CHEM-E4109

MODERN METHODS IN BIOCATALYSIS

chapter #10: enzymes in non-natural reactions

Jan Deska
Bioorganic
Chemistry

1.4.2022

That's nice, right? But how far can you go?

Synthetic Biology & Metabolic Engineering can...

- ✓ effectively regulate pathways that are intrinsic in life
- ✓ access structures that already found somewhere in nature

Synthetic Biology & Metabolic Engineering fails to...

- provide solutions for truly synthetic targets
- offer bio-based solutions for many traditional chemistries
- engage in anything that lacks precedence in biosynthesis

SCOPE & LIMITATIONS OF ENZYMES

The bright side...

- ✓ highly productive enzymatic systems for the preparation of chiral building blocks, e.g. alcohols, amines, amino acids,...
- ✓ mild reaction conditions in absence of hazardous solvents or reagents
- ✓ modern biotechnology allows for effective catalysts tuning
- ✓ easy to combine multiple biocatalysts one-pot: design of catalytic cascades

SCOPE & LIMITATIONS OF ENZYMES

The dark side...

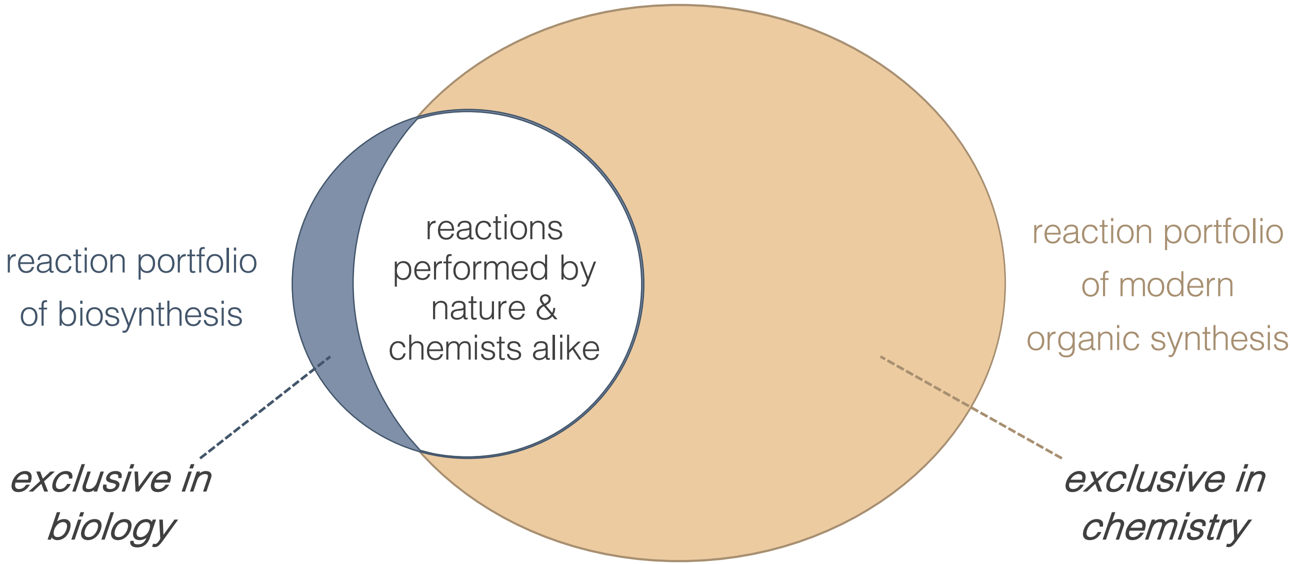
- ✓ **few** privileged enzymatic transformations of synthetic relevance
- ✓ **some** biosynthetically encoded but inadequately applicable reactions
- ✓ **many** abiotic purely synthetic molecular manipulations

= limited reaction portfolio results in limited applicability in synthetic strategies

Biosynthesis vs chemical synthesis

- **few** privileged enzymatic transformations of synthetic relevance
- **some** biosynthetically encoded but inadequately applicable reactions
- **many** abiotic purely synthetic molecular manipulations

= limited reaction portfolio results in limited applicability in synthetic strategies

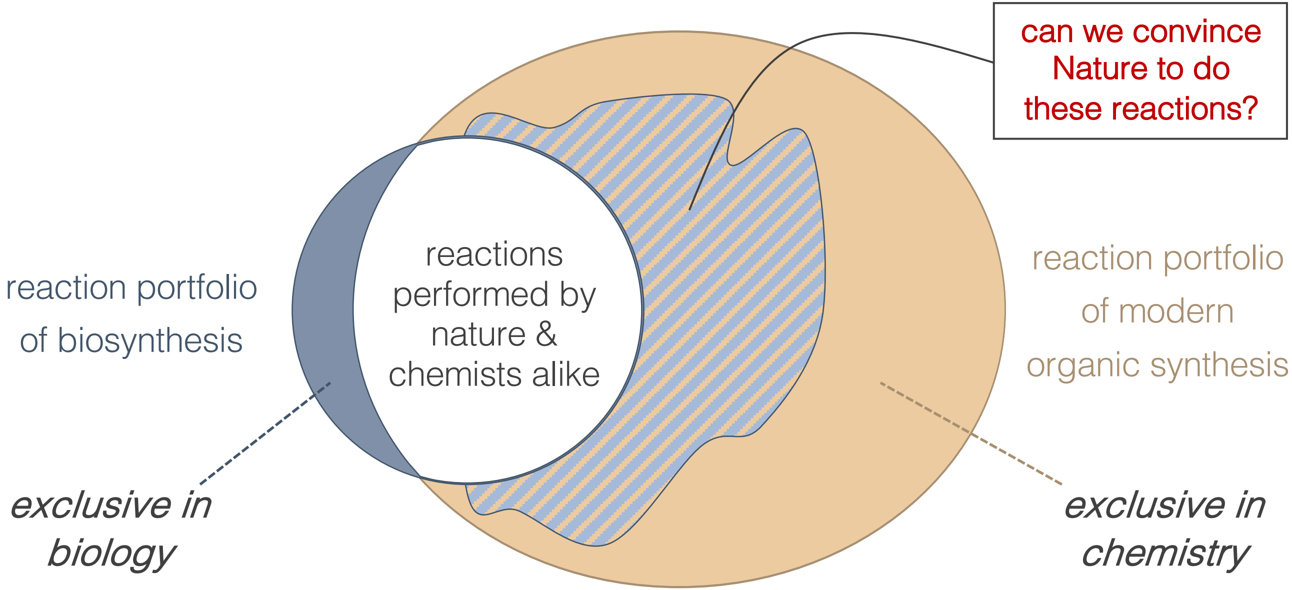


Jan Deska
Bioorganic
Chemistry

Biosynthesis vs chemical synthesis

- **few** privileged enzymatic transformations of synthetic relevance
- **some** biosynthetically encoded but inadequately applicable reactions
- **many** abiotic purely synthetic molecular manipulations

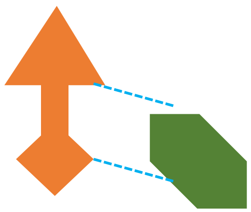
= limited reaction portfolio results in limited applicability in synthetic strategies



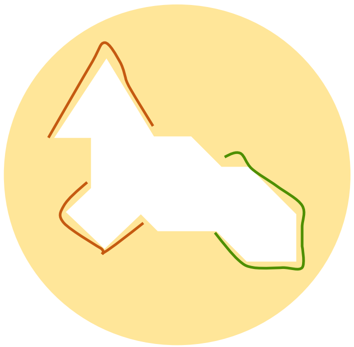
Jan Deska
Bioorganic Chemistry

De novo design

- identification of configurations of low-energy transition states
- mapping of a general binding pocket architecture



generic transition state



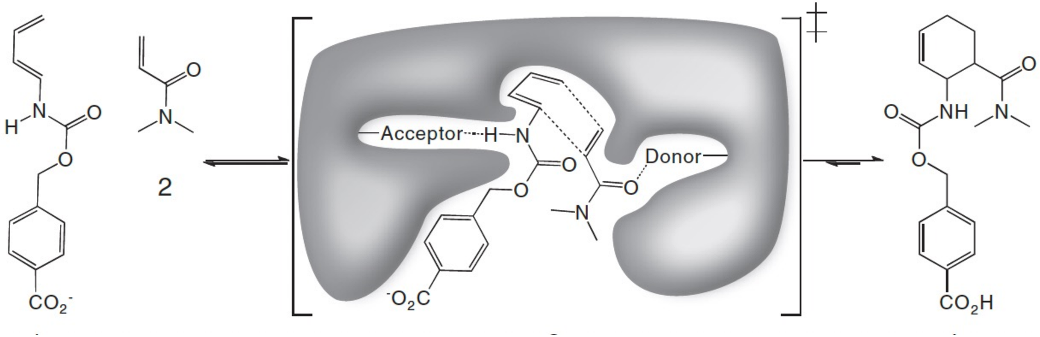
functional groups and orientation to
stabilize the envisioned transition state

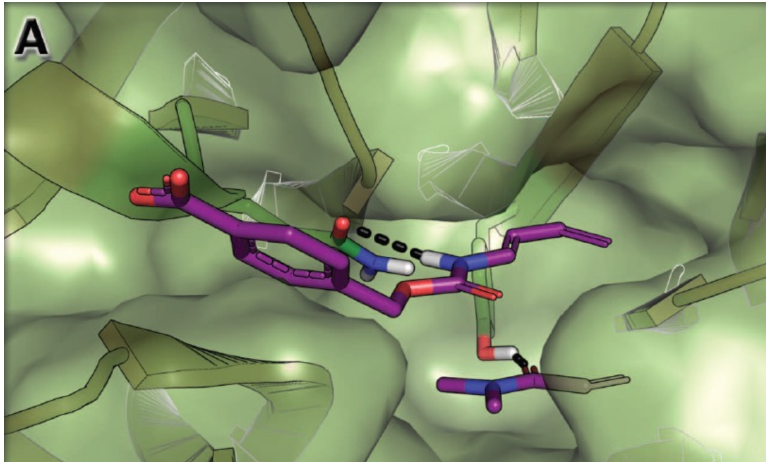
Jan Deska
Bioorganic
Chemistry

De novo design

- identification of configurations of low-energy transition states
- mapping of a general binding pocket architecture

✓ computer-guided selection of stable protein scaffolds to match the necessary shape
✓ introduction of required amino acid functionalities to the positions





Baker et al, *Science* **2010**, *329*, 309

Nature as Inspiration

Ronald Breslow

"In Biomimetic Chemistry, we take what we have observed in Nature and apply its principles to the invention of novel synthetic compounds that can achieve the same goals... As an analogy, we did not simply make larger versions of birds when we invented airplanes, but we did take the idea of the wing from Nature, and then used the aerodynamic principles in our own way to build a jumbo jet."

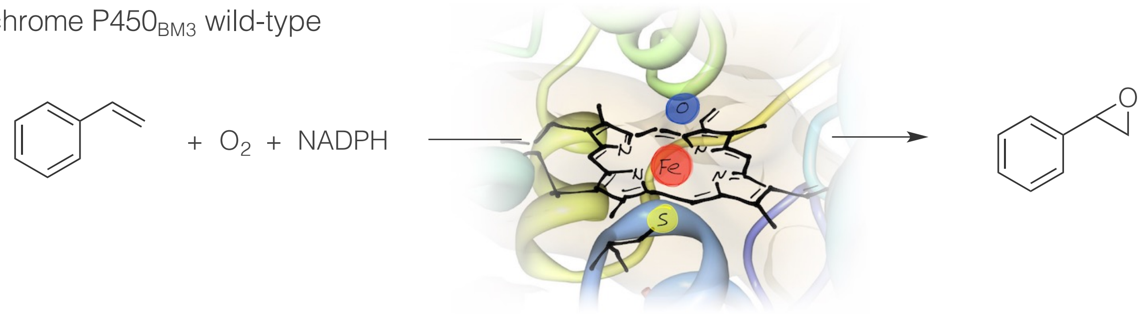
Synthetic compounds that mimic biological materials' functions or properties:

- bioactivity (medicinal chemistry)
- light-activated (photovoltaics & photocatalysis)
- natural binding modes (organocatalysis)

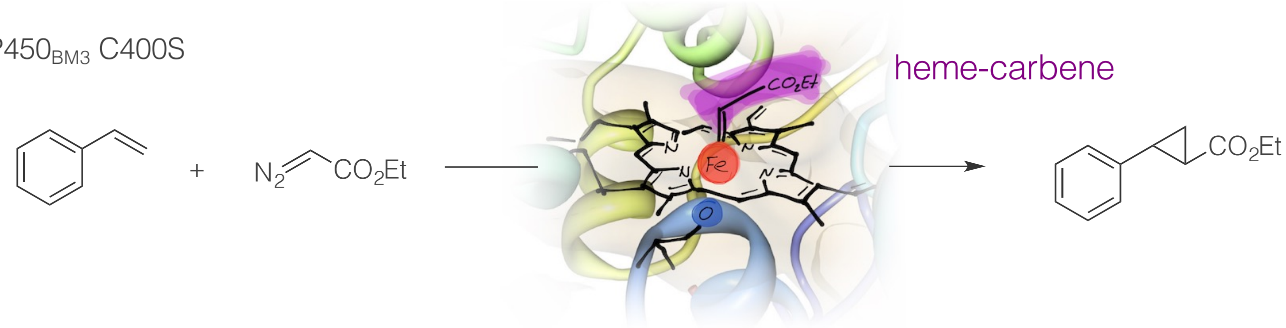
Reverting biomimetics?

- modern synthetic organic chemistry as a blueprint for natural catalysts
- chemoinspired artificial functions of biocatalysts

Cytochrome $P450_{BM3}$ wild-type



Cytochrome $P450_{BM3}$ C400S

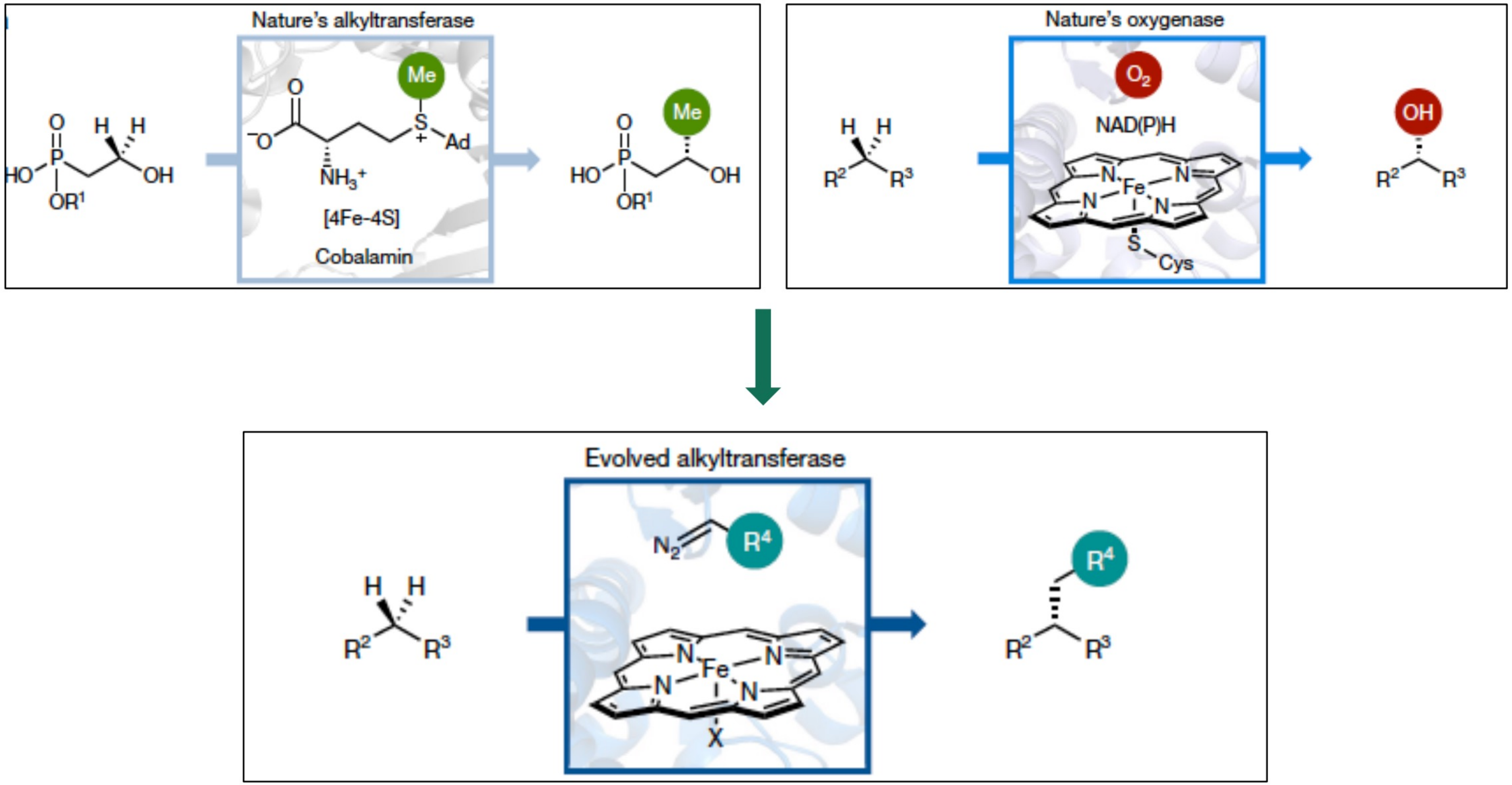


Frances Arnold

Nobel Prize in Chemistry, 2018

a) Coelho, Brustad, Kannan, Arnold, *Science* **2013**, *339*, 307-310; b) Coelho, Wang, Ener, Baril, Kannan, Arnold, Brustad, *Nature Chem. Biol.* **2013**, *9*, 485-487.

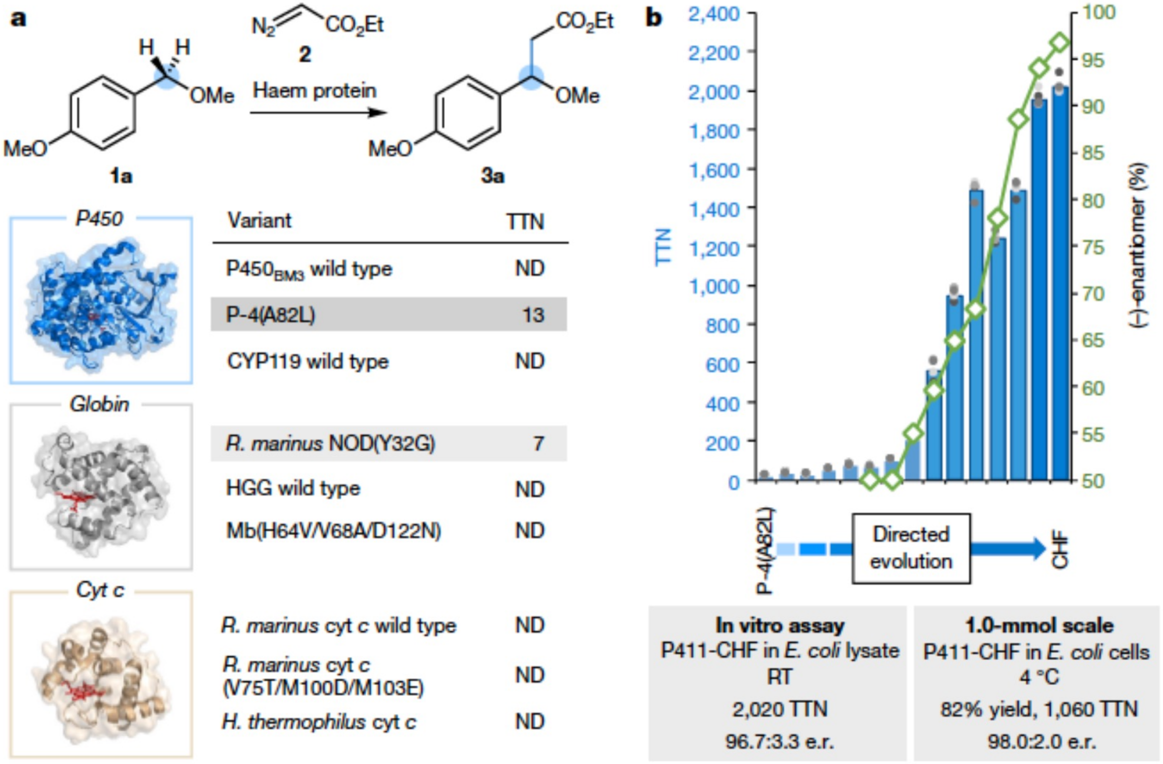
General concept for cyclopropanation, alkylation, amination…



Zhang et al. *Nature* **2019**, 67

Jan Deska
Bioorganic
Chemistry

Directed evolution as key for effective biocatalysts

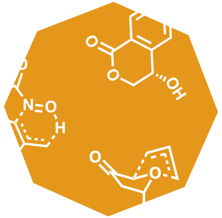


Zhang et al. *Nature* **2019**, 67

Jan Deska
Bioorganic
Chemistry

Sure… but why bother

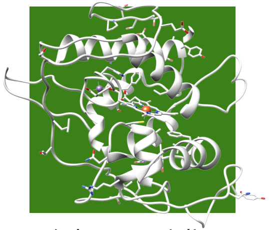
key chemical transformations



unprecedented in nature, yet template for the design of new enzymes

targeted activity mining & evolution

novel enzymatic activities

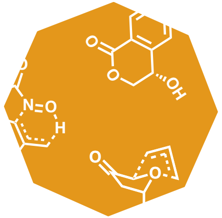


artificial biocatalytic modules enabling non-natural transformations

Jan Deska
Bioorganic
Chemistry

Sure… but why bother

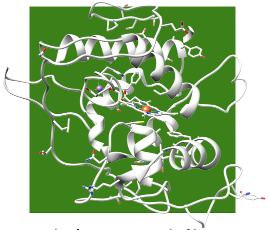
key chemical transformations



unprecedented in nature, yet template
for the design of new enzymes

targeted activity
mining & evolution

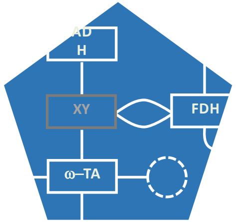
novel enzymatic activities



artificial biocatalytic modules enabling
non-natural transformations

incorporation into
multi-enzyme
scenarios

ex vivo cascade design

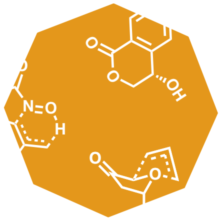


synthetic tools combining
native and abiotic functions

Jan Deska
Bioorganic
Chemistry

Sure… but why bother

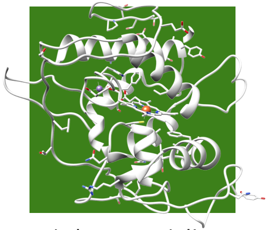
key chemical transformations



unprecedented in nature, yet template
for the design of new enzymes

*targeted activity
mining & evolution*

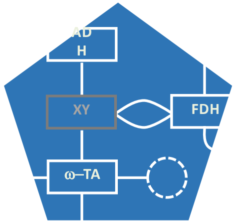
novel enzymatic activities



artificial biocatalytic modules enabling
non-natural transformations

*incorporation into
multi-enzyme
scenarios*

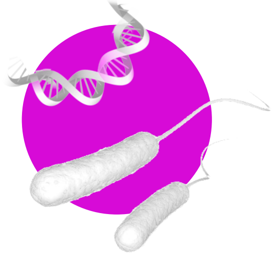
ex vivo cascade design



synthetic tools combining
native and abiotic functions

*encoding of
abiotic function into
microbial host*

*next generation
metabolic pathway design*

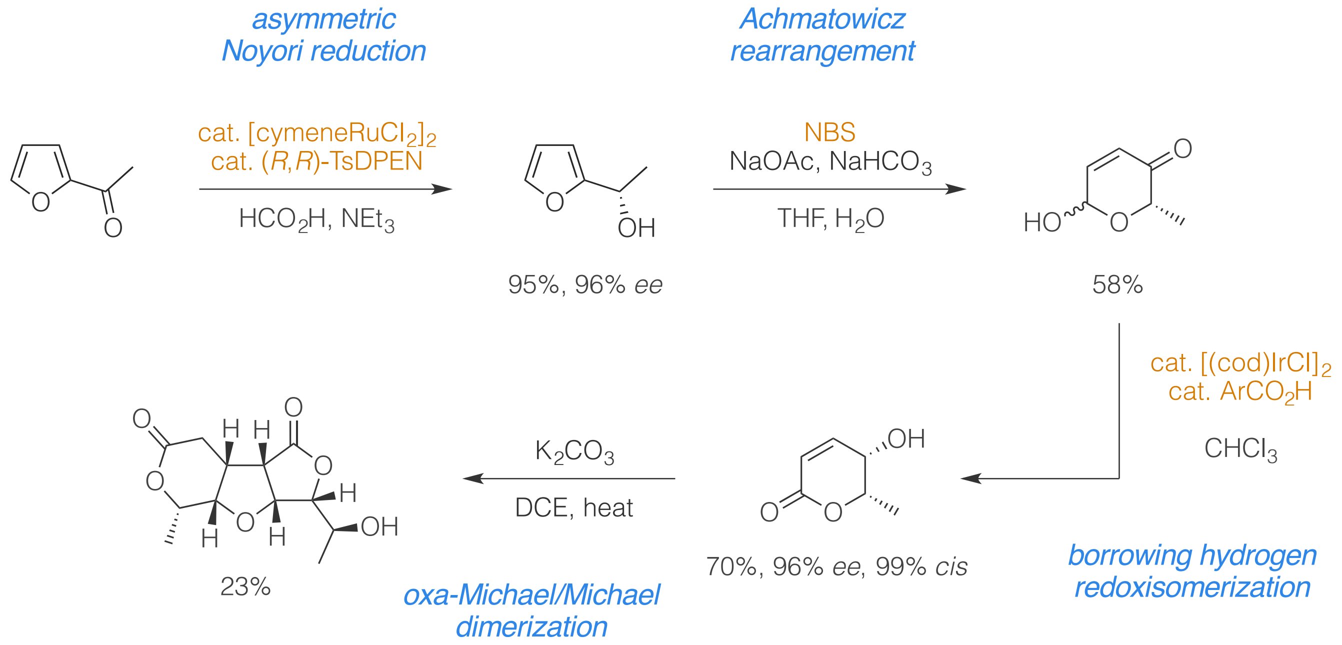


tailor-made cellular factories based
on traditional chemical strategies

Jan Deska
Bioorganic
Chemistry

Traditional organic synthesis

- 2017: Independent syntheses of Angiopterlactone B by Lawrence and Bhattacharya

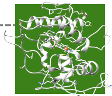


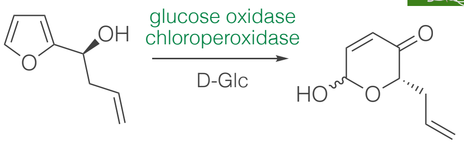
Thomson, Nichol, Lawrence, *Org. Lett.* **2017**, *19*, 2199-2201
Kottamagari, Gonnade, Bhattacharya, *Org. Lett.* **2017**, *19*, 3564-3567

Jan Deska
Bioorganic
Chemistry

Our assembly line in action

Achmatowicz'ase

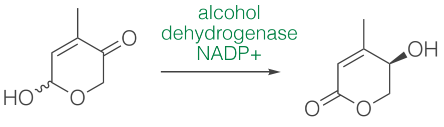




- purely enzymatic method for conversion of biorefinery furans

Thiel, Doknic, Deska, *Nature Commun.* 2014, *5*, 5278

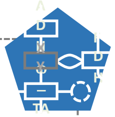
Borrowing Hydrogen'ase

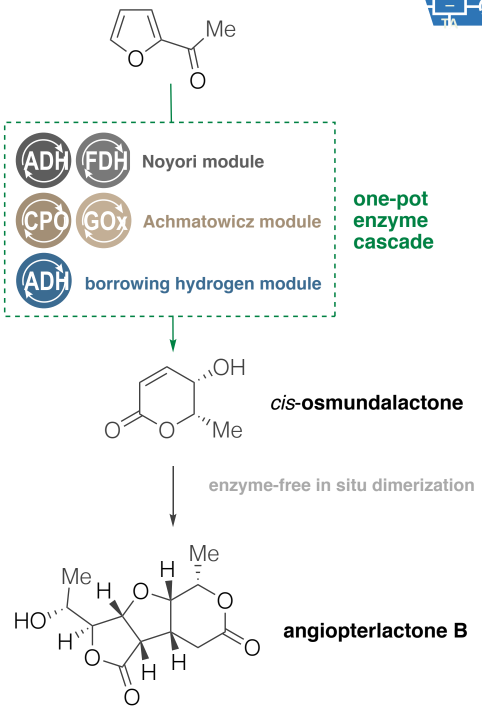


- enantioconvergent isomerization of Achmatowicz pyranones

Liu, Merten, Deska, *Angew. Chem. Int. Ed.* 2018, *57*, 12151

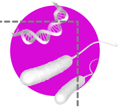
Artificial Metabolism

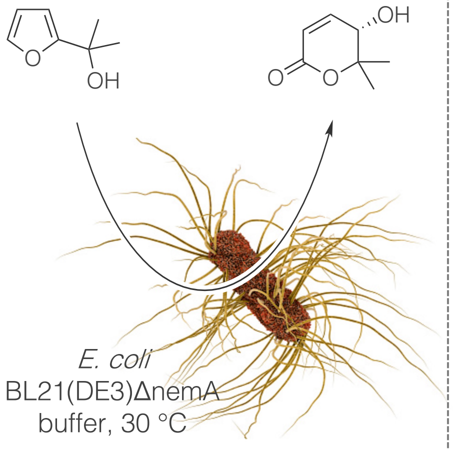


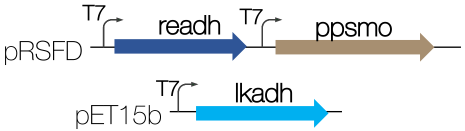


Kiefer, Jäger, Gummerer, Deska, *ChemRXiv*, 2021, 14679738

in vivo Pathway design







up to 90%, >99% *ee*

Liu, Wu, Deska, *ChemRXiv* 2021, 14564919

Jan Deska
Bioorganic
Chemistry

What are interesting reactions to aim for?

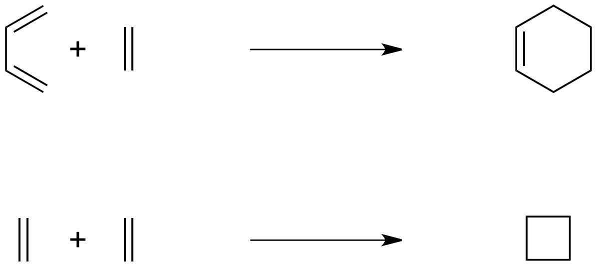
(no need to agree with me on that one)

Pericyclic reactions?

Three kinds of pericyclic reactions

- Common features
  - ✓ all pericyclic reaction are concerted (= electron reorganization takes place in one single step)
  - ✓ either thermal or photochemical reactions
  - ✓ highly selective and generally not affected by catalysts or solvents

- Cycloaddition reactions
  - ✓ reaction between two pi-containing molecules to form a cyclic product



- ✓ two fewer pi bonds in the product
- ✓ two additional sigma bonds in the product

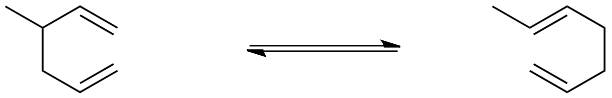
Jan Deska
Bioorganic
Chemistry

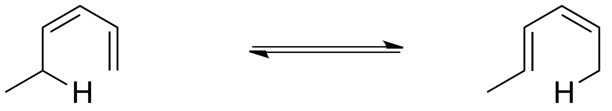
Pericyclic reactions?

Three kinds of pericyclic reactions

- Common features
  - ✓ all pericyclic reaction are concerted (= electron reorganization takes place in one single step)
  - ✓ either thermal or photochemical reactions
  - ✓ highly selective and generally not affected by catalysts or solvents

- Sigmatropic rearrangements
  - ✓ intramolecular migration of groups by rearrangement of pi bond(s)





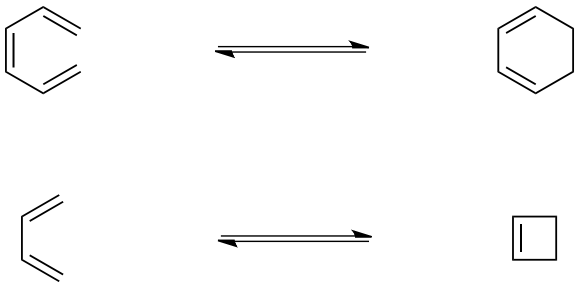
- ✓ a sigma bond is broken while a new sigma bond forms
- ✓ a pi bond, or a conjugated pi system in the product
- ✓ reversibility! check equilibrium position

Pericyclic reactions?

Three kinds of pericyclic reactions

- Common features
  - ✓ all pericyclic reaction are concerted (= electron reorganization takes place in one single step)
  - ✓ either thermal or photochemical reactions
  - ✓ highly selective and generally not affected by catalysts or solvents

- Electrocyclic reactions
  - ✓ intramolecular reaction where a conjugated linear pi system is closed at the ends



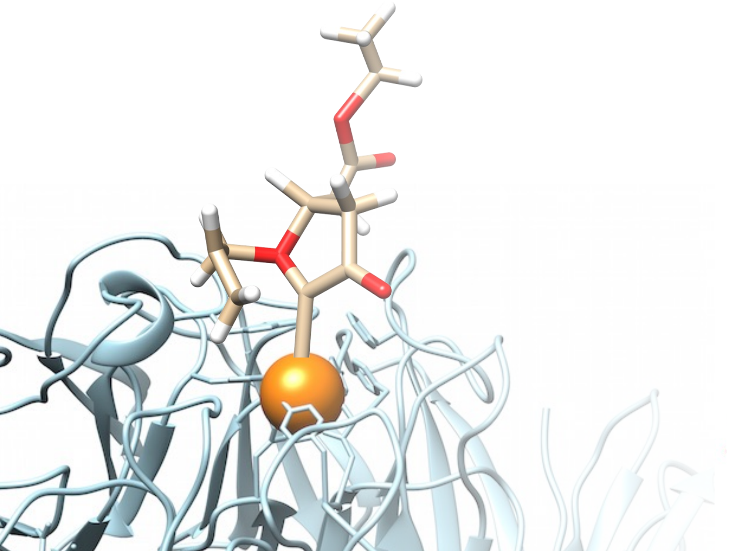
- ✓ a new sigma bond is formed, one pi bond less in the product
- ✓ reversibility! check equilibrium position

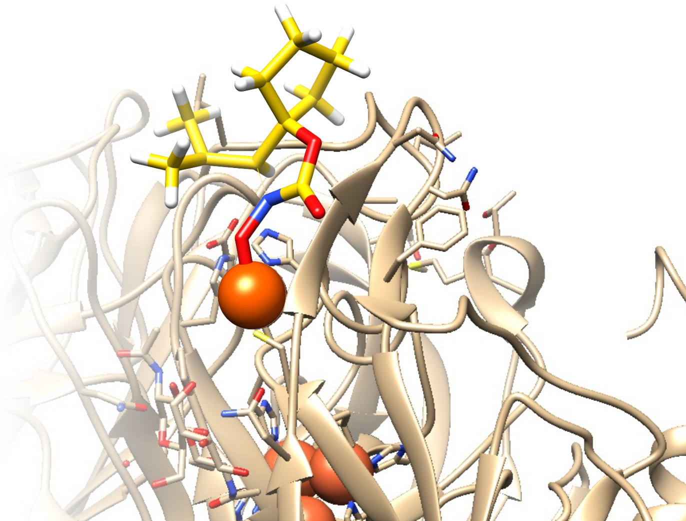
Jan Deska
Bioorganic
Chemistry

Biological mimicry of catalytic principles relevant for pericyclic reactions

Here: two conceptional work-in-progress projects

- ✓ Sigmatropic rearrangements by hijacking copper-dependent monooxygenases
- ✓ CH amination by a biocatalytic alternative to allylpalladium- or nitrene insertion-type C-N bond formation

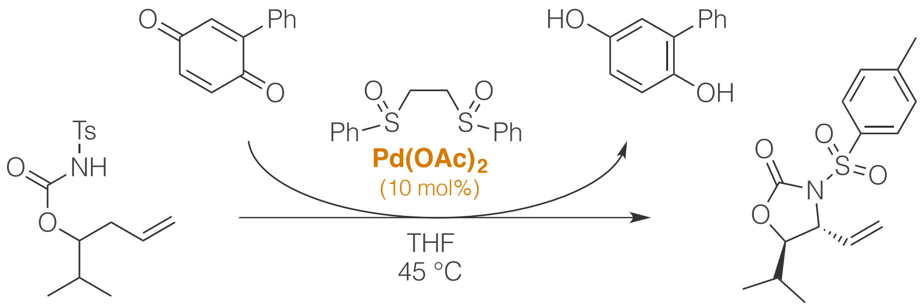




Jan Deska
Bioorganic
Chemistry

Oxidative sp_3–CH amination strategies

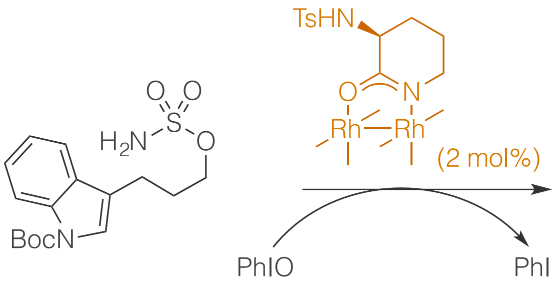
- via allylpalladium species



72%
86% *trans*

biochemically challenging!

- via nitrene insertion



98%, 92% *ee*

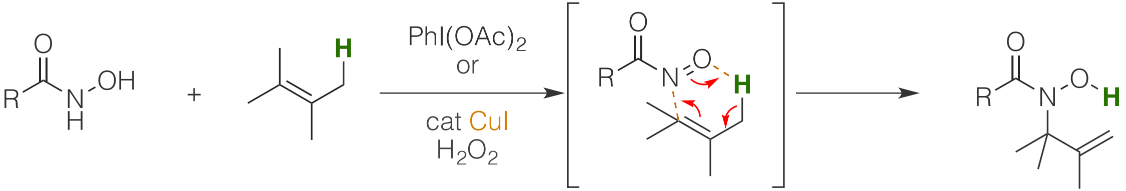
enzymatically implemented
by using sulfonyl azides

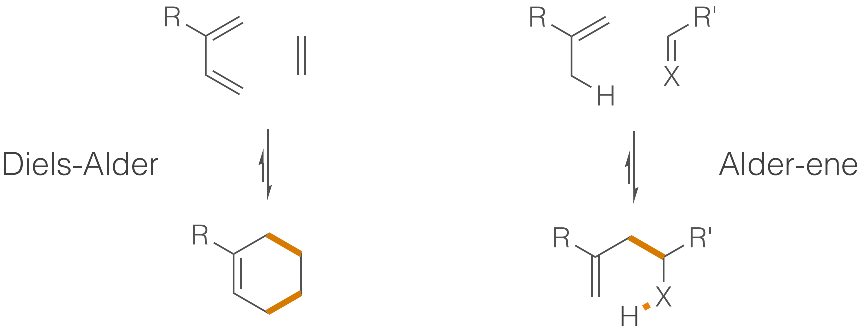
Arnold et al., *Angew. Chem. Int. Ed.* **2013**, *52*, 9309-9312

Frauenhoffer, White, *J. Am. Chem. Soc.* **2007**, *129*, 7274-7276.
Zalatan, Du Bois, *J. Am. Chem. Soc.* **2008**, *130*, 9220-9221.

Oxidative sp³–CH amination strategies

- via Alder-ene reaction of N-containing enophiles



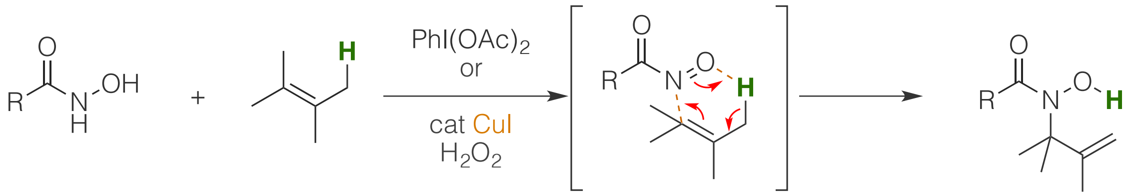


Palmer, Frazier, Read de Alaniz, *Synthesis* **2014**, *46*, 269-280 (review).
Morozova, Shumakovich, Shleev, Yaropolov, *Appl. Biochem. Microbiol.* **2007**, *43*, 523-535 (review).

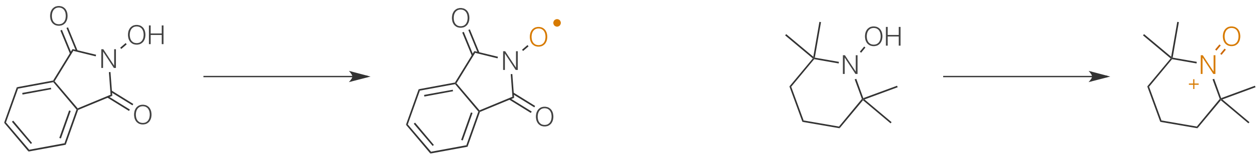
Jan Deska
Bioorganic
Chemistry

Oxidative sp^3–CH amination strategies

- via Alder-ene reaction of N-containing enophiles

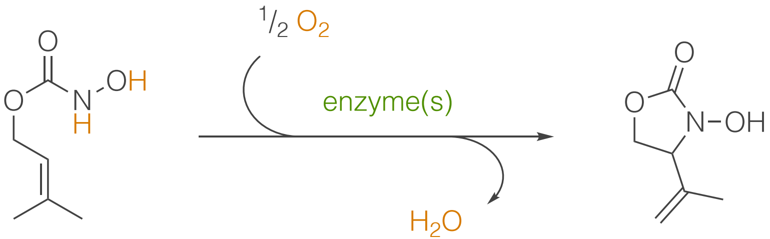


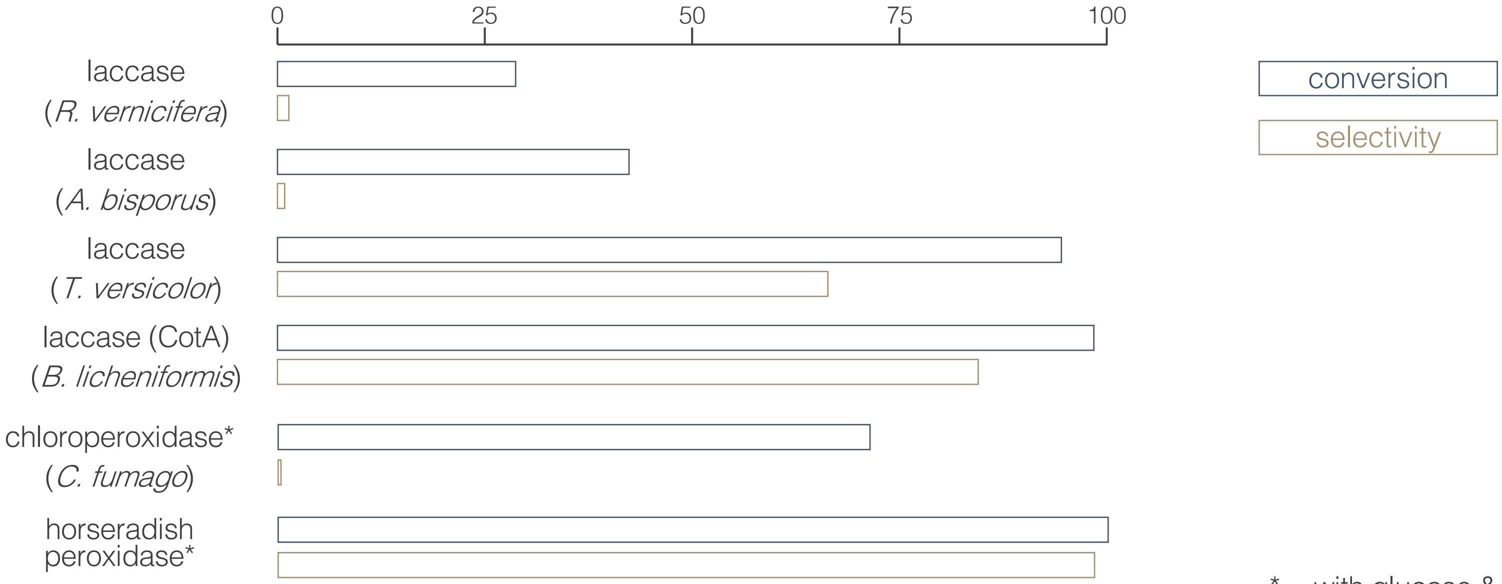
- ✓ hydroxamic acid derivatives and hydroxylamines commonly used as redox mediator in enzyme-driven aerobic oxidation reactions



Palmer, Frazier, Read de Alaniz, *Synthesis* 2014, *46*, 269-280 (review).
Morozova, Shumakovich, Shleev, Yaropolov, *Appl. Biochem. Microbiol.* 2007, *43*, 523-535 (review).

A Copper-dependent Alder-Enase?

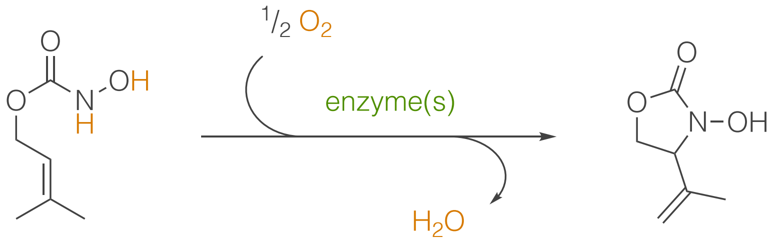


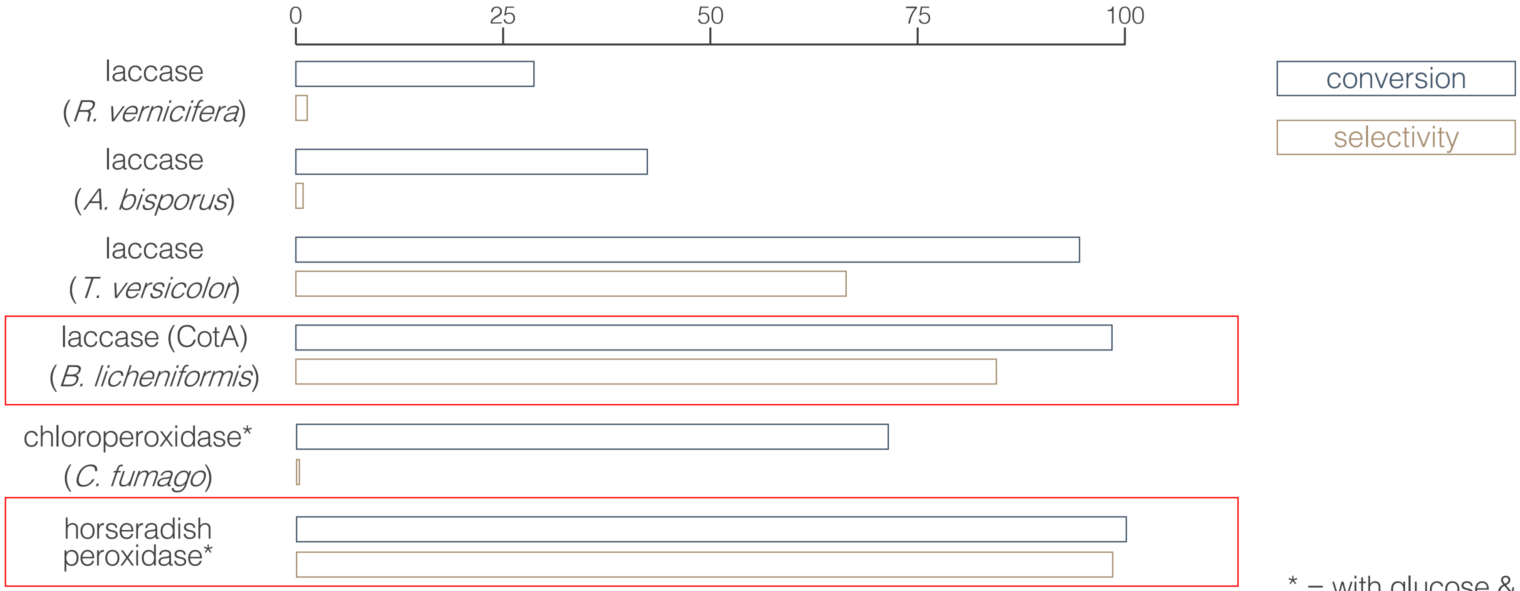


* = with glucose & glucose oxidase

Jan Deska
Bioorganic
Chemistry

A Copper-dependent Alder-Enase?

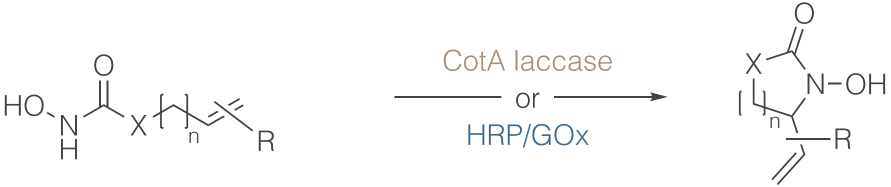


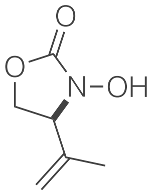


* = with glucose & glucose oxidase

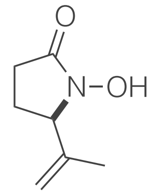
Jan Deska
Bioorganic Chemistry

Two potent biocatalysts for C–N bond formations

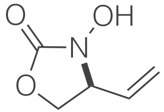




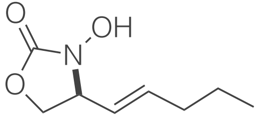
HRP yield: 97%
CotA yield: 81%



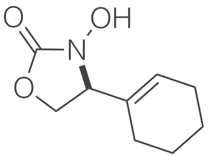
HRP yield: 77%
CotA yield: 67%



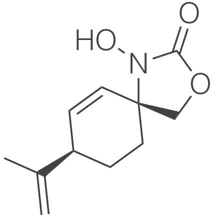
HRP yield: 53%
CotA yield: 44%



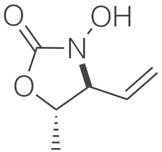
HRP yield: 66%
CotA yield: 48%



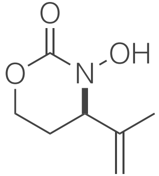
HRP yield: 77%
CotA yield: 63%



HRP yield: 94%



HRP yield: 52%
CotA yield: 51%

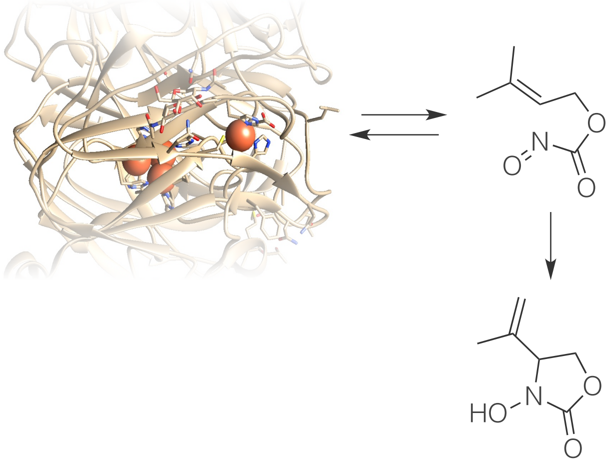


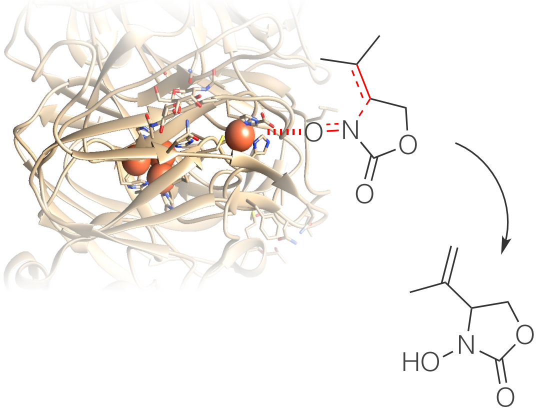
CotA yield: 59%

Jan Deska
Bioorganic
Chemistry

Towards a mechanistic understanding

- enzyme-induced vs enzyme-assisted





diffusion of nitroso species?

small molecules as electron mediators?

intimate copper-substrate interaction

SET's vs 2e⁻ processes?

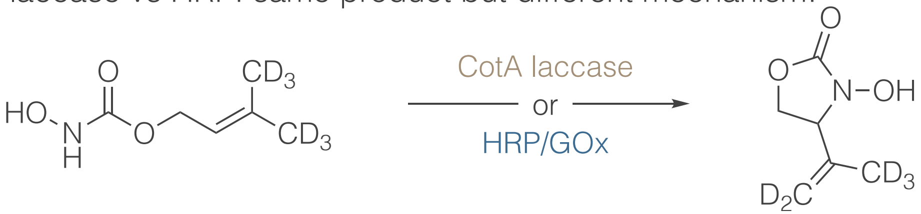
Pericyclic vs addition/elimination

Kinetic & stereochemical studies

- regioselectivity: defined rearrangement, no free radical intermediates

>99% CD_3 (both CotA & HRP)

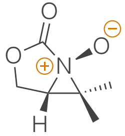
- laccase vs HRP: same product but different mechanism!



kinetic isotope effect (k_H vs k_D)

HRP = 0.79, CotA = 0.98

in line with free aziridinium oxide

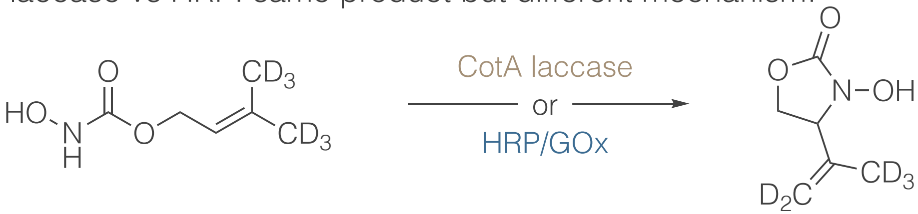


Kinetic & stereochemical studies

- regioselectivity: defined rearrangement, no free radical intermediates

>99% CD_3 (both CotA & HRP)

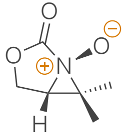
- laccase vs HRP: same product but different mechanism!



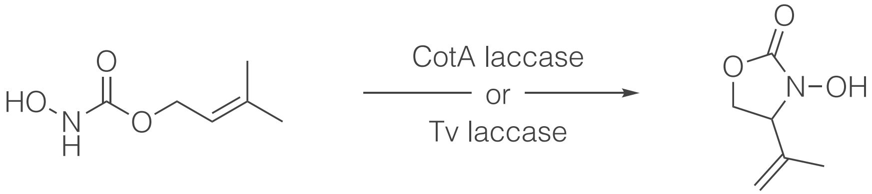
kinetic isotope effect (k_H vs k_D)

HRP = 0.79, CotA = 0.98

in line with free aziridinium oxide



- enantioselectivity: not awesome (but that's what we have directed evolution for, right?)



pH 7.0: CotA = 2% *ee*, Tv = 5% *ee*

pH 5.5: CotA = 27% *ee*, Tv = 43% *ee*

Jan Deska
Bioorganic
Chemistry

Summary 'Alder enase'

- ✓ exploiting intrinsic oxidase activity for generation of reactive intermediates
- ✓ highly effective biocatalyst for C-N bond forming reactions without biosynthetic precedence
- ✓ rational activity mining based on knowledge of laccase's reactivity principles

- ○ directed evolution required to solve existing challenges (e.g. enantioselectivity)

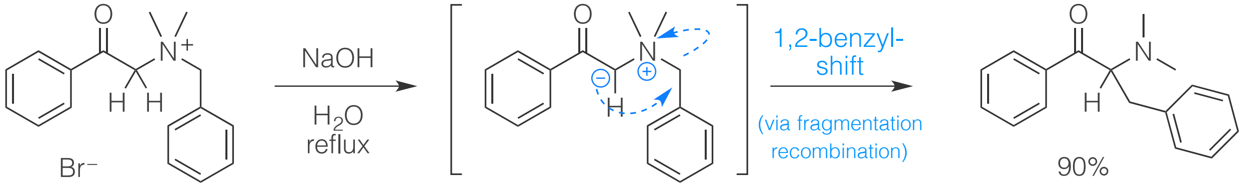
Sigmatropic reactions beyond chorismate mutase

(hopefully more versatile than native mutases)

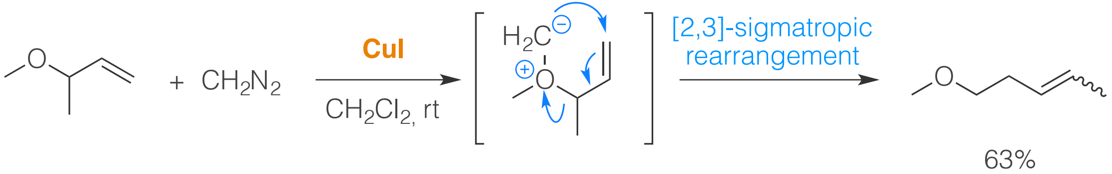
Jan Deska
Bioorganic
Chemistry

Onium ylide formation as interesting target reaction

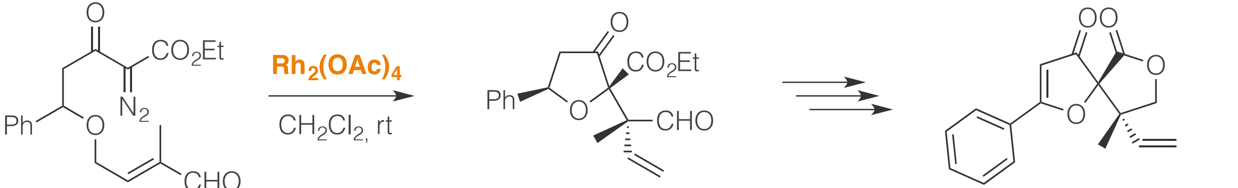
- historical, Stevens (1928): 1,2-benzyl shift of ammonium ylides



- Kirmse (1968): oxonium ylide formation from metal carbenes: the basics...



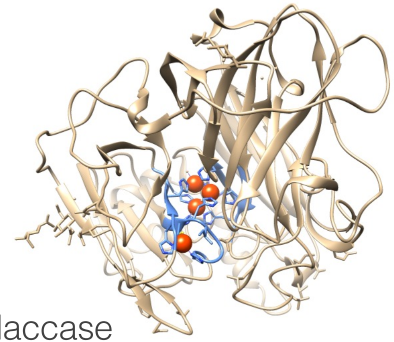
- ... and synthetic applications



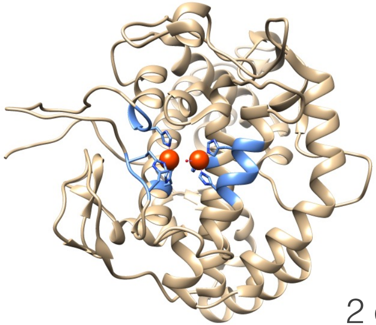
Hyperolactone C

1) Stevens, Creighton, MacNicol, *J. Chem. Soc.* **1928**, 3193; 2) Kirmse, Kamps, *Chem. Ber.* **1968**, *101*, 949; 3) Hodgson, Angrish, Erickson, Kloesges, Lee, *Org. Lett.* **2008**, *10*, 5553.

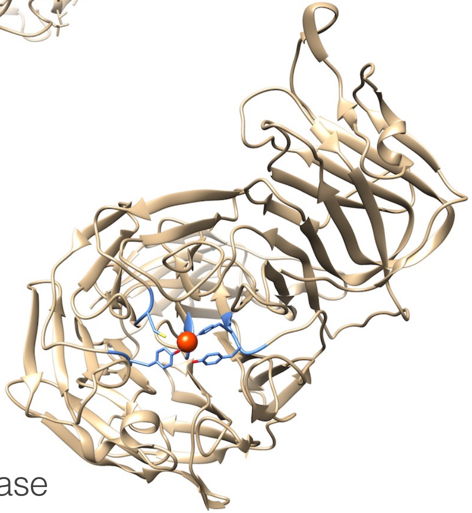
Copper oxidases as artificial Kirmse mutases?



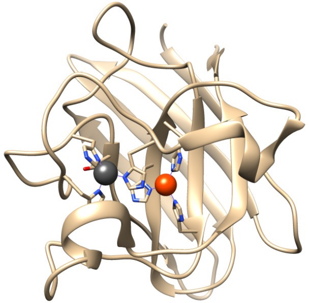
laccase
4 copper centres



tyrosinase
2 copper centres



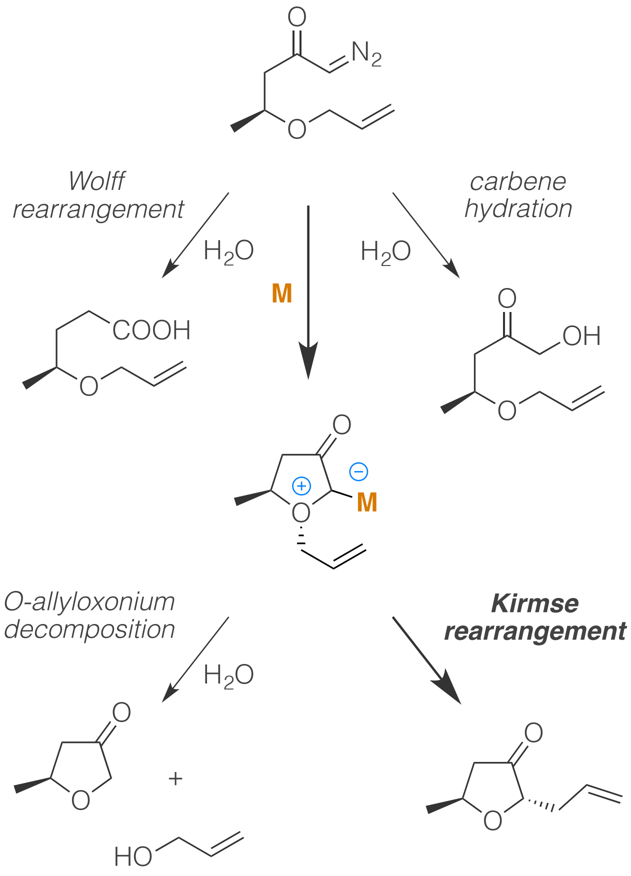
galactose oxidase
1 copper centre



superoxide dismutase
1 copper centre

Jan Deska
Bioorganic
Chemistry

Pathways for diazo activation

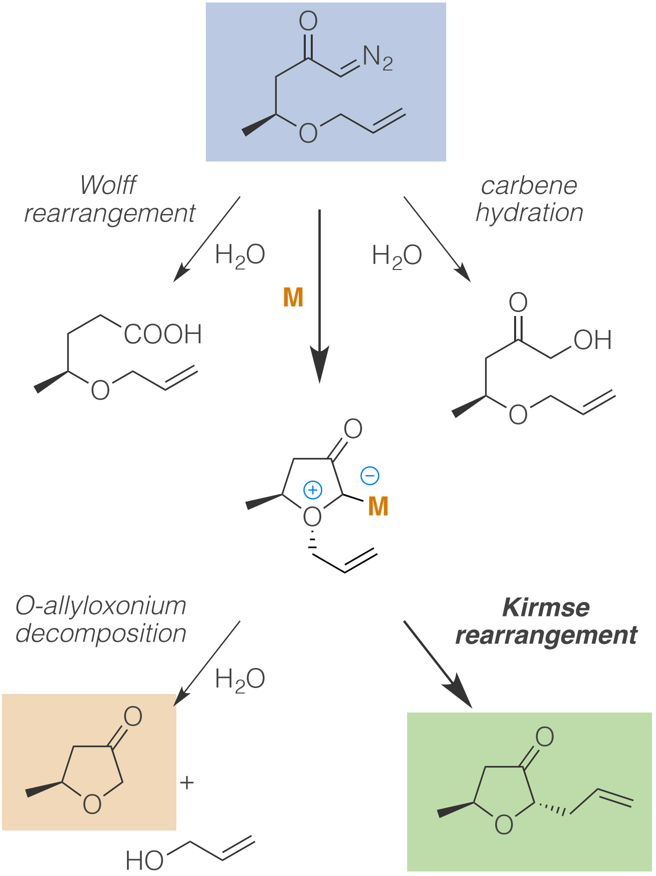


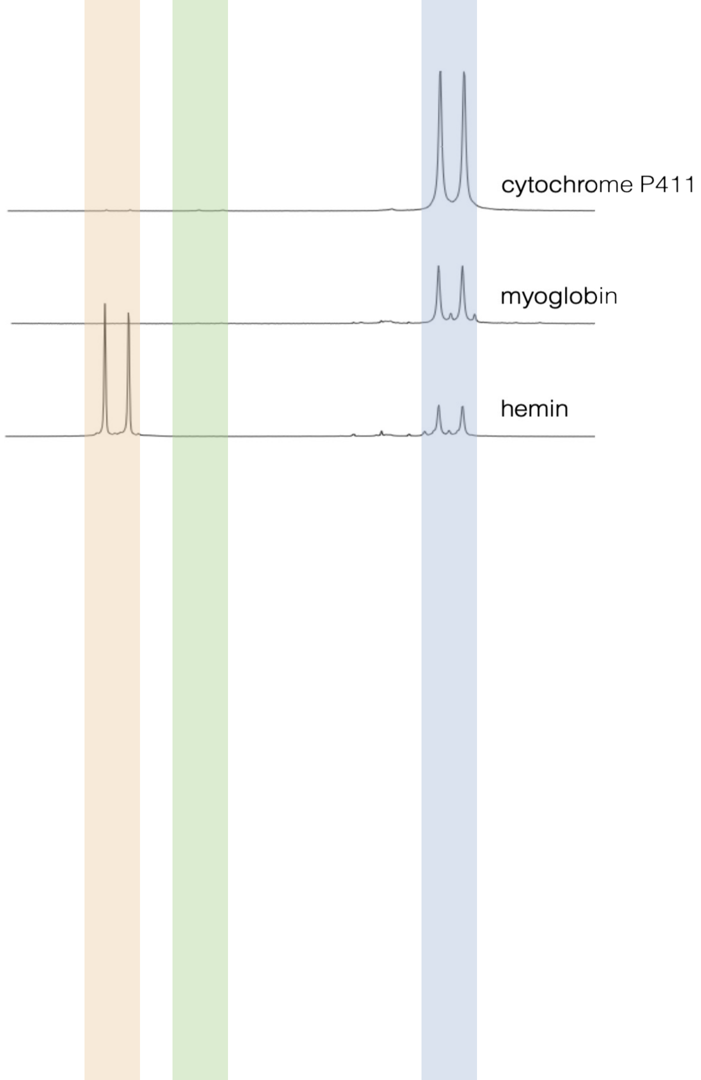
major challenge:

- oxonium ylides not necessarily compatible with aqueous media
- with water being an absolute prerequisite for those kinds of proteins

Jan Deska
Bioorganic
Chemistry

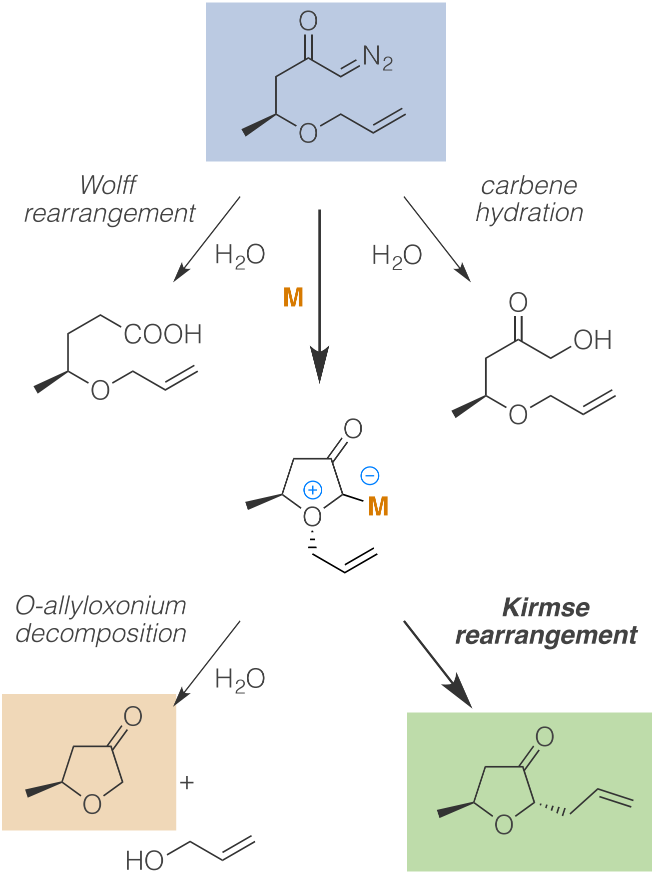
Pathways for diazo activation

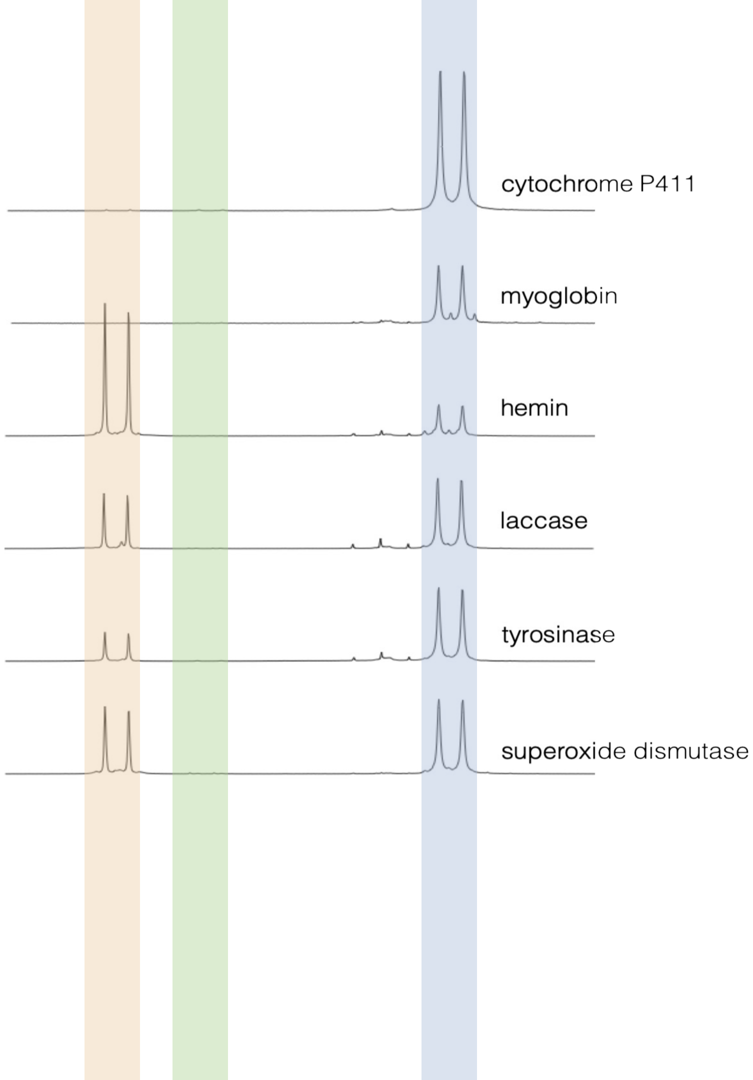




Jan Deska
Bioorganic
Chemistry

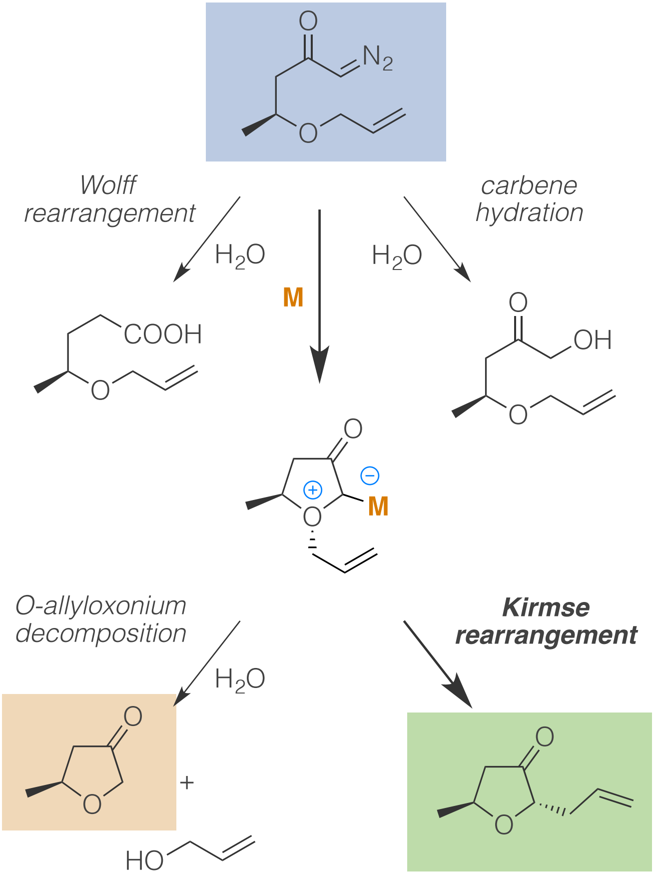
Pathways for diazo activation

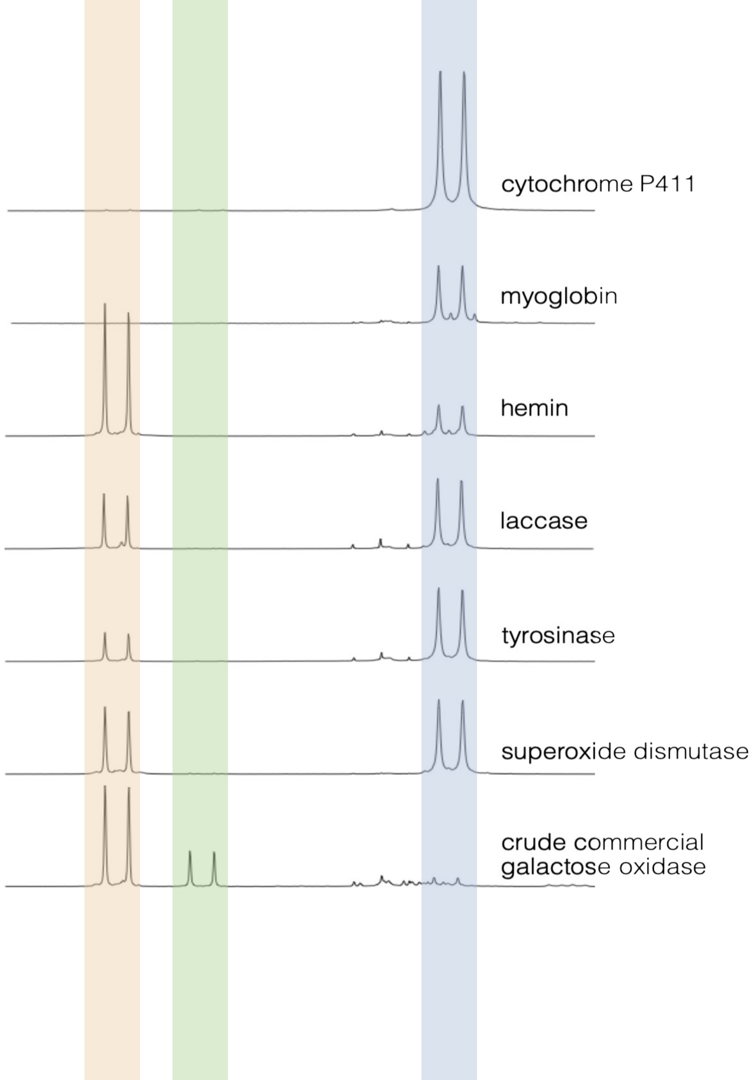




Jan Deska
Bioorganic
Chemistry

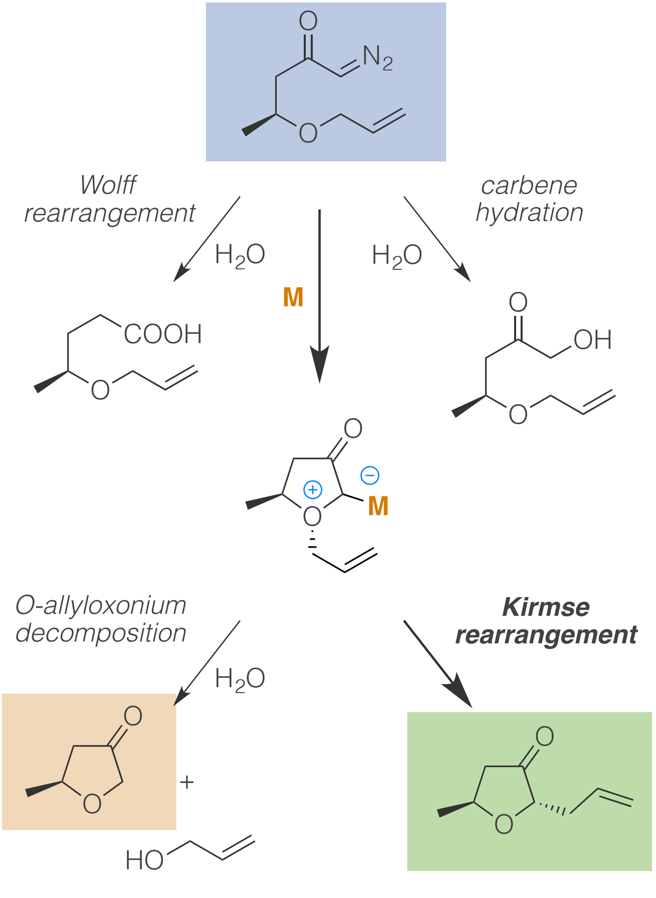
Pathways for diazo activation

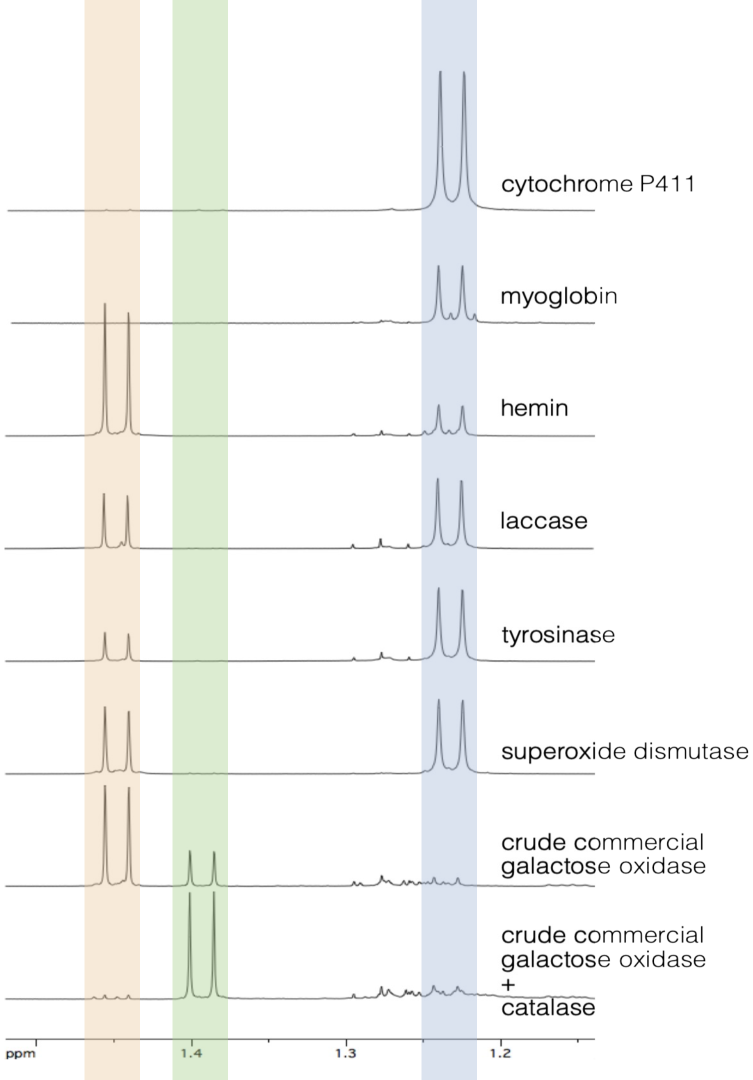




Jan Deska
Bioorganic
Chemistry

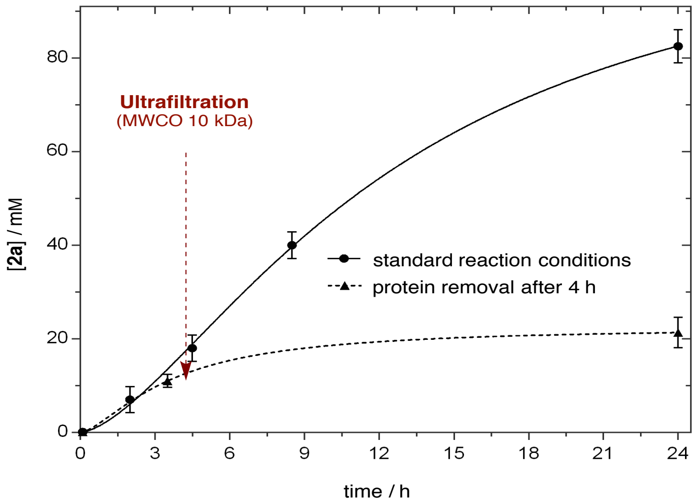
Pathways for diazo activation

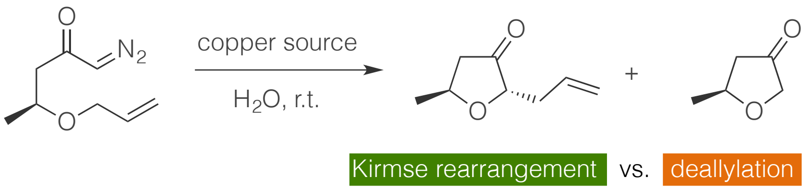


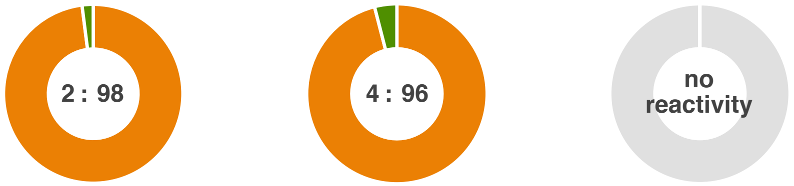


Jan Deska
Bioorganic
Chemistry

Role of free copper or other proteins





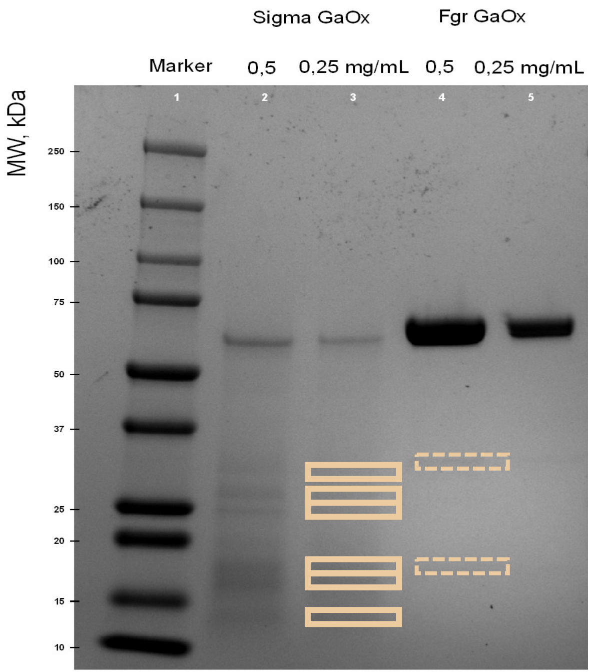


$CuSO_4$	laccase (*T. versicolor*)	galactose oxidase (*F. graminearum*)

- protein removal leads to complete shutdown of product formation
- ✓ no free copper ions responsible for observed effect
- ✓ inverted selectivity with free copper or Cu-leaching proteins
- ✓ no reactivity with recombinant galactose oxidase !!!

Jan Deska
Bioorganic
Chemistry

What else could be involved



- ✓ rich impurity pattern in commercial GalOX
- ✓ no similarities to recombinant *F. gr.* GalOx

- other low MW Copper Oxidase?

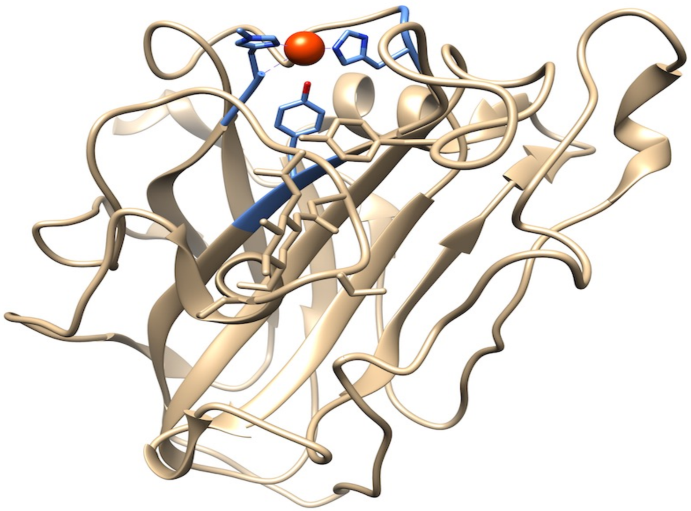
Collection of protein fractions by UPLC

- ✓ GalOx region shows no Kirmse activity
- ✓ only the low retention first fractions exhibit the previously observed effect from the crude GalOx mixture

Jan Deska
Bioorganic
Chemistry

The most likely candidate

- *Fusarium graminearum* encodes for at least 18 lytic polysaccharide monooxygenases
- carbohydrate-active mononuclear copper enzymes with *auxiliary activity* (AA9, AA10, AA11)
- cleave cellulose and related biopolymers (chitin, chitosan, xyloglucan,...)

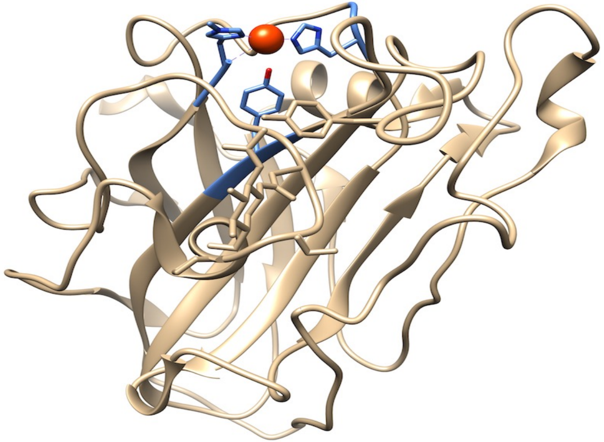


Vaaje-Kolstad, Westereng, Horn, Liu, Zhai, Sørlie, Eijsink, *Science* **2010**, *330*, 219-222.

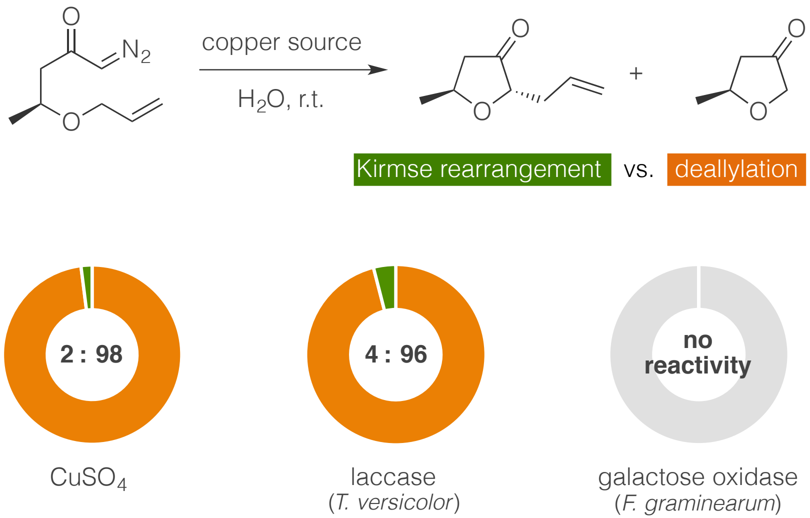
Jan Deska
Bioorganic
Chemistry

The most likely candidate

- *Fusarium graminearum* encodes for at least 18 lytic polysaccharide monooxygenases
- carbohydrate active mononuclear copper enzymes with *auxiliary activity* (AA9, AA10, AA11)
- cleave cellulose and related biopolymers



Vaaje-Kolstad, Westereng, Horn, Liu, Zhai, Sørlie, Eijsink, *Science* **2010**, *330*, 219-222.

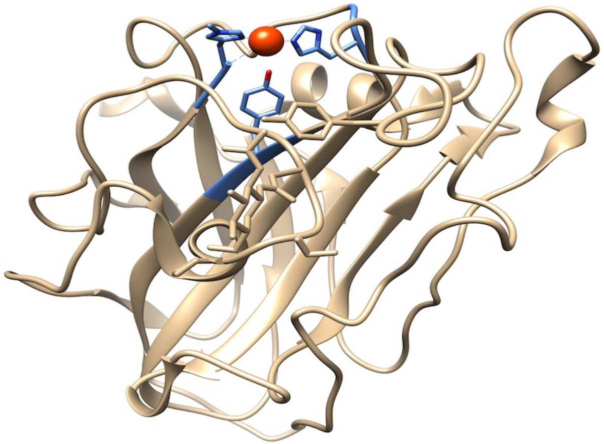


So how would a random LPMO perform?

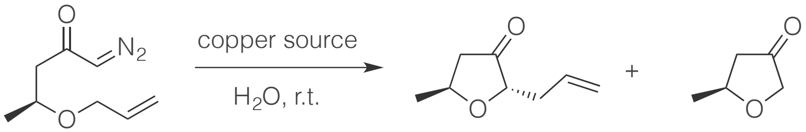
Jan Deska
Bioorganic
Chemistry

The most likely candidate

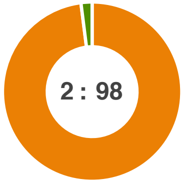
- *Fusarium graminearum* encodes for at least 18 lytic polysaccharide monooxygenases
- carbohydrate active mononuclear copper enzymes with *auxiliary activity* (AA9, AA10, AA11)
- cleave cellulose and related biopolymers



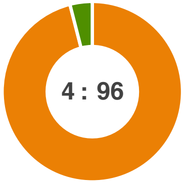
Vaaje-Kolstad, Westereng, Horn, Liu, Zhai, Sørlie, Eijsink, *Science* 2010, *330*, 219-222.



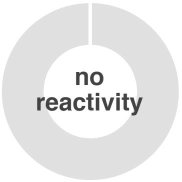
Kirmse rearrangement vs. deallylation



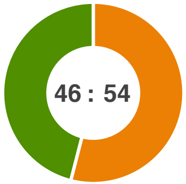
$CuSO_4$



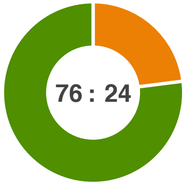
laccase
(*T. versicolor*)



galactose oxidase
(*F. graminearum*)



lytic polysaccharide monooxygenase
(*P. chrysosporium* K-3)



lytic polysaccharide monooxygenase
(*P. chrysosporium* K-3)
+
galactose oxidase
(*F. graminearum*)
+
catalase
(*C. glutamicum*)

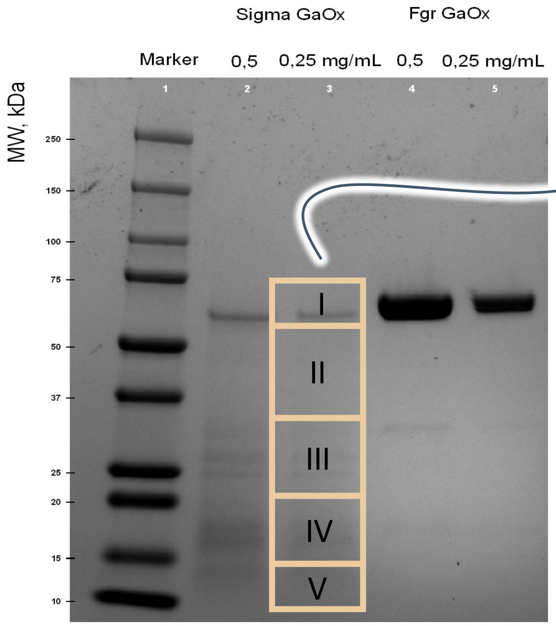
Jan Deska
Bioorganic
Chemistry

Chasing the actual catalyst

✓ reconstruction of initial reactivity by recombinant & purified enzymes

but

- selectivity and activity still behind the crude enzyme system



1 selection of gel cut-outs with characteristic protein fractions

2 tryptic digest to yield peptide fragments

3 MS/MS analysis & bioinformatics

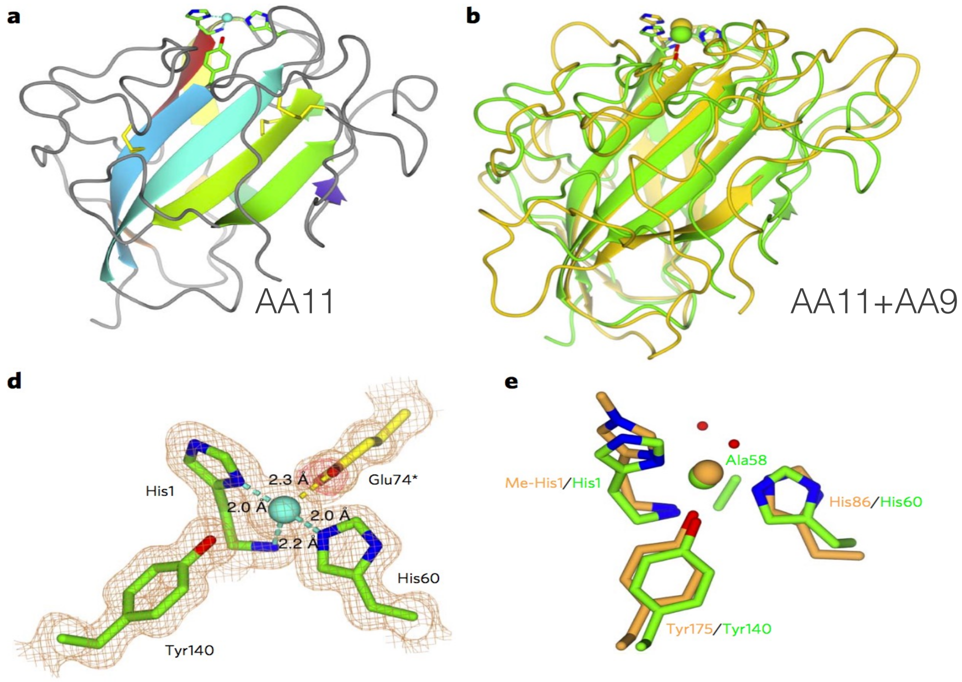
hits in section III with high level of confidence for:
cellulose-active LPMO (AA9) & chitin-active LPMO (AA11)

production of two new family 11 LPMOs in *P. pastoris*

Jan Deska
Bioorganic
Chemistry

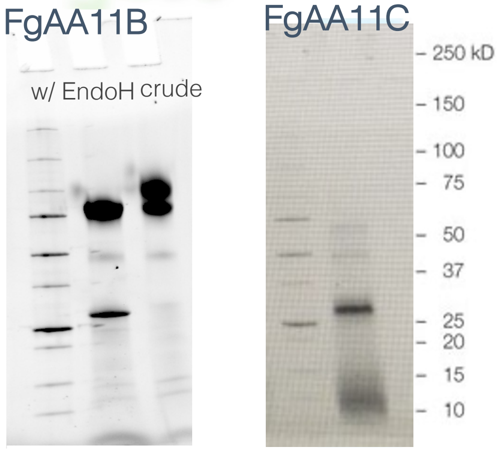
Chitinase as the real biocatalyst?

- chitinase closely related to the tested PcLPMO9



Hemsworth, Henrissat, Davies, Walton, *Nat. Chem Biol.* **2014**, *10*, 122-126.

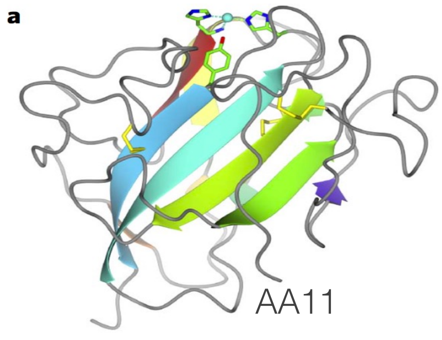
structural insights
still missing!

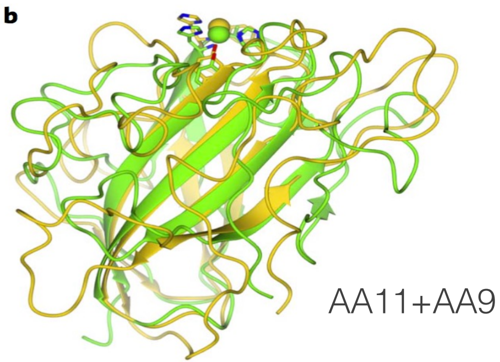


Jan Deska
Bioorganic
Chemistry

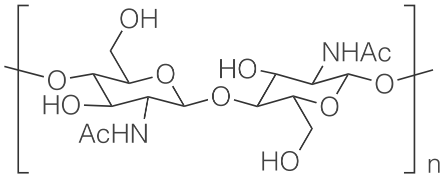
Chitinase as the real biocatalyst?

- chitinase closely related to the tested PcLPMO9

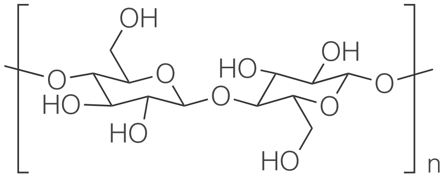




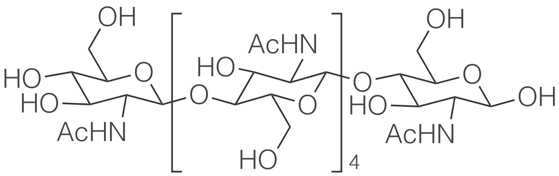
structural insights
still missing!



prefers polymeric chitin



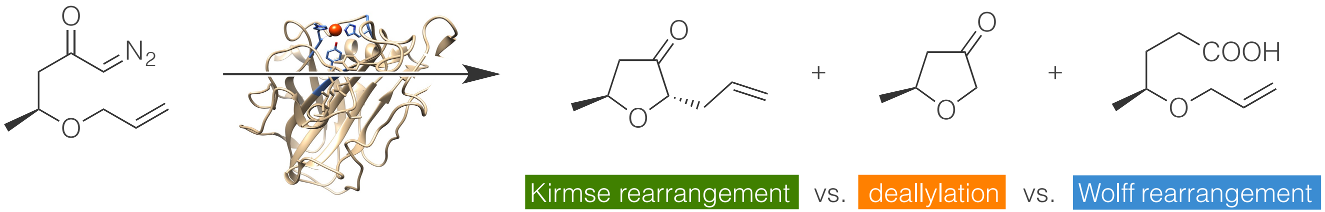
prefers polymeric cellulose

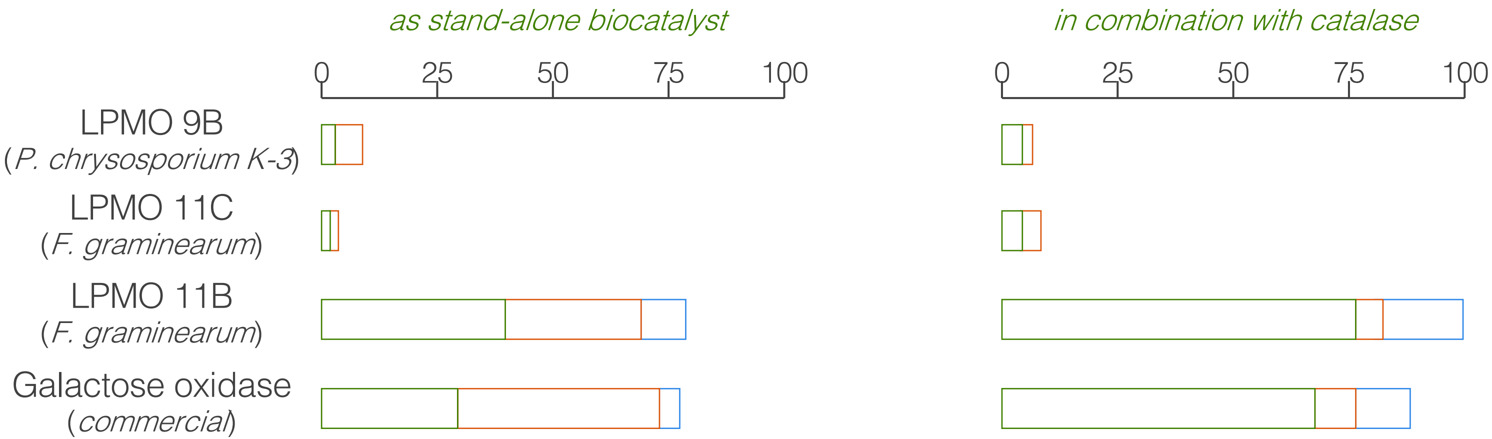


prefers small soluble chitin oligos!

Jan Deska
Bioorganic
Chemistry

Chitin-active monooxygenases as Kirmse mutase

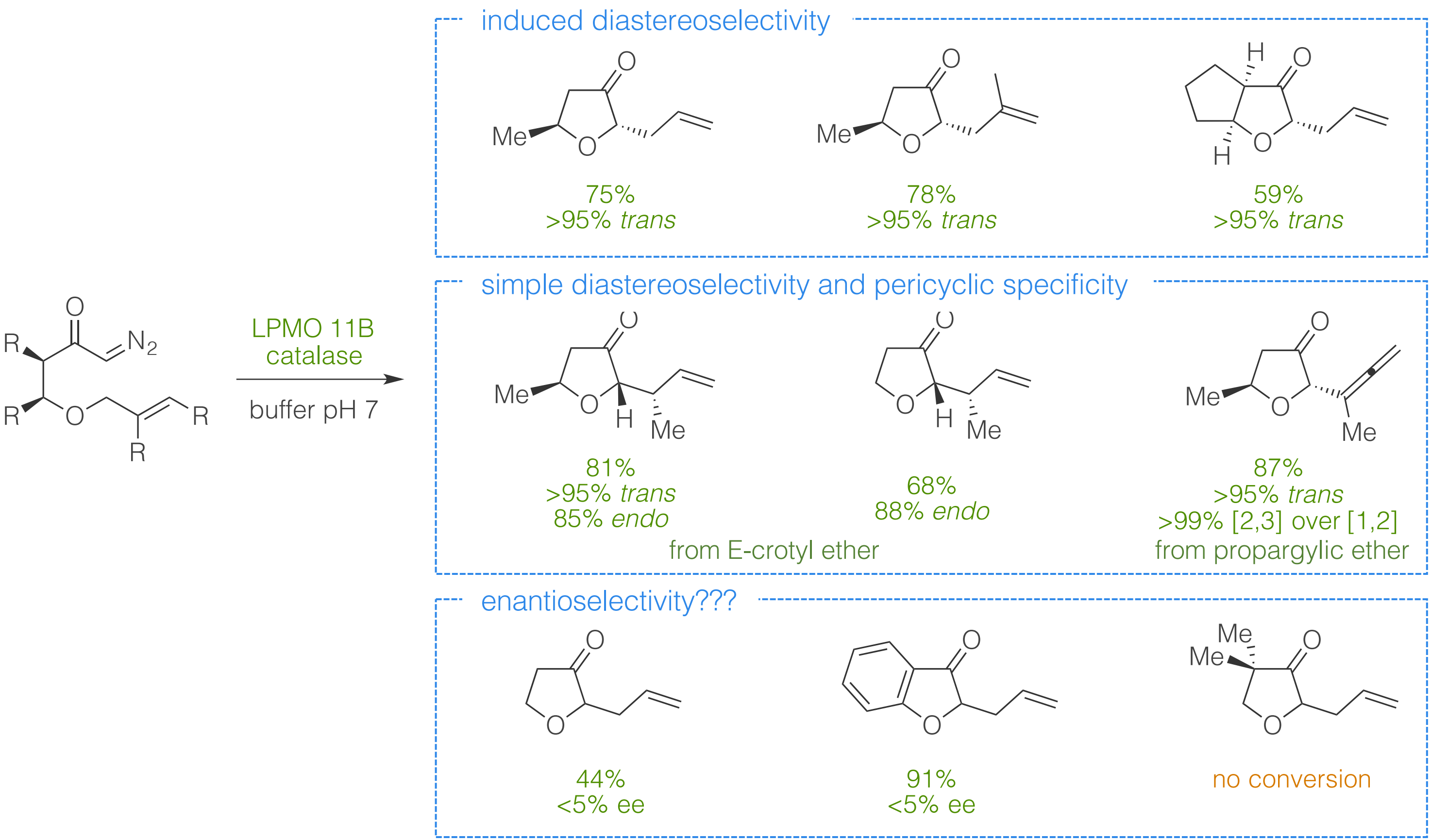




- ✓ recombinant system matches the previously successful commercial black box almost spot-on
- ✓ highly effective (TTN ~ 20 000)

Jan Deska
Bioorganic
Chemistry

Substrate scope



Jan Deska
Bioorganic
Chemistry

Summary 'Kirmse mutase'

- ✓ oxidative enzyme as catalyst in non-oxidative transformation
- ✓ stabilization of highly reactive intermediates that seem incompatible with aqueous media

- ✓ prime example for serendipity in science
- ✓ complete failure of rational design

That's it... We're almost done

Thank you for your participation and your input through the learning diaries

What's left to do?

- ✓ prepare your seminar presentations
- ✓ join the seminar on your day of choice (13.4. or 14.4.)
- ✓ please answer the Webropol feedback survey (available now, until 27.4.2021)

Jan Deska
Bioorganic
Chemistry