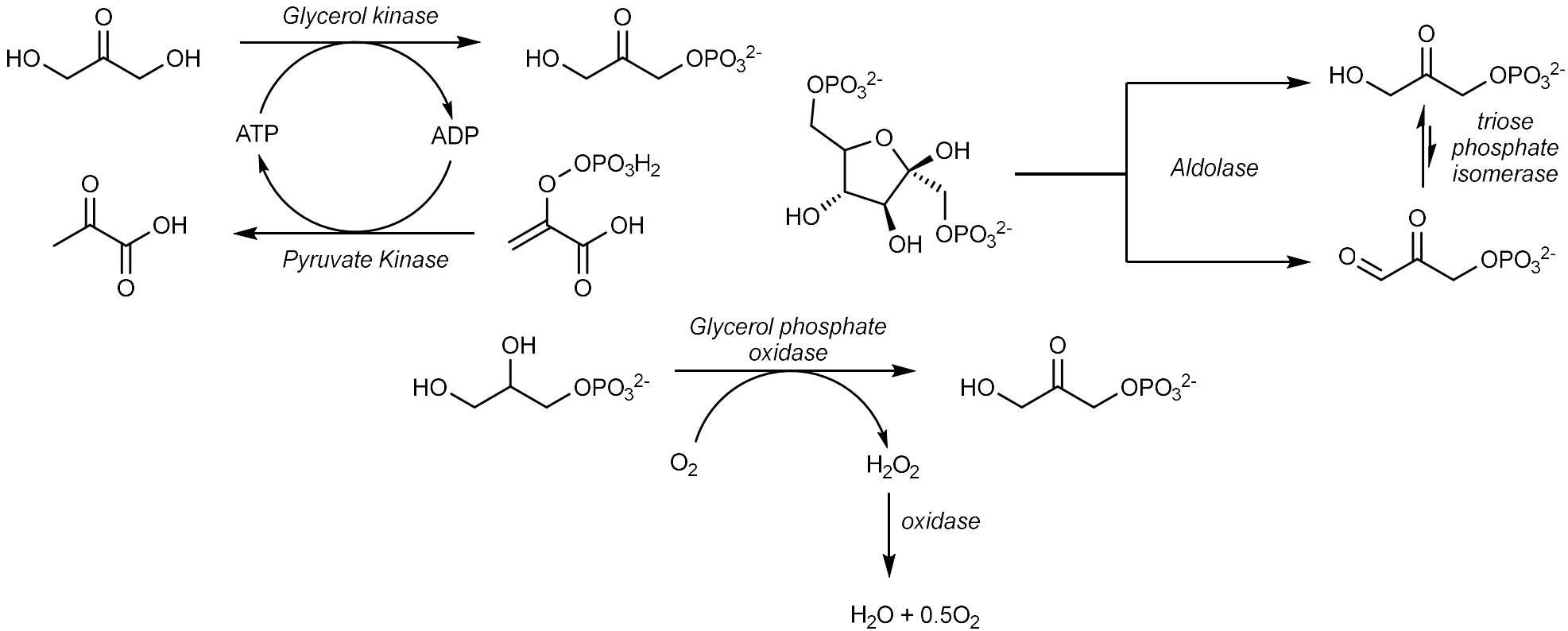
Adv. Synth. Catal. **2008**, *350*, 2761-2766.

Aldolases

Generalized schemes:

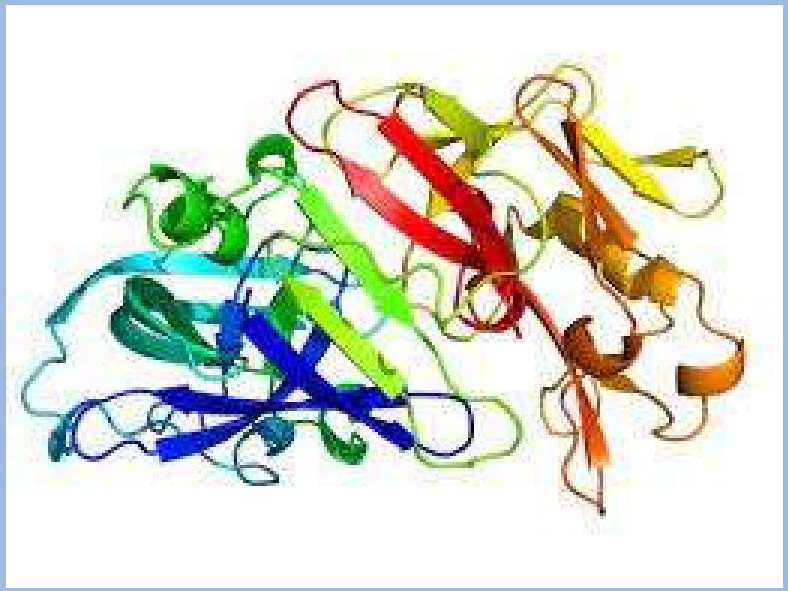
- Range of acceptors is typical broad
- Donors are typically limited to dihydroxyacetone phosphate (DHAP) derivatives

In Situ Preparation of DHAP:

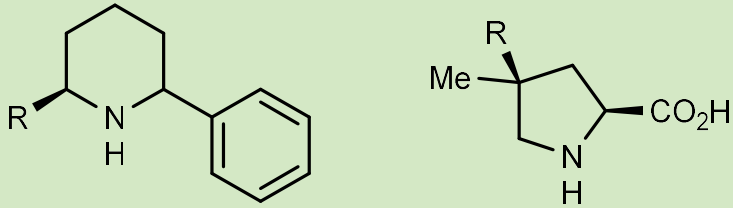


Adv. Synth. Catal. **2011**, *353*, 2239-2262. *Adv. Synth. Calal.* **2019**, *361*, 2421-2432.

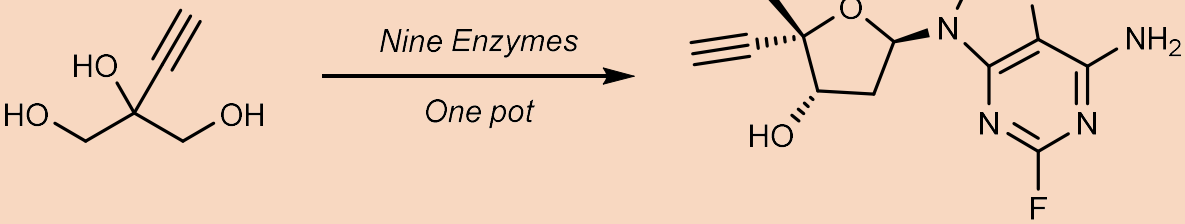
Outline



Enzyme classifications



Biocatalytic cascades for the synthesis of nitrogen heterocycles



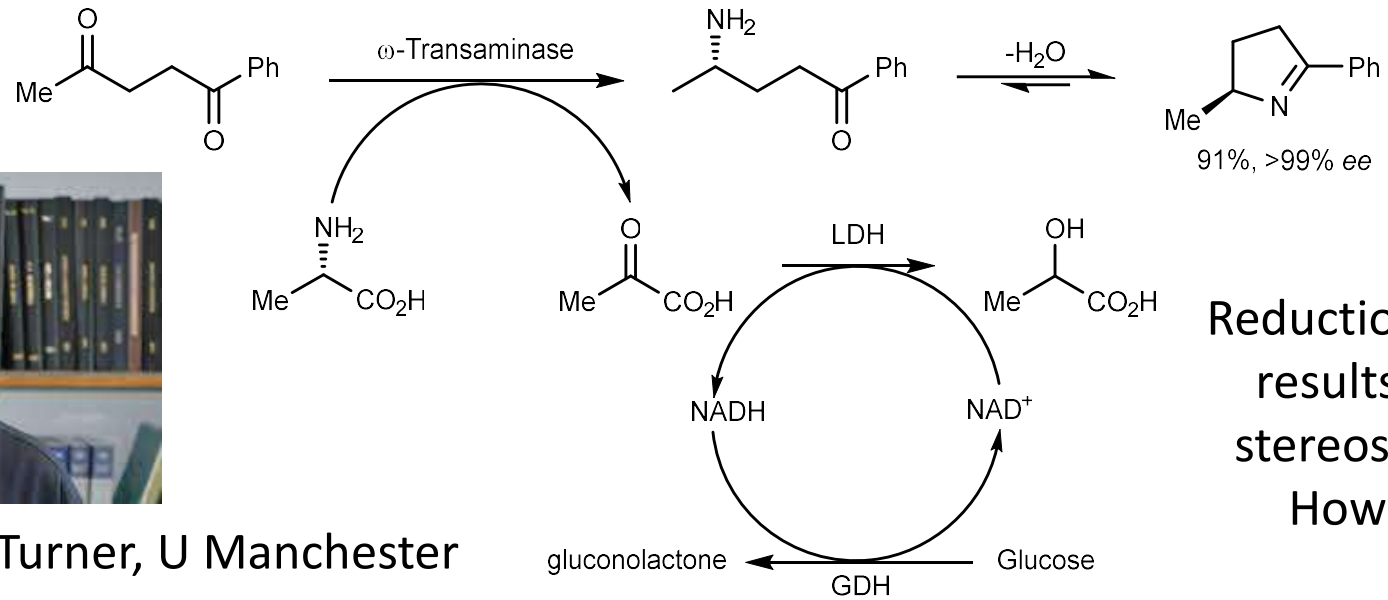
MERCK INVENTING FOR LIFE

CODEXIS®

Pharmaceuticals prepared by biocatalytic cascades

Preparation of Disubstituted Pyrrolidines

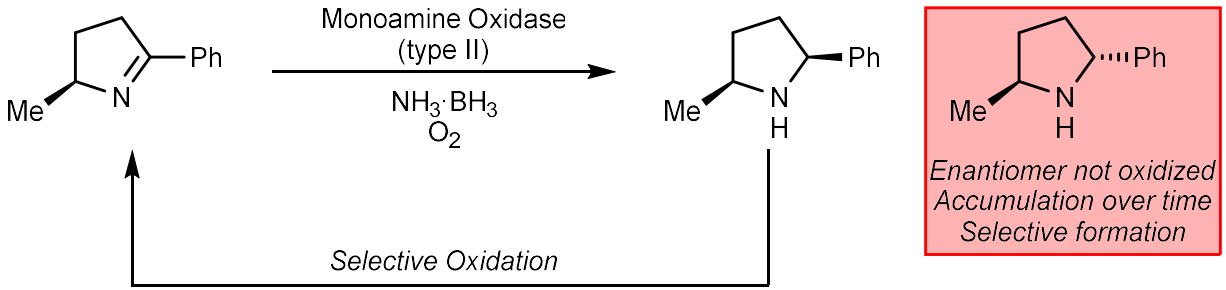
Cyclization with transaminases:



Prof. Nicholas Turner, U Manchester

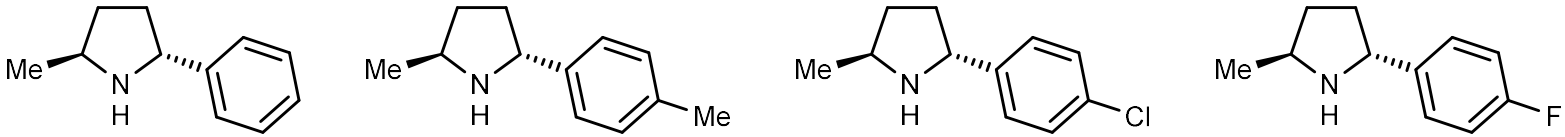
Reduction of imine results in poor stereoselectivity
How to fix?

