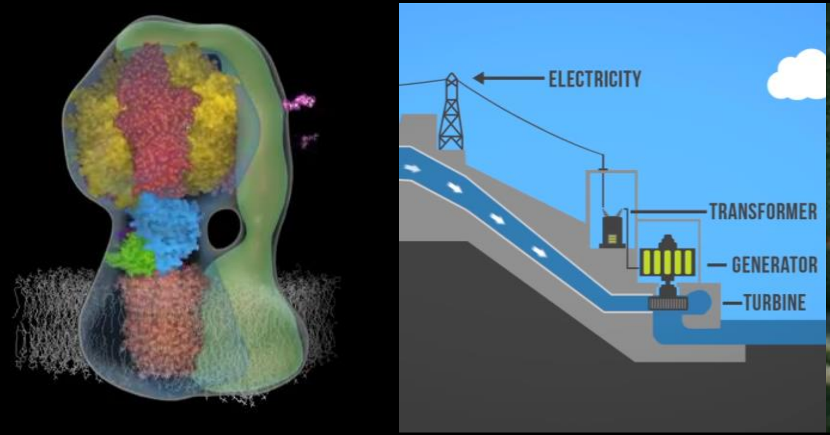
Engineering the tryptophan synthase β-subunit for synthesis of non-canonical amino acids

ARNOLD LAB
EXPLORING THE NON-NATURAL WORLD

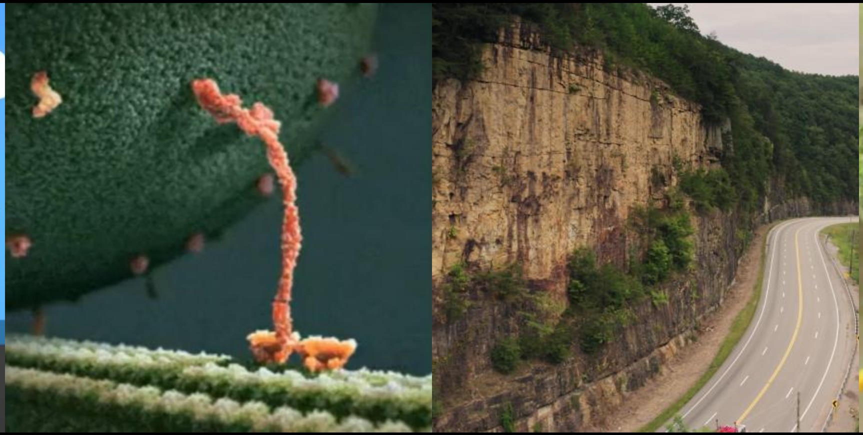
Ella Watkins-Dulaney
Thesis defense
210610

CALIFORNIA INSTITUTE OF TECHNOLOGY
1891

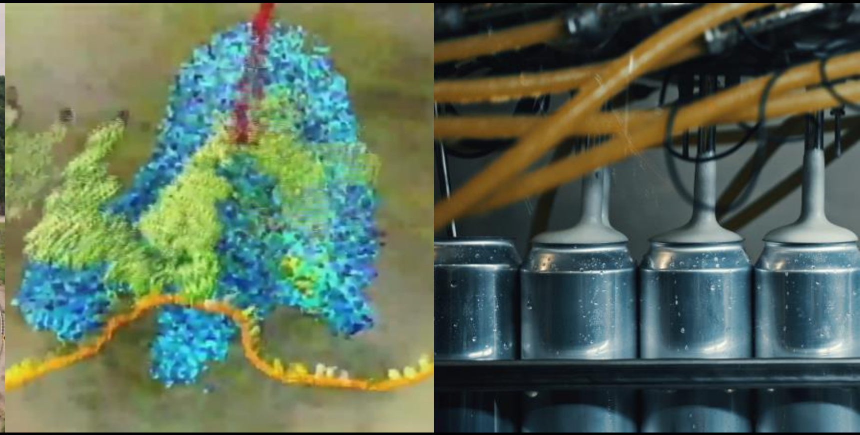
Proteins are incredible molecular machines



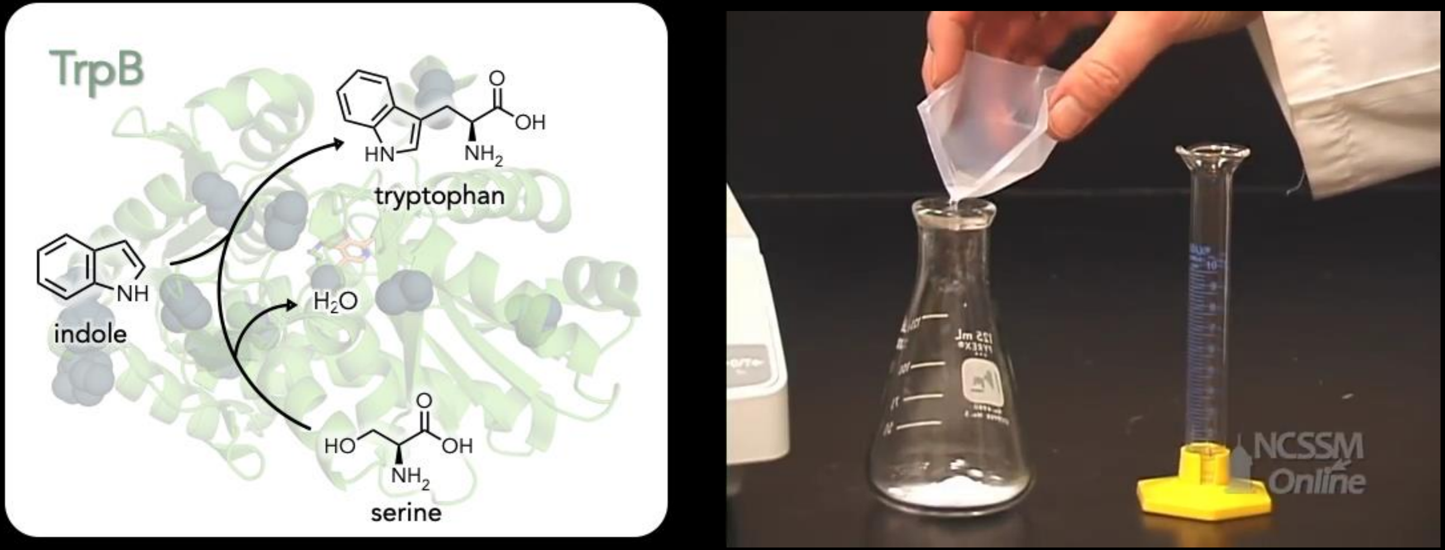
ATP synthase works just like a hydroelectric dam



Kinesin shuttles cargo across the cell



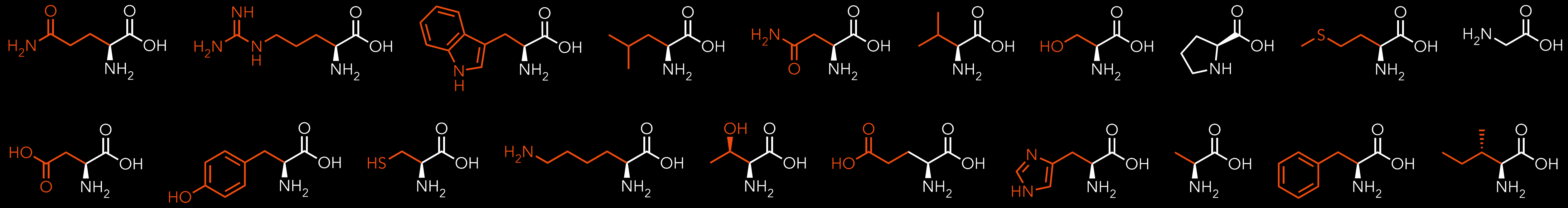
The ribosome is like an assembly line for protein synthesis

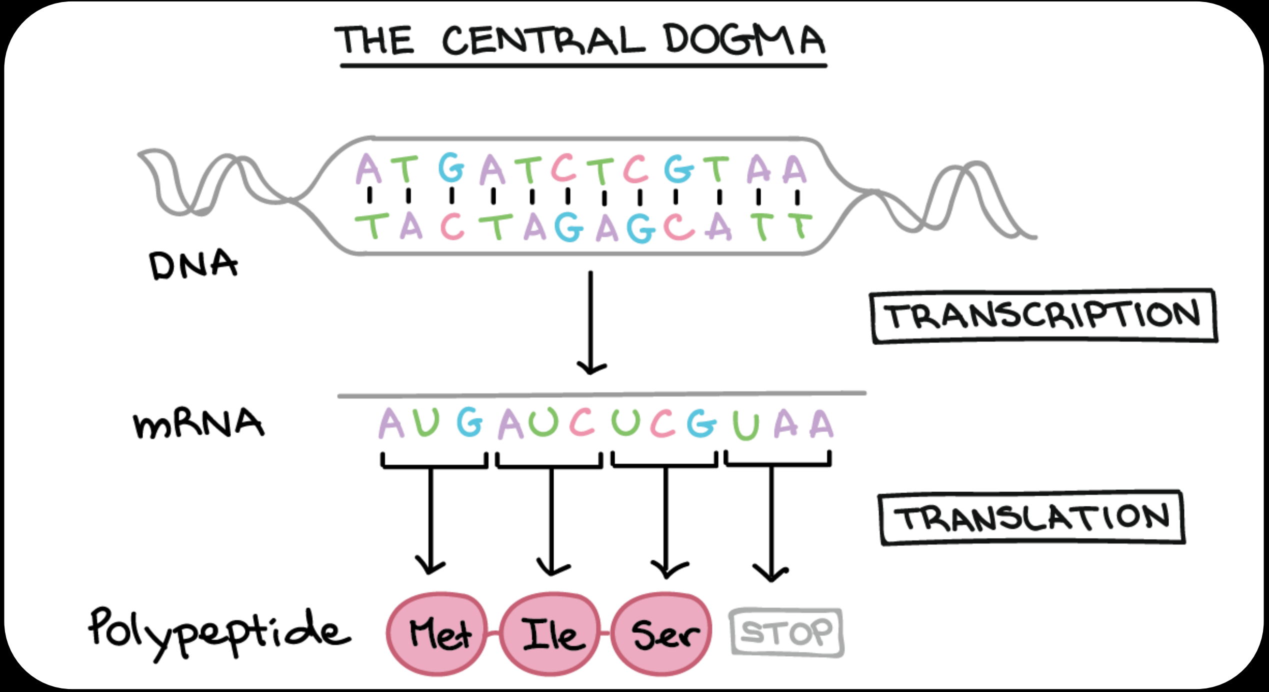


Enzymes are proteins that catalyze chemical reactions

Proteins are made of 20 canonical amino acids

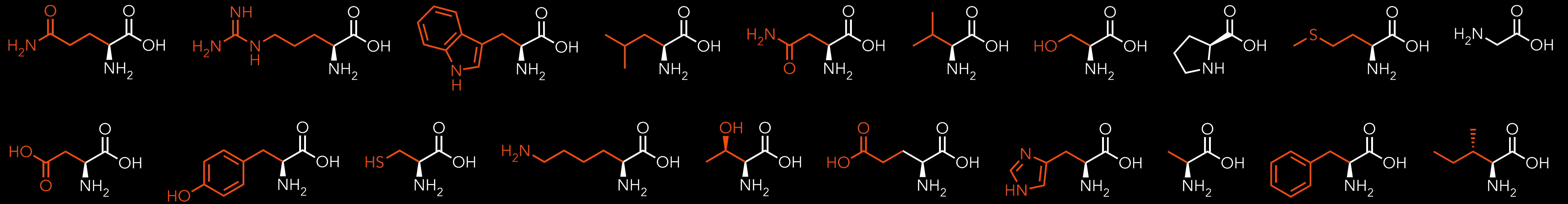
Canonical amino acids





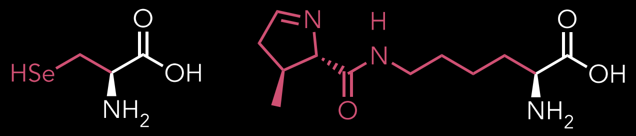
To infinity and beyond: non-canonical amino acids (ncAAs)

Canonical amino acids

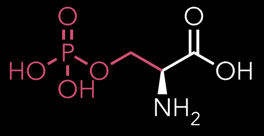


Naturally occurring non-canonical amino acids and modifications

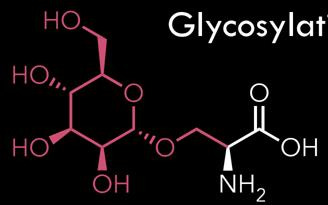
Genetic code expansion

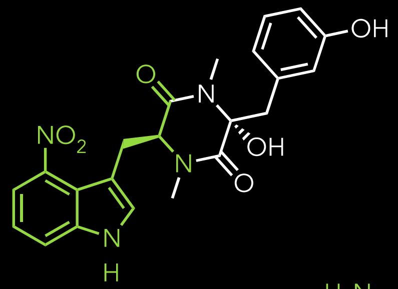


Phosphorylation

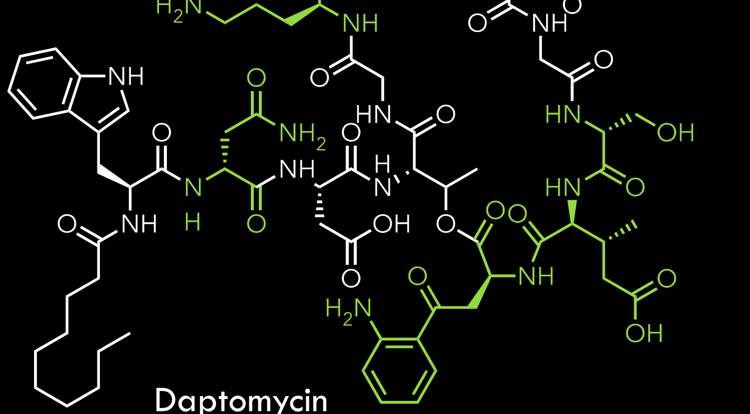


Glycosylation





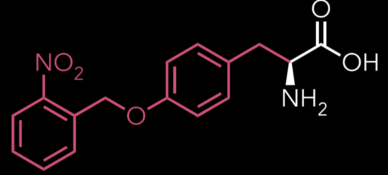
Thaxtomin A
(herbicide)



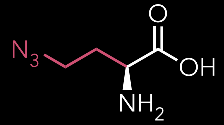
Daptomycin
(antibiotic)

Unnatural non-canonical amino acids

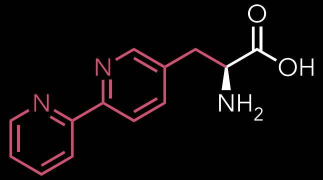
Photocaged



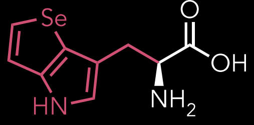
Bioorthogonal chemistry



Metal binding