Cyclization with transaminases:



Strategy used previously to prepare monosubstituted pyrrolidines

Substrates prepared in one-pot:



> 99% Conv.
> 99% *de*

> 99% Conv.
> 99% *de*

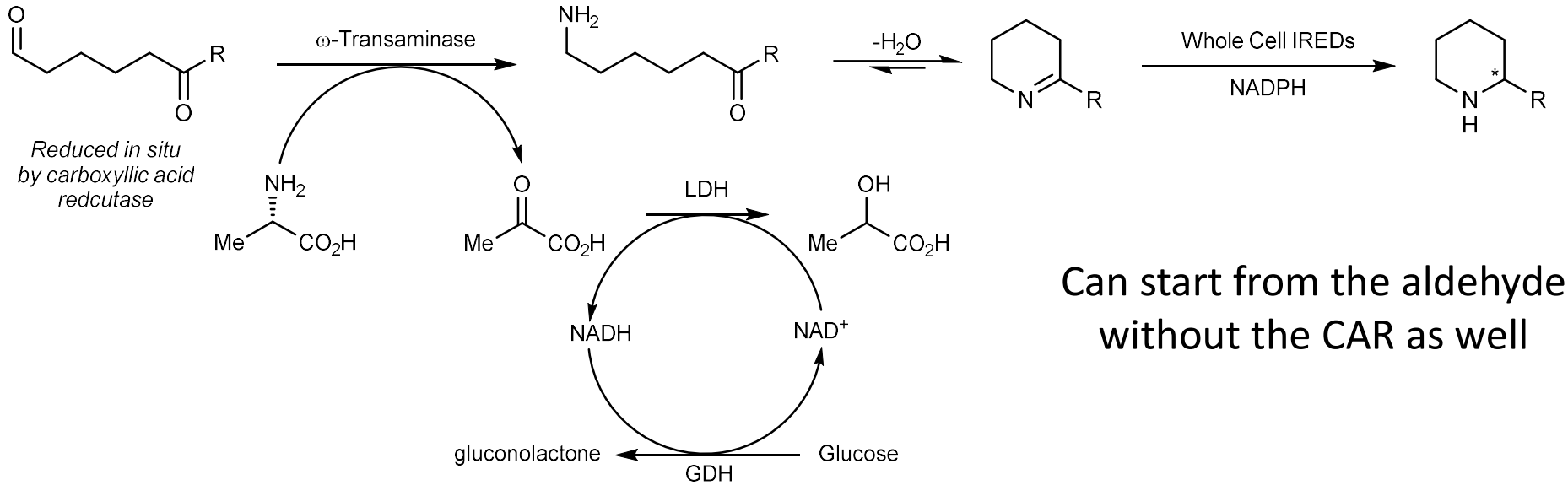
> 99% Conv.
> 99% *de*

> 99% Conv.
> 99% *de*

ACIE **2014**, *53*, 2447-2450.; *J. Am. Chem. Soc.* **2006**, *128*, 2224-2225.

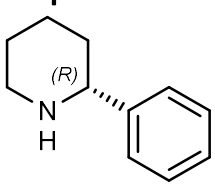
Synthesis of Substituted Piperidines

Access to piperidines *via* IREDs and a carboxylic acid reductase:

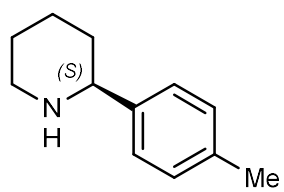


Can start from the aldehyde without the CAR as well

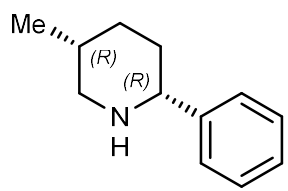
Selected Examples:



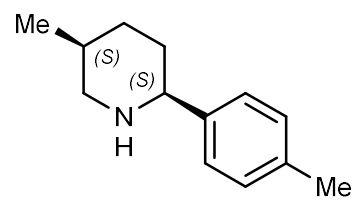
> 98% Conv.
93% *e.e.*
CAR, ATA, (*S*)-IRED



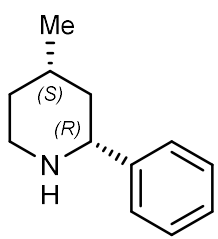
> 98% Conv.
98% *e.e.*
CAR, ATA, (*R*)-IRED



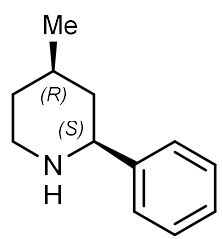
95% Conv.
>98% *e.e.*, >98% *d.e.*
ATA, (*S*)-IRED



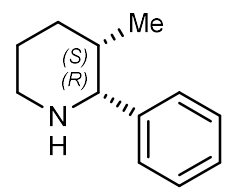
96% Conv.
>98% *e.e.*, 29% *d.e.*
ATA, (R)-IRED



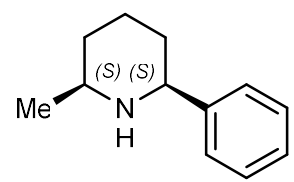
98% Conv.
>98% *e.e.*, 95% *d.e.*
CAR, ATA, (*S*)-IRED



74% Conv.
>98% *e.e.*, 92% *d.e.*
CAR, ATA, (*R*)-IRED



89% Conv.
81% *e.e.*, 81% *d.e.*
CAR, ATA, (*S*)-IRED

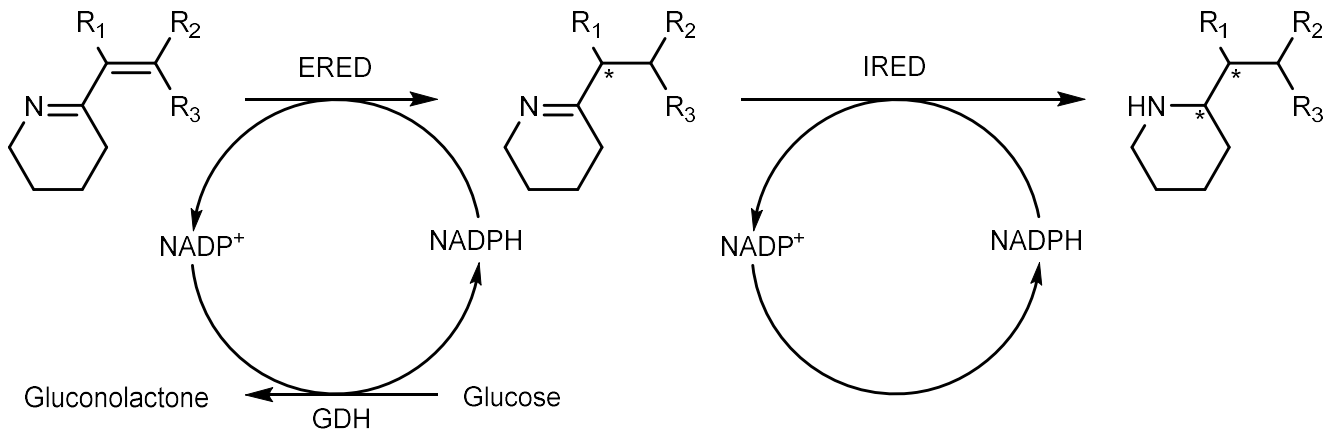


81% Conv.
98% *e.e.*, 98% *d.e.*
ATA, (*R*)-IRED
Sequential Protocol

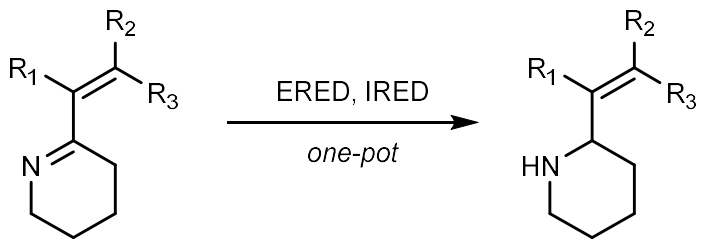
ACS Catal. **2016**, *6*, 3753-3759.

Further Substitution

Contiguous stereocenters:



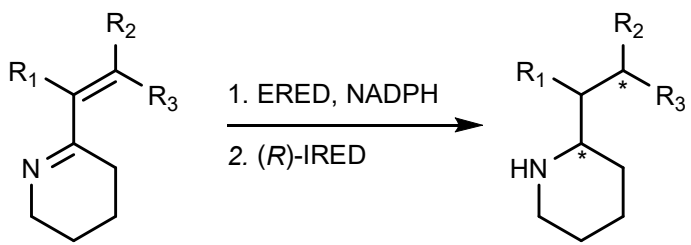
Challenge:
rate of competing reductions

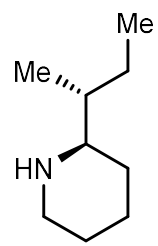


$k_{IRED} >>> k_{ERED}$

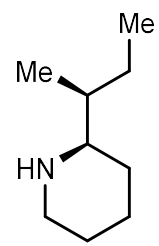
Allylic amines are not acceptors for EREDS

Sequential Protocol:

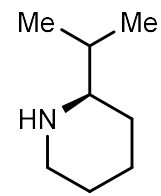




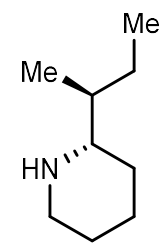
> 99% conv.
93% *ee*, 72:28 *d.r.*



> 99% conv.
93% *ee*, 85:15 *d.r.*



> 99% conv.
>99% *ee*



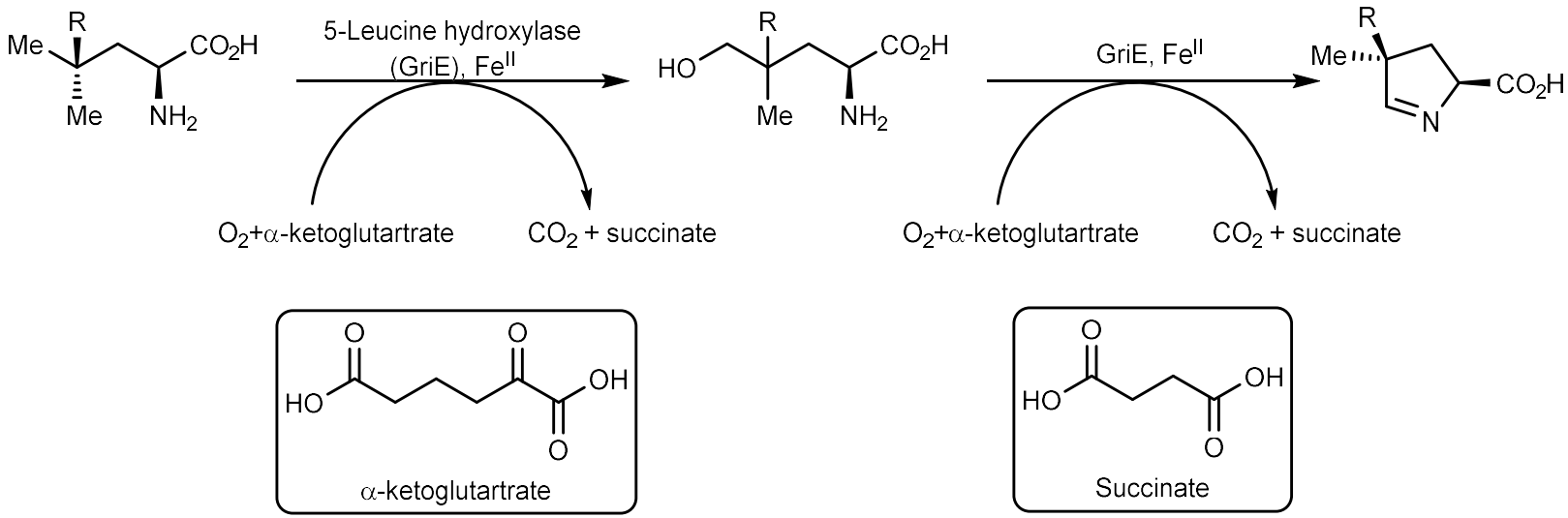
> 99% conv.
55% *ee*, 73:27 *d.r.*

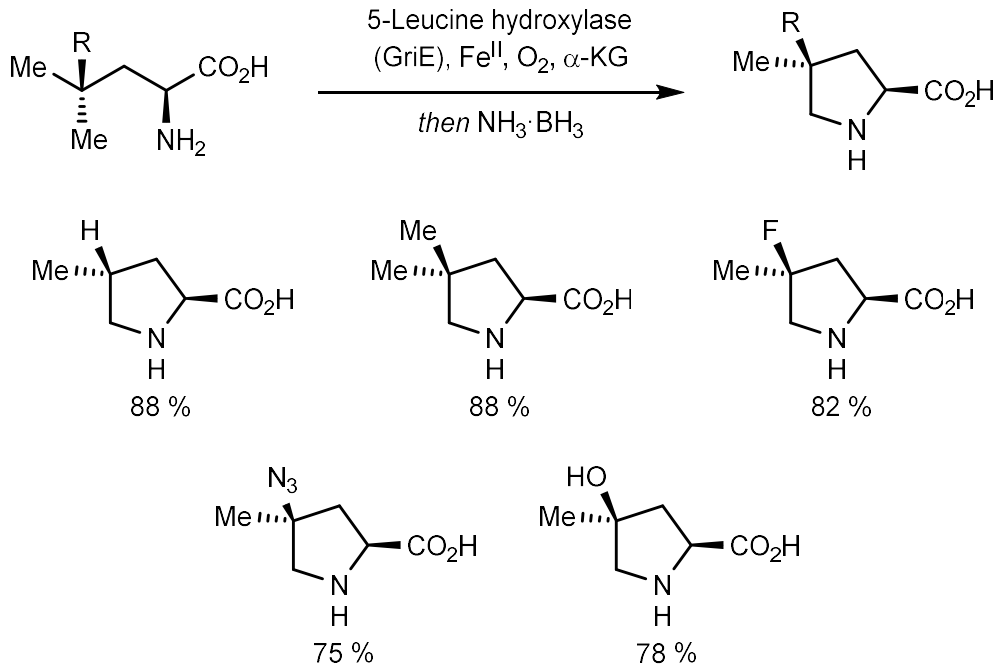
5 and 7 membered rings also reported,

J. Am. Chem. Soc. **2019**, *141*, 19208-19213

Synthesis of Substituted Prolines

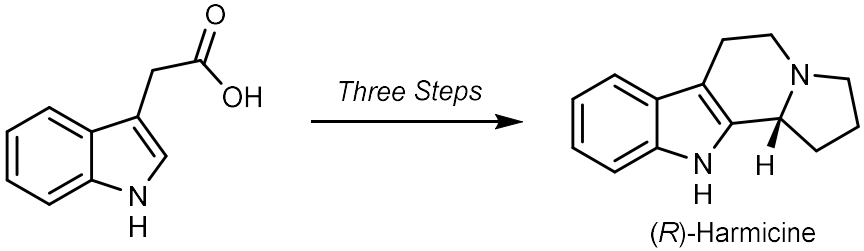
Leucine-hydroxylases:





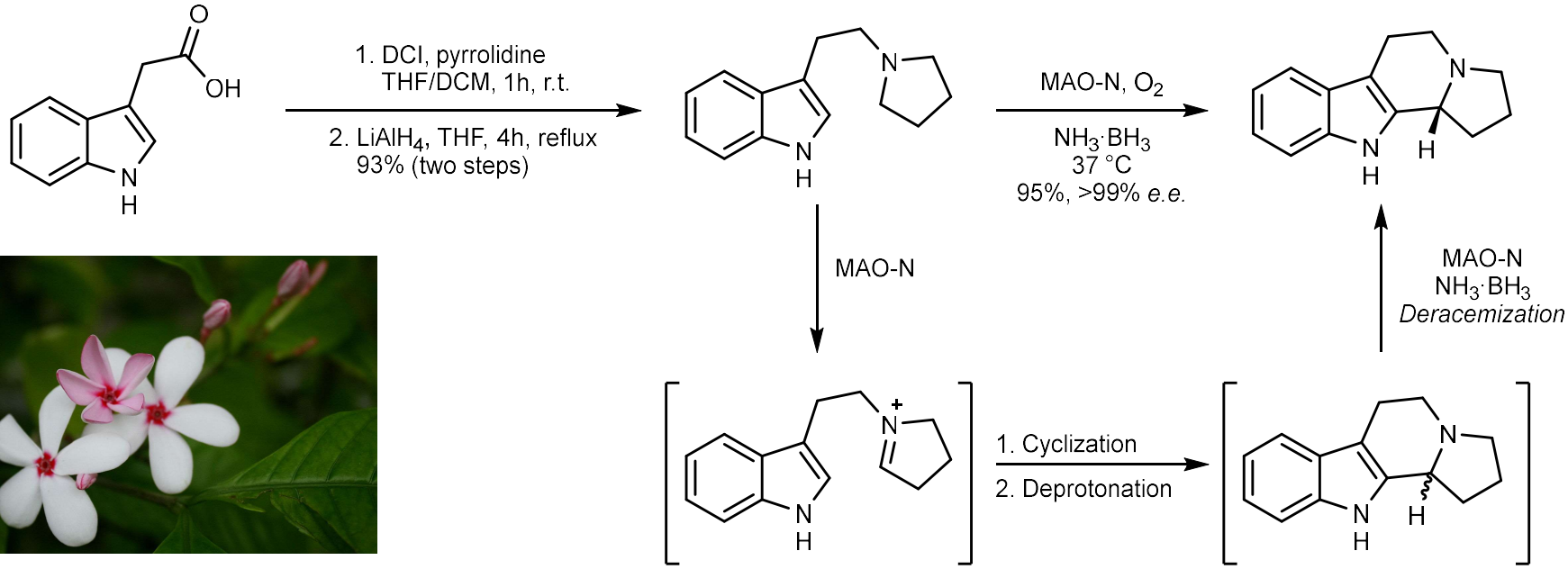
J. Am. Chem. Soc. **2018**, *140*, 1165-1169.

Group Problem #3



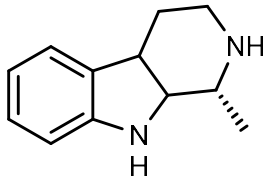
How would you prepare this natural product using biocatalysis?

Turner's Synthesis of Harmicine:

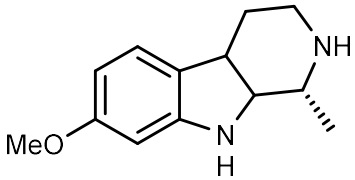


Kopsia griffithii

Similarly prepared by deracemization:



elgegnine

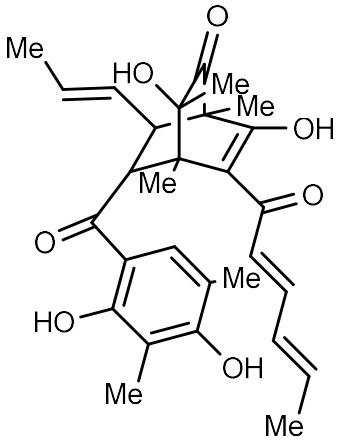


leptaflorin

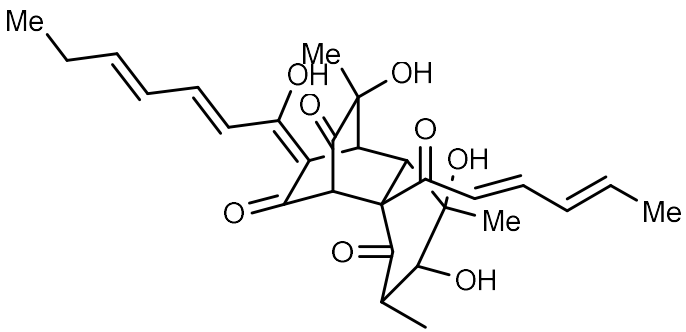
J. Am. Chem. Soc. **2013**, *135*, 10863-10869.

Interlude: NP Synthesis

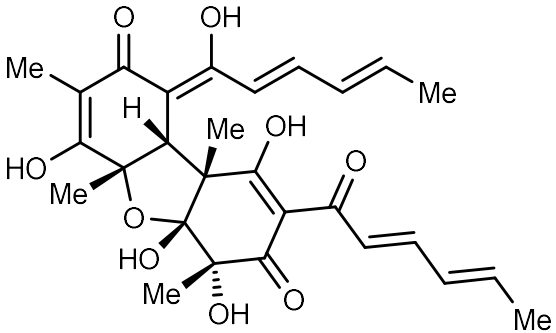
Natural products formed by biocatalytic cascades (Oxidative dearomatization, OQ-methide):



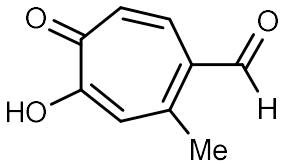
sorbiquniol
Gulder, 2017



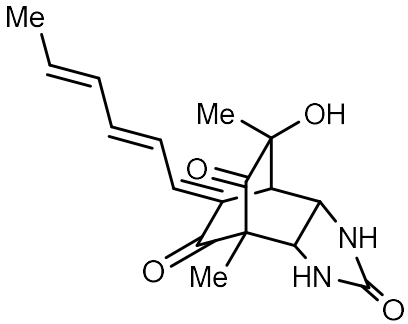
trichodimerol
Gulder, 2017



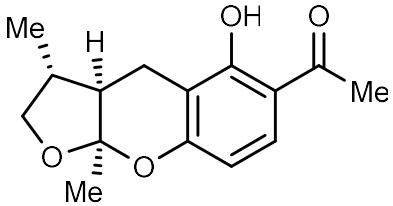
bisvertinolone
Gulder, 2018



stipitatic aldehyde
Narayan, 2018



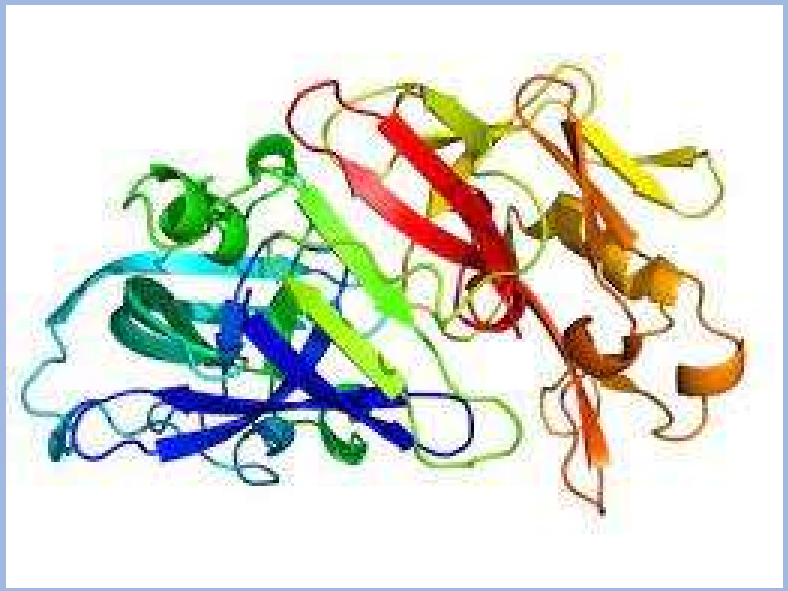
urea sorbicillinoid
Narayan, 2018



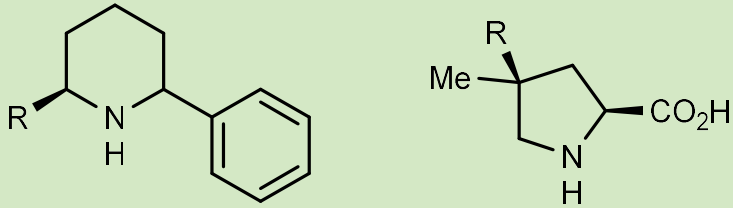
(-)-xyloketal D
Narayan, 2019

Nat. Chem. **2018**, *10*, 119-125.; *ACIE* **2017**, *56*, 12888-12891.; *ACIE* **2018**, *57*, 14650-14653. *J. Am. Chem. Soc.* ***2019***, *141*, 20269-20277.

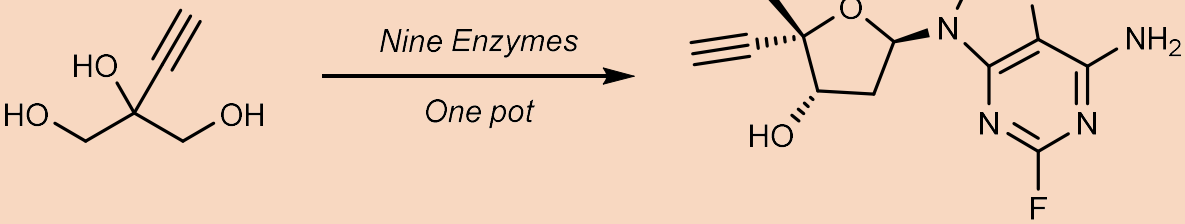
Outline



Enzyme classifications



Biocatalytic cascades for the synthesis of nitrogen heterocycles



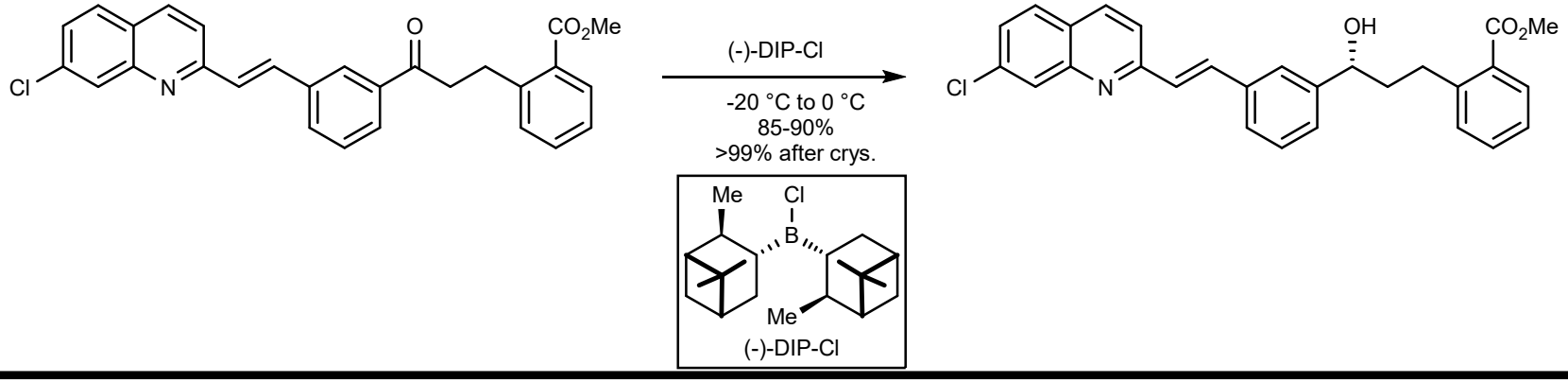
MERCK INVENTING FOR LIFE

CODEXIS®

Pharmaceuticals prepared by biocatalytic cascades

Montelukast

Original synthesis:



Biocatalysis approach:

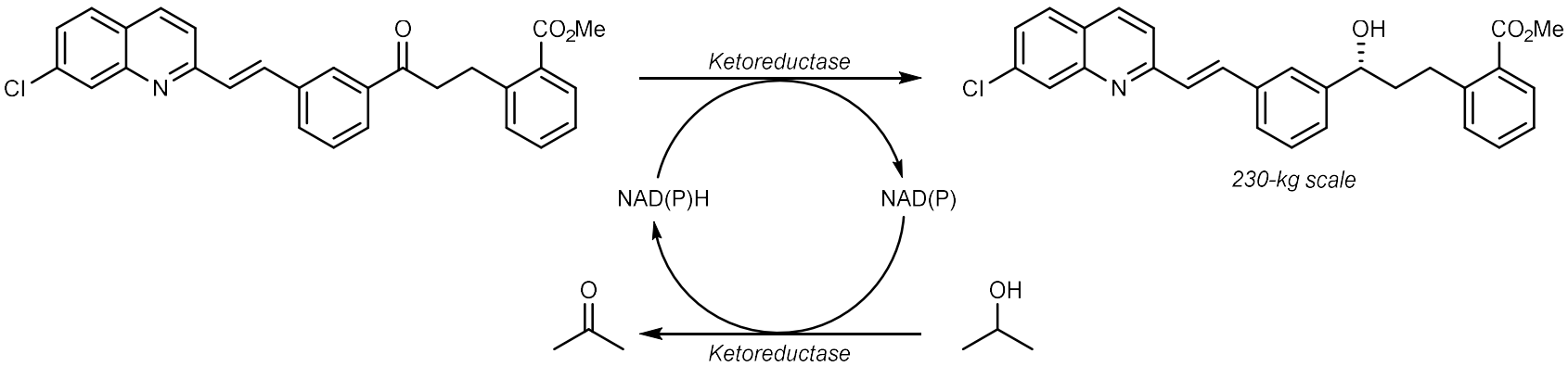
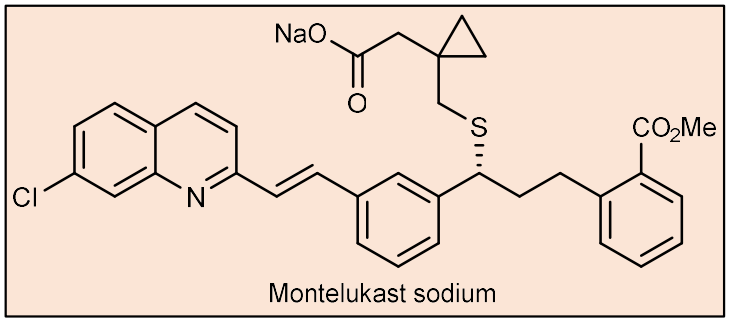


Table 5. **Process conditions and performances of the DIP-Cl process vs the KRED process**

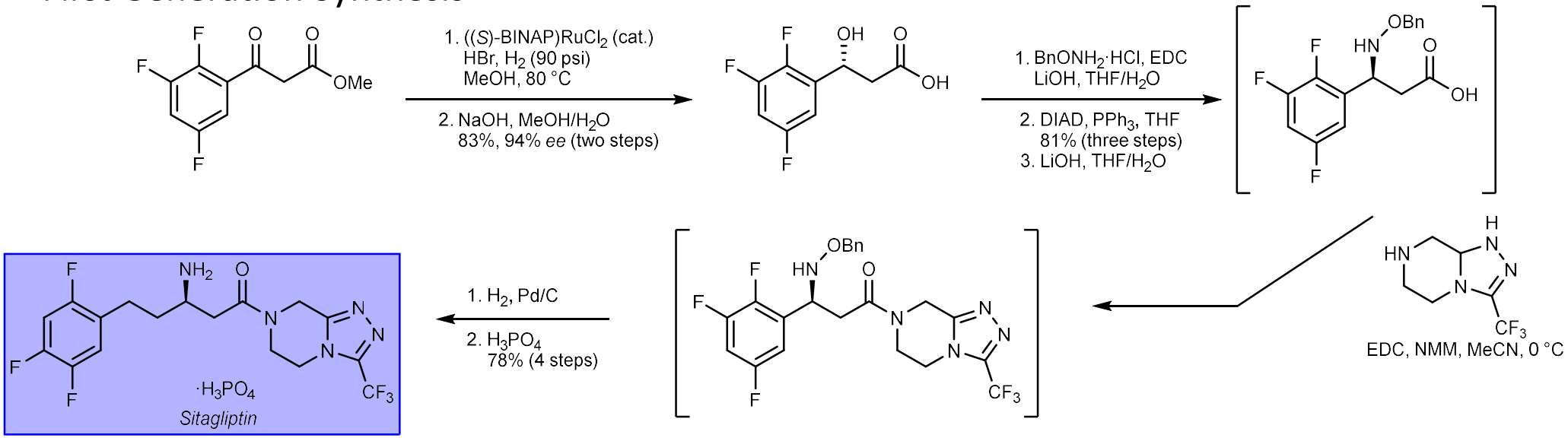
	(−)-DIP-Cl	KRED
substrate loading	~100 g/L	100 g/L
reaction conditions	moisture sensitive	aqueous
reagent or catalyst loading	at least 1.8 equiv (at least 150 wt %)	3−5 wt %
optical purity	crystallization needed to upgrade >99% ee	crude product is >99.9% ee (upgrade not needed)
yield	85−90%	90−98%



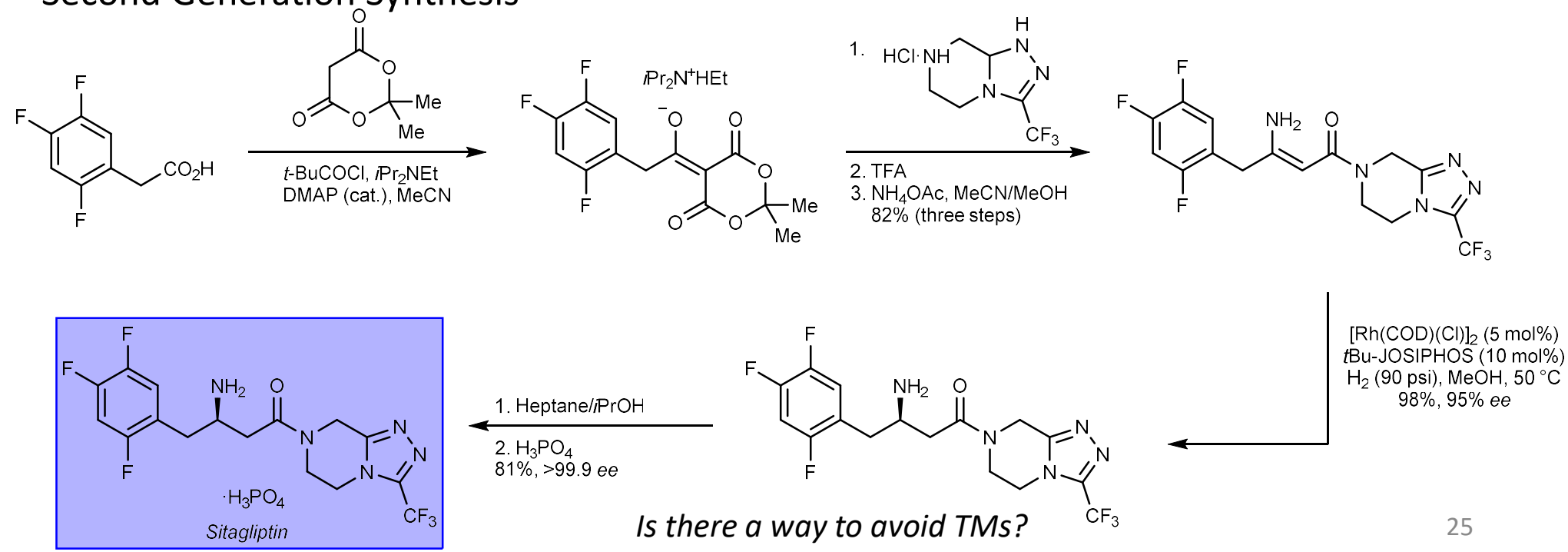
OPRD **2010**, *14*, 193-198.

Sitagliptin

First Generation Synthesis



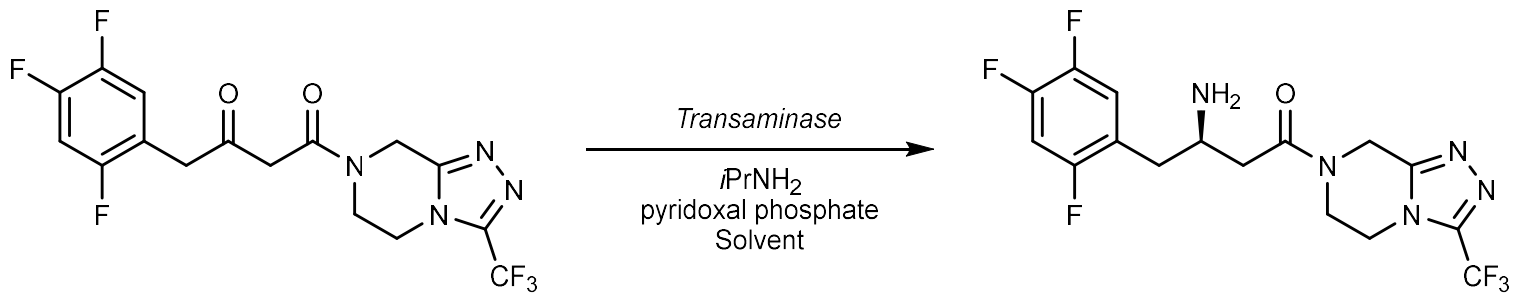
Second Generation Synthesis



Is there a way to avoid TMs?

ORPD **2005**, *9*, 634-639. *J. Am. Chem. Soc.* **2009,** *131*, 8798-8804.

Sitagliptin-Optimization



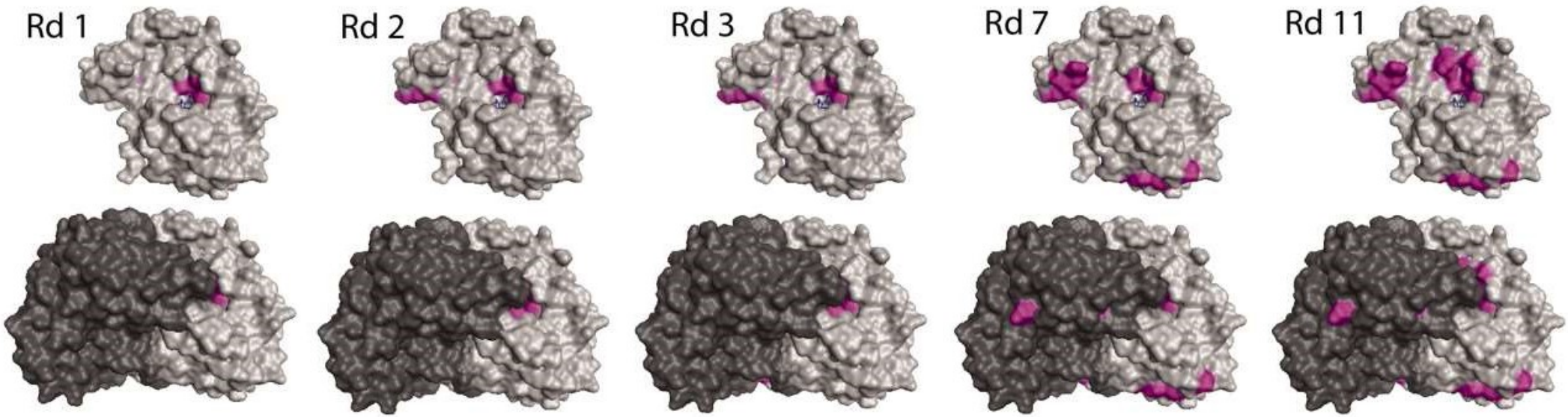
Added Mutations*	[Substrate] in g/l	Assay changes	Round identified	Improvement over parent†
S223P	2		1a	Not active
Y26H;‡ V65A;‡ V69G; F122I; A284G	2		1b	first active
H62T; G136Y; E137I; V199I; A209L; T282S	2		2	75
S8P; H26Y; G69C; M94I; I137T; G215C	5	5% DMSO to 5% MeOH; RT to 30°C	3	9
L61Y; C69T; Y136F; T137E	10	0.5 to 1 M iPrNH$_2$; pH 7.5 to pH 8.5	4	4
D81G; I94L; I96L; T178S; L269P; P297S; S321P	40	5 to 10% MeOH; 30 to 45°C	5	1.4
Y60F; L94I; A169L; S178T; G217N; L273Y	100	10 to 20% MeOH	6	1.6
S124H	100	20% MeOH to 25% DMSO	7	1.1
I122M; H124N	100		8	1.1
Q329H	100		9	1.9
N124T; Y150S; V152C; H329Q	50	25 to 50% DMSO; 0.5% acetone	10	2
S126T	50		11	1.4

0.7% conversion/24h

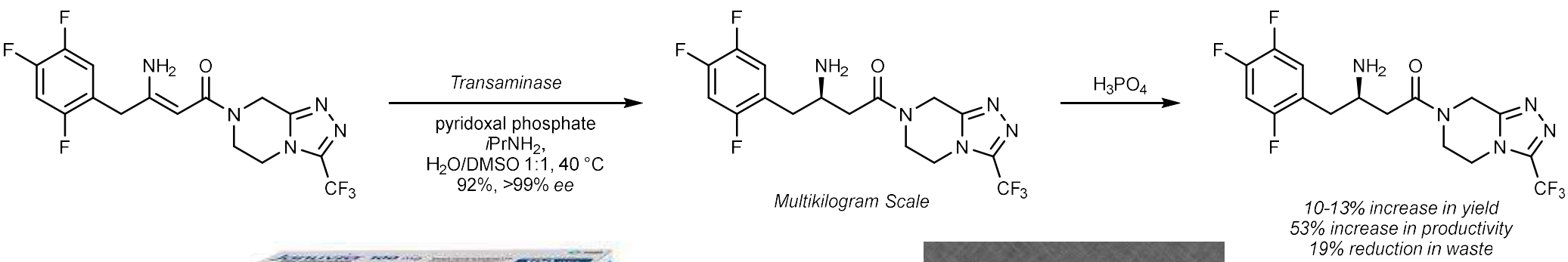
Science **2010**, *329*, 305-309.

Sitagliptin —a "Formal" Cascade

Effect of directed evolution on the binding site

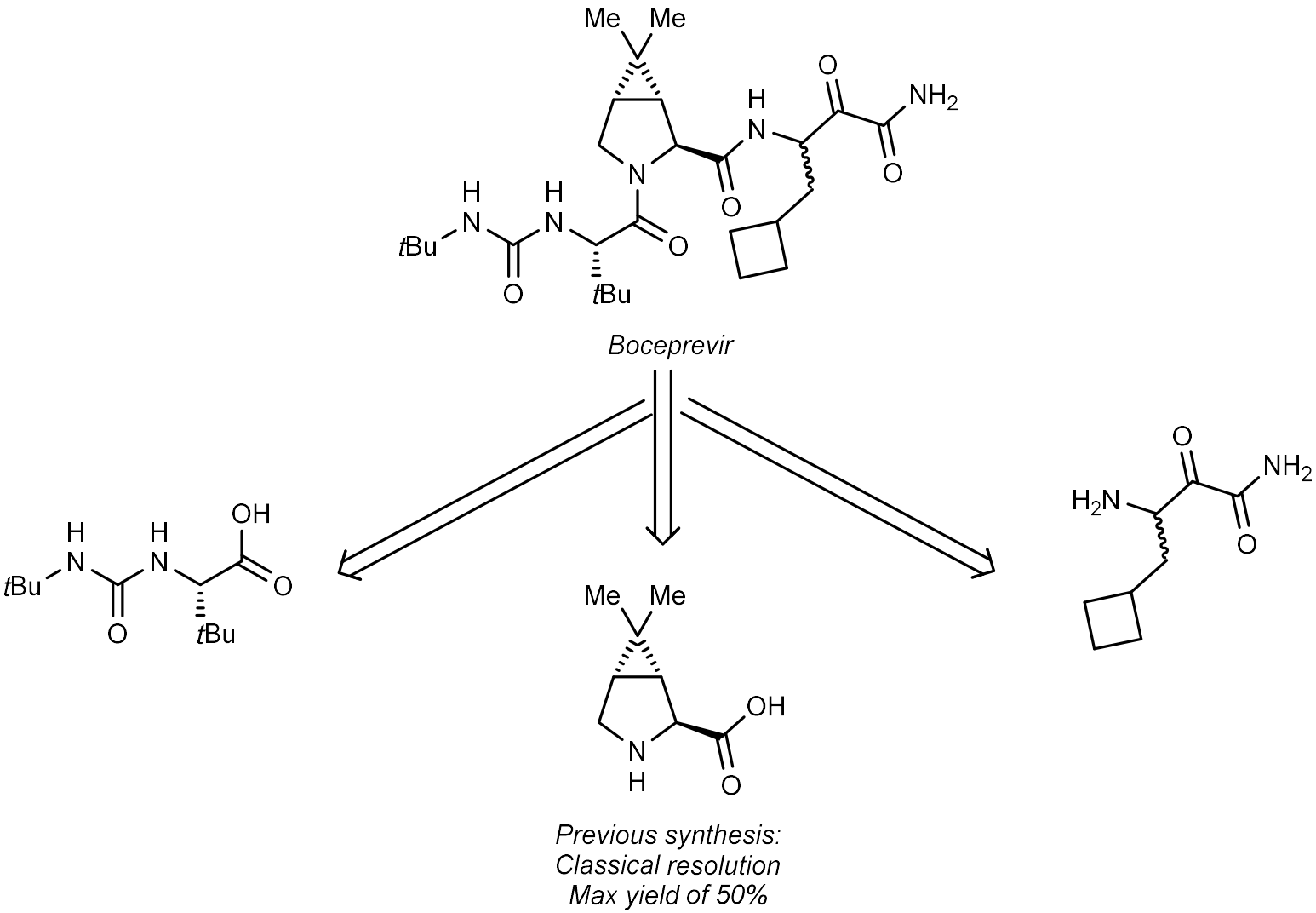


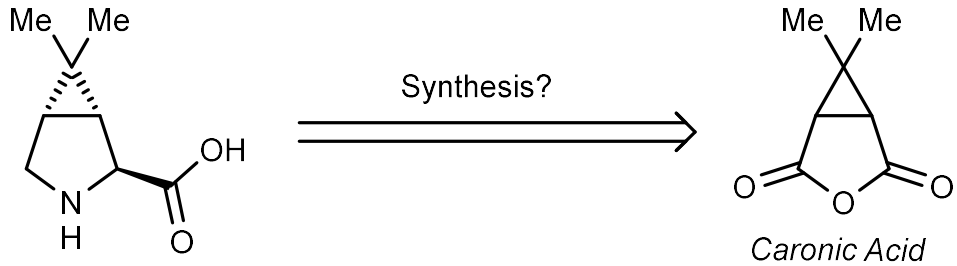
Final Route:



Science **2010**, *329*, 305-309.

Boceprevir





J. Am. Chem. Soc. **2012**, *134*, 6467-6472.

Boceprevir

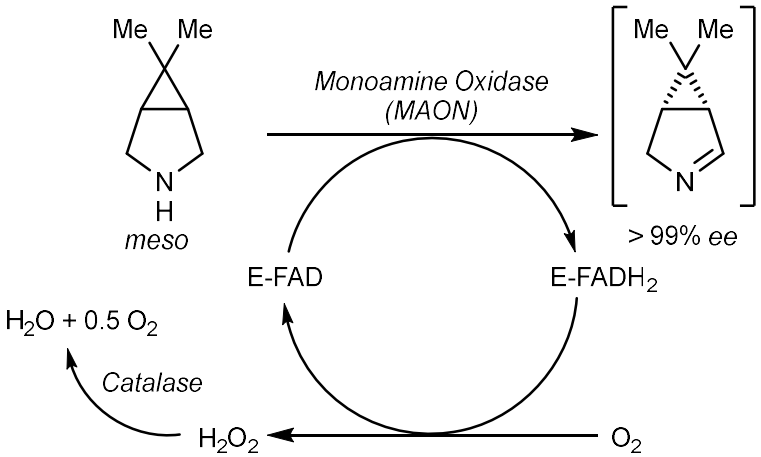
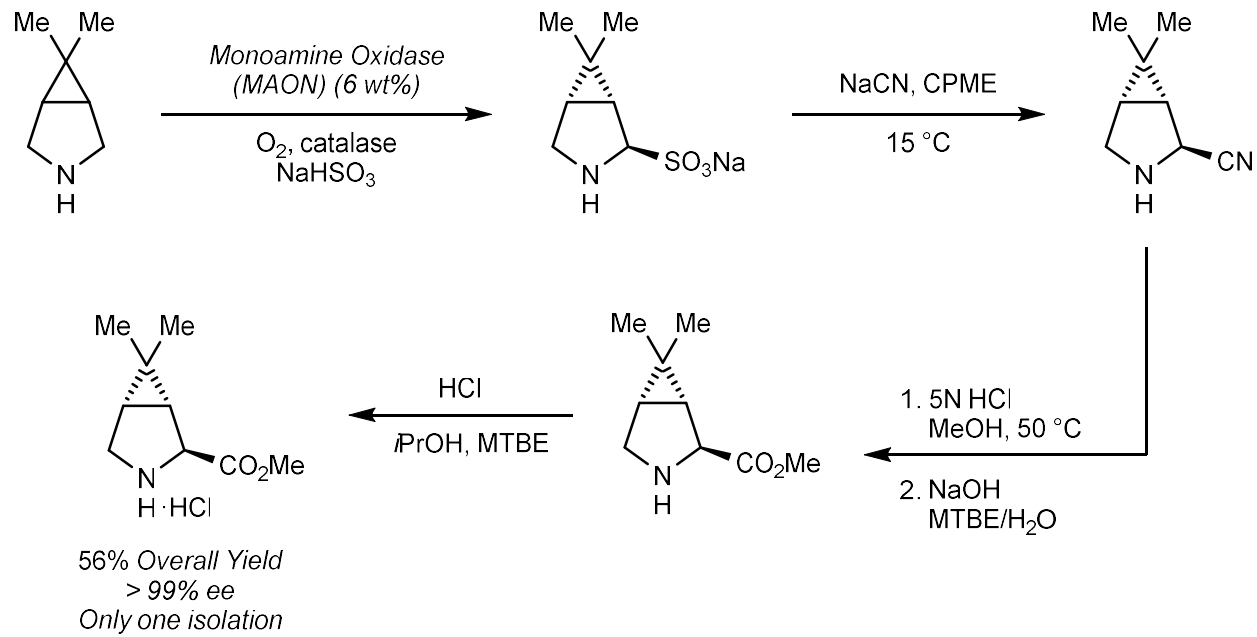


Table 1. Summary of MAON Improvement over the Four Rounds of Evolution

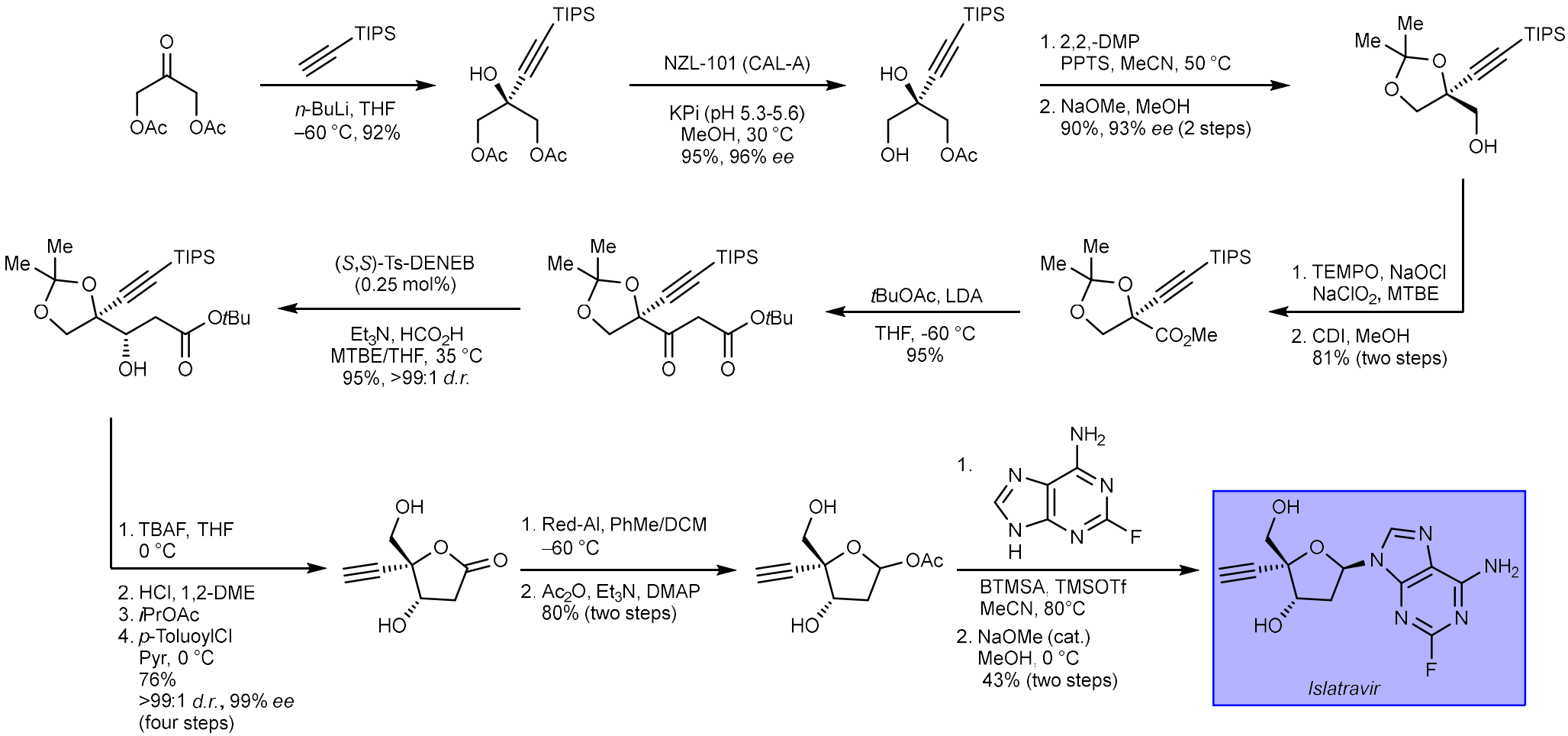
round	MAON variant	mutation	diversity source	improvement
	wild type	*A. niger* wild type		
1	156	*A. niger* + A289V, K348Q	active site targeted library	activity
2	274	many (see the Supporting Information)	*A. niger* and *A. oryzae*	thermal stability at 40 °C
2	291	150 + S465G	diversity from round 1	activity and solubility
3	301/304/308	many (see the Supporting Information)	MAON274 and MAON291	thermal stability at 50 °C
4	401	304 + F382L	active site library	8-fold increase in activity



J. Am. Chem. Soc. **2012**, *134*, 6467-6472.

Islatravir

First Generation:



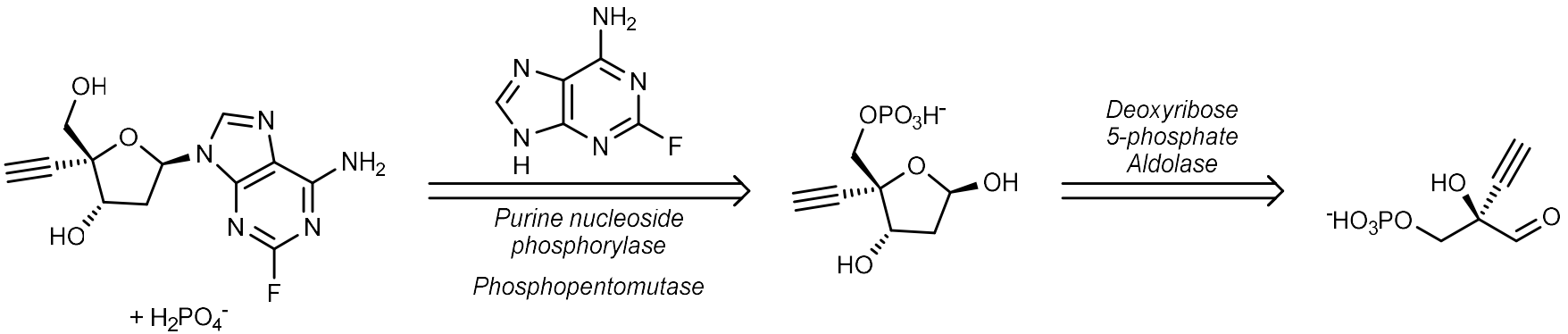
Disadvantages:

1. Multiple protecting group manipulations
2. Anomeric bond-forming step has poor yield
3. Long route—17 steps!

Org. Lett. **2017**, *19*, 926-929.

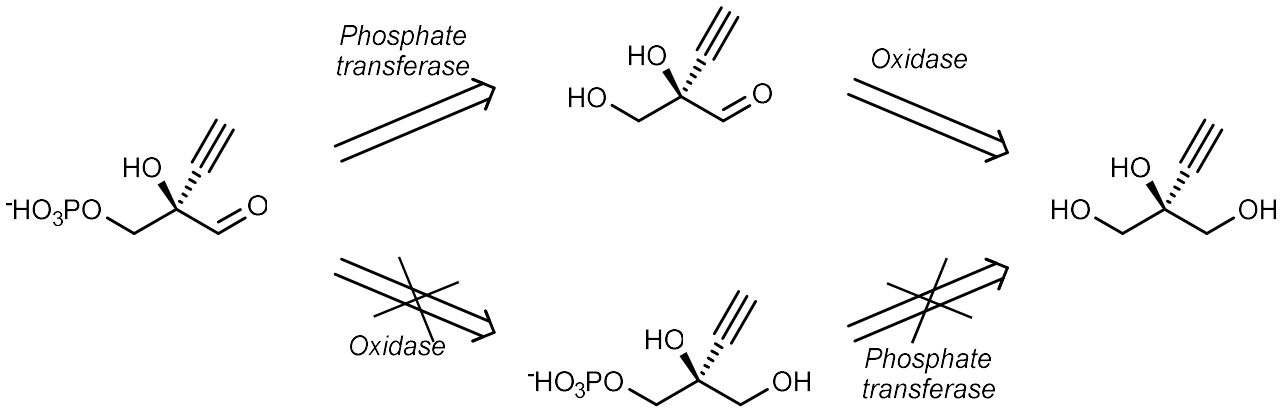
Islatravir

The Bacterial Nucleoside Salvage Pathway—Applied to Islatravir:



Could this cascade work in reverse to give the product? Accept unnatural substrates?

Retrosynthesis of the triol:

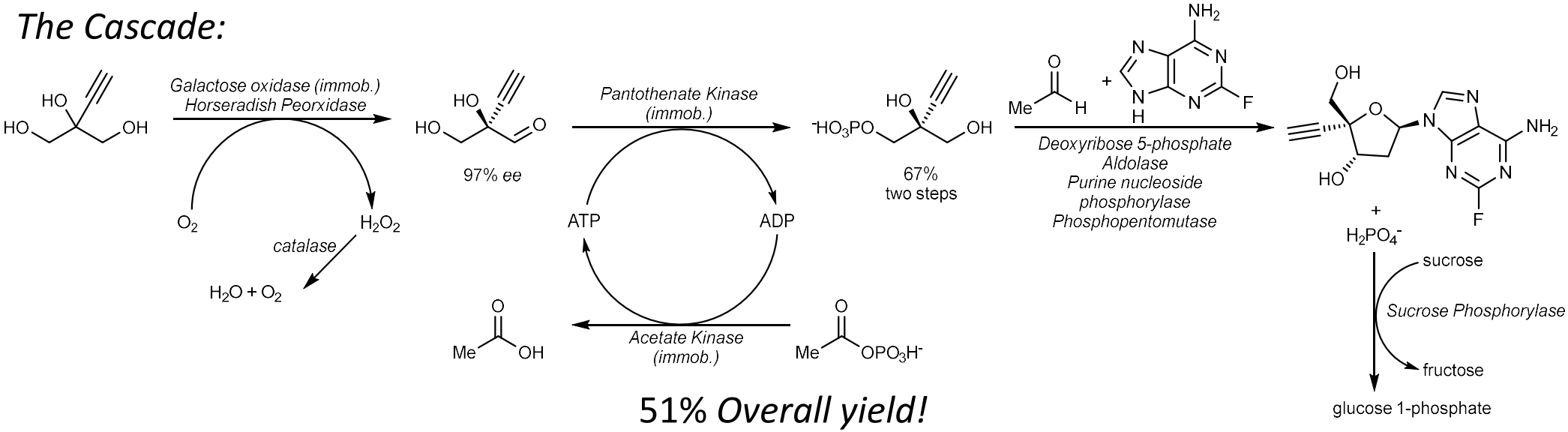


- Enzymes had been shown to work individually with unnatural substrates, not in sequence
- Major equilibrium challenges were anticipated
- How to setup a process like this?

Science **2019**, *366*, 1255-1259. For synthesis of the nucleoside: *OPRD* **2021**, *25*, 395-404. For synthesis of the triol: *ChemRxiv* **2021.** 10.26434/chemrxiv.14502744.v1

Islatravir

The Cascade:



51% *Overall yield!*

Table 1. Properties and performance of evolved enzymes used in the biocatalytic pathway.

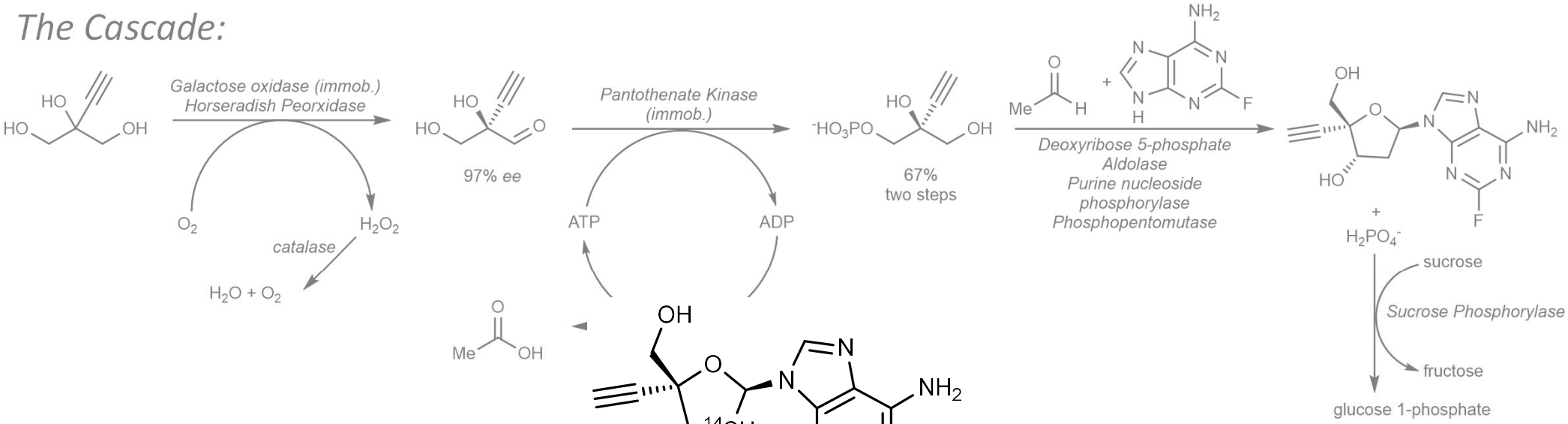
					Starting enzyme		Evolved variant	
Enzyme	Source organism	Evolution focus	Rounds of evolution	Global amino acids changed (no.)	Conversion (selectivity)	Loading (%)*	Conversion (selectivity)	Loading (%)*
Oxidase (GOase)	*Fusarium graminearum*	Stereoselectivity Activity	12	34	Variant M1:33%†‡ (8:92 *R*:*S*) Variant F2: 8%†‡ (40:60 *R*:*S*)	100	80%†§ (90:10 *R*:*S*)	20
PanK	*E. coli*	Activity	3	10	<1% (5:1 *R*:*S*)†¶	10	>95% (10:1 *R*:*S*)†#	10
DERA	*S. halifaxensis*	Acetaldehyde tolerance	2	11	97%**[>98:1:1 (3*S*4*R*):(3*R*4*R*):(3*S*4*S*)]	5	97%**[>98:1:1 (3*S*4*R*):(3*R*4*R*):(3*S*4*S*)]	0.2
PPM	*E. coli*	Activity	2	5	0.5%††	0.5	34%††	0.5
PNP	*E. coli*	Activity	4	7	0.18%‡‡ (>99.5:0.5 *dr*)	0.125	62%‡‡ (>99.5:0.5 *dr*)	0.125

*Enzyme loading refers to the mass of lyophilized clarified cell lysate relative to the mass of the reaction substrate. Results may reflect improvements in enzyme expression as well as activity. †Reaction with nonimmobilized enzymes. ‡GOase-M1-Strep and F2-Strep: 172 mM **6**, pH 7.5, 0.2 mM CuSO4, 25°C, 4 hours. §GOAse-13BB-His: 258 mM **6**, pH 7.5, 0.2 mM CuSO4, 25°C, 4 hours. ¶44 mM **7**, pH 7.5, 20°C, 18 hours. #235 mM **7**, pH 6.4, 20°C, 18 hours. **142 mM **5**, 420 mM acetaldehyde, pH 7.2, 30°C, 24 hours. ††15 mM **4**, 5 mM $MnCl_2$, pH 7.5, 40°C, 18 hours. ‡‡13 mM **1b**, pH 7.5, 40°C, 16 hours.

Science **2019**, *366*, 1255-1259.

Islatravir

The Cascade:



Prepared using cascade
34.1% radiochemical yield
OPRD **2021**, *25*, 516-521.

Table 1. Properties and performance of evolved enzy

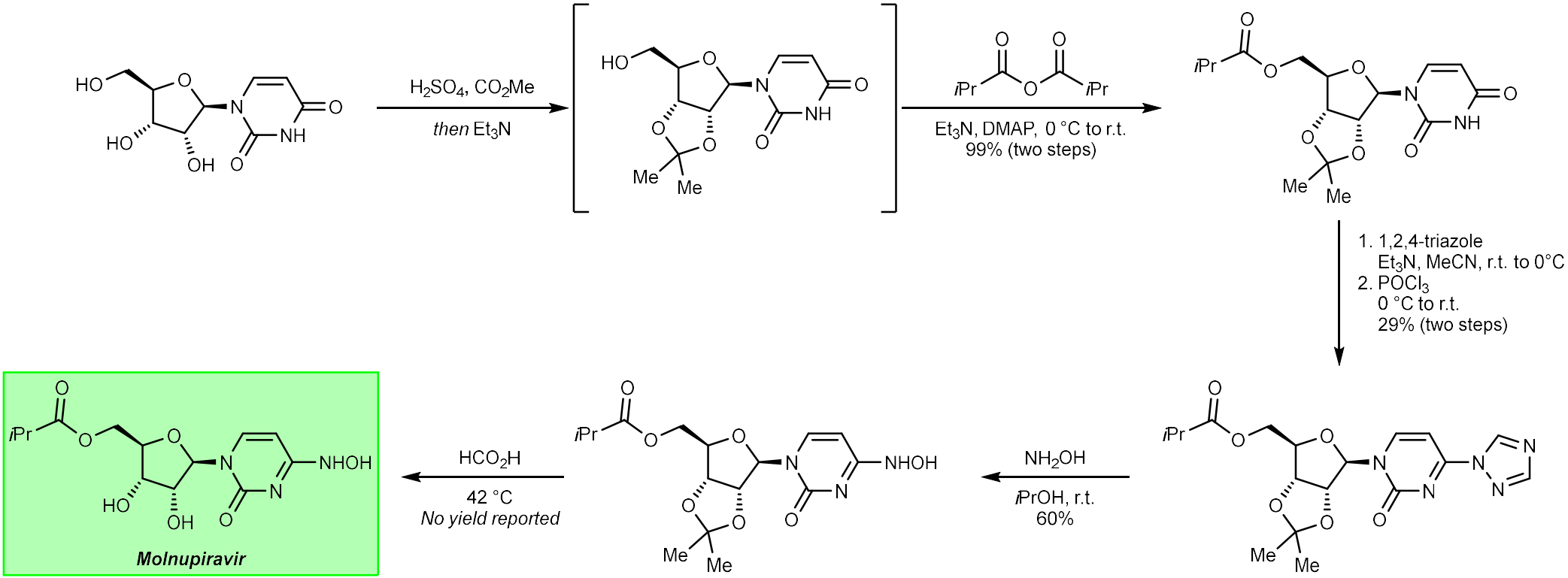
Enzyme	Source organism	Evolution focus	Roun evolution	acids changed (no.)	ting enzyme rsion (selectivity)	Loading (%)*	Evolved variant Conversion (selectivity)	Loading (%)*
Oxidase (GOase)	*Fusarium graminearum*	Stereoselectivity Activity	12	34	Variant M1:33%†‡ (8:92 *R*:*S*) Variant F2: 8%†‡ (40:60 *R*:*S*)	100	80%†§ (90:10 *R*:*S*)	20
PanK	*E. coli*	Activity	3	10	<1% (5:1 *R*:*S*)†¶	10	>95% (10:1 *R*:*S*)†#	10
DERA	*S. halifaxensis*	Acetaldehyde tolerance	2	11	97%**[>98:1:1 (3*S*4*R*):(3*R*4*R*):(3*S*4*S*)]	5	97%**[>98:1:1 (3*S*4*R*):(3*R*4*R*):(3*S*4*S*)]	0.2
PPM	*E. coli*	Activity	2	5	0.5%††	0.5	34%††	0.5
PNP	*E. coli*	Activity	4	7	0.18%‡‡ (>99.5:0.5 *dr*)	0.125	62%‡‡ (>99.5:0.5 *dr*)	0.125

*Enzyme loading refers to the mass of lyophilized clarified cell lysate relative to the mass of the reaction substrate. Results may reflect improvements in enzyme expression as well as activity. †Reaction with nonimmobilized enzymes. ‡GOase-M1-Strep and F2-Strep: 172 mM **6**, pH 7.5, 0.2 mM CuSO4, 25°C, 4 hours. §GOAse-13BB-His: 258 mM **6**, pH 7.5, 0.2 mM CuSO4, 25°C, 4 hours. ¶44 mM **7**, pH 7.5, 20°C, 18 hours. #235 mM **7**, pH 6.4, 20°C, 18 hours. **142 mM **5**, 420 mM acetaldehyde, pH 7.2, 30°C, 24 hours. ††15 mM **4**, 5 mM $MnCl_2$, pH 7.5, 40°C, 18 hours. ‡‡13 mM **1b**, pH 7.5, 40°C, 16 hours.

Science **2019**, *366*, 1255-1259.

Molnupiravir

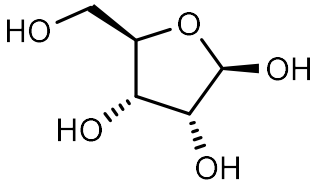
Patent route:



Disadvantages of route:

- Relies on supply-chain-dependent uridine
- Maximum of 17% yield
- Multiple isolation steps
- Protecting groups
- Not supply chain friendly

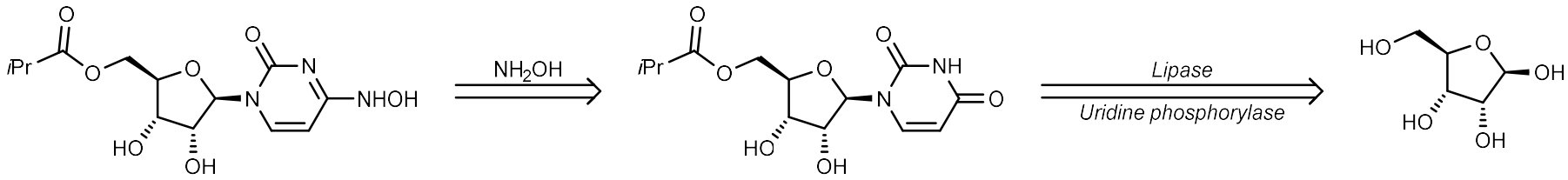
Could a route be prepared from ribose?



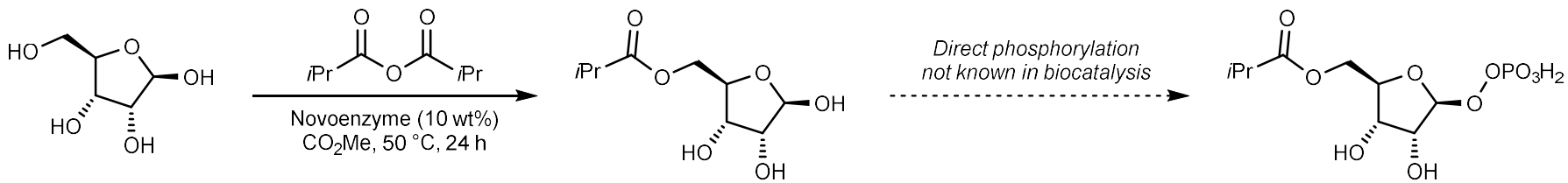
Chem. Commun. **2020**, *56*, 13363-13364. *Chem Rxiv*. **2020**, 10.26434/chemrxiv.13472373.v1.

Molnupiravir

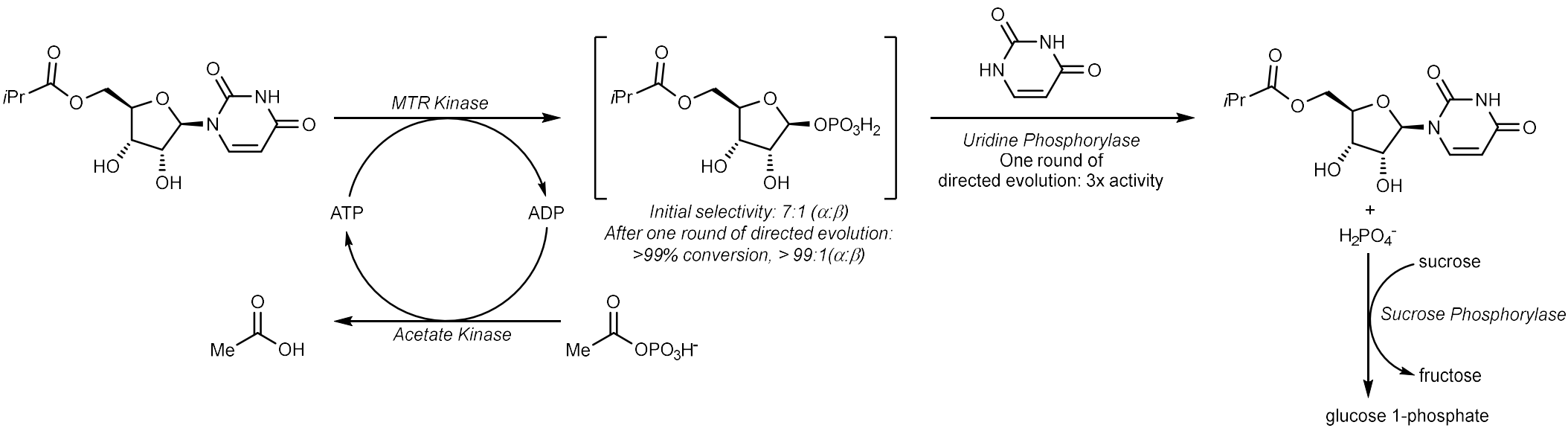
Retrosynthetic Analysis:



Initial Route:



Development of a novel biocatalytic cascade:

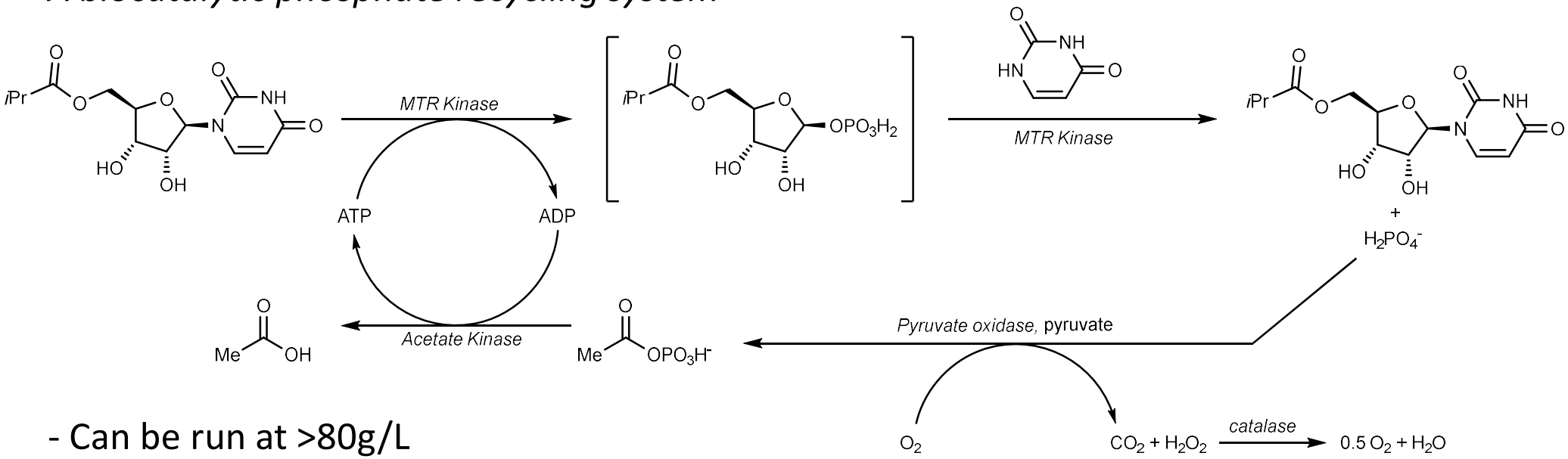


Could further improvements be made by recycling the inorganic phosphate?

Chem Rxiv. **2020**, 10.26434/chemrxiv.13472373.v1.

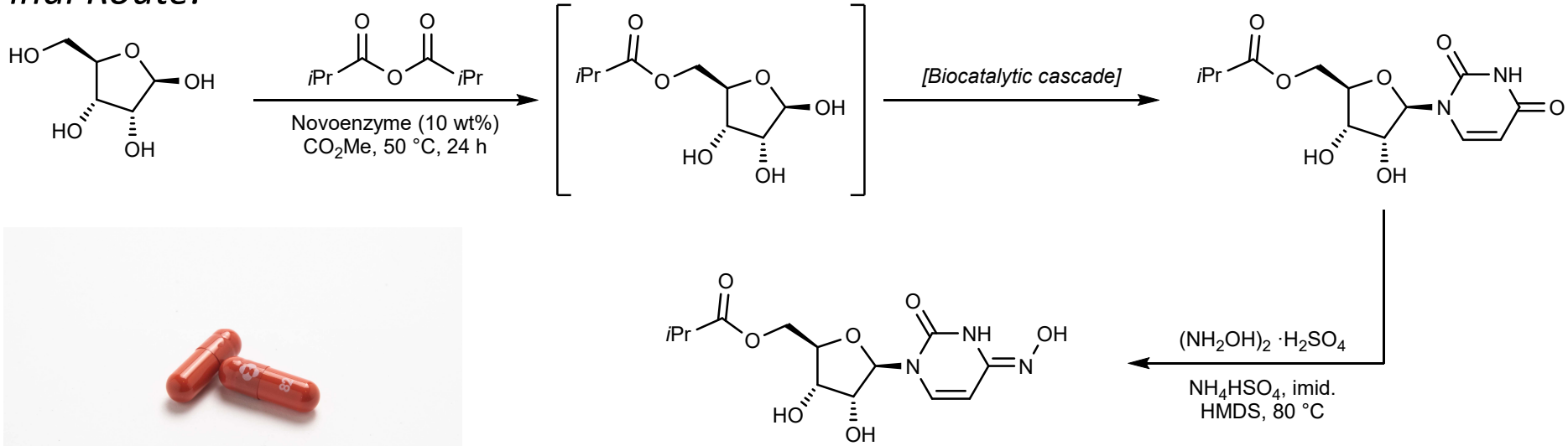
Molnupiravir

A biocatalytic phosphate recycling system



- Can be run at >80g/L

Final Route:



3 Steps
Decagram Scale
69% overall yield
70% shorter; ~7x yield improvment

Chem Rxiv. **2020**, 10.26434/chemrxiv.13472373.v1.

Conclusions

Conclusions:

- Biocatalytic cascades are fantastic at:
  - Performing protecting group free synthesis
  - Performing exceptionally chemoselective and stereoselective transformations
  - Obtaining high-value chemicals from cheap starting materials

- Limitations
  - Evolution of enzymes is often necessary for scaling-up
  - Finding the “right” enzyme can be very tricky
  - Biocatalysis is not non-specialist friendly

Future Directions:

- Cascades with P450s
- Evolving or discovering aldolases to expand possible donors
- Preparation of artificial metallenzymes in cascades (few examples)
- Implementation of a directed evolution system for non-specialists
- Combining chemocatalysis and biocatalysis (beyond reductions)
- Designing systems that can be done on meaningful scale (beyond industry)

Further Reading

ChemSusChem
Chemistry–Sustainability–Energy–Materials

Chemistry Europe
European Chemical Societies Publishing

Review | Full Access

Broadening the Scope of Biocatalysis in Sustainable Organic Synthesis

Prof. Roger A. Sheldon, Prof. Dean Brady

First published: 01 April 2019 | https://doi.org/10.1002/cssc.201900351 | Citations: 102

RETURN TO ISSUE | < PREV REVIEW NEXT >

Artificial Biocatalytic Linear Cascades for Preparation of Organic Molecules

Joerg H. Schrittwieser[†], Stefan Velikogne[‡], Mélanie Hall[†], and Wolfgang Kroutil[*†‡]

View Author Information

Cite this: *Chem. Rev.* 2018, 118, 1, 270−348
Publication Date: May 8, 2017
https://doi.org/10.1021/acs.chemrev.7b00033
Copyright © 2017 American Chemical Society
RIGHTS & PERMISSIONS

Article Views	Altmetric	Citations
13328	-	301

LEARN ABOUT THESE METRICS

Review | Free Access

Biocatalysis: A Pharma Perspective

Joseph P. Adams, Murray J. B. Brown, Alba Diaz-Rodriguez, Richard C. Lloyd, Gheorghe-Doru Roiban

First published: 05 May 2019 | https://doi.org/10.1002/adsc.201900424 | Citations: 43

SECTIONS PDF TOOLS SHARE

Constructing Biocatalytic Cascades: In Vitro and in Vivo Approaches to de Novo Multi-Enzyme Pathways

Scott P. France[†], Lorna J. Hepworth[†], Nicholas J. Turner[*], and Sabine L. Flitsch[*]

View Author Information

Cite this: *ACS Catal.* 2017, 7, 1, 710−724
Publication Date: December 12, 2016
https://doi.org/10.1021/acscatal.6b02979
Copyright © 2016 American Chemical Society
RIGHTS & PERMISSIONS Subscribed

Article Views	Altmetric	Citations
5063	4	220

LEARN ABOUT THESE METRICS

Share Add to Export RIS

PDF (3 MB) Discover

SUBJECTS: Chemical reactions, Alcohols, Peptides and proteins, Amines, Genetics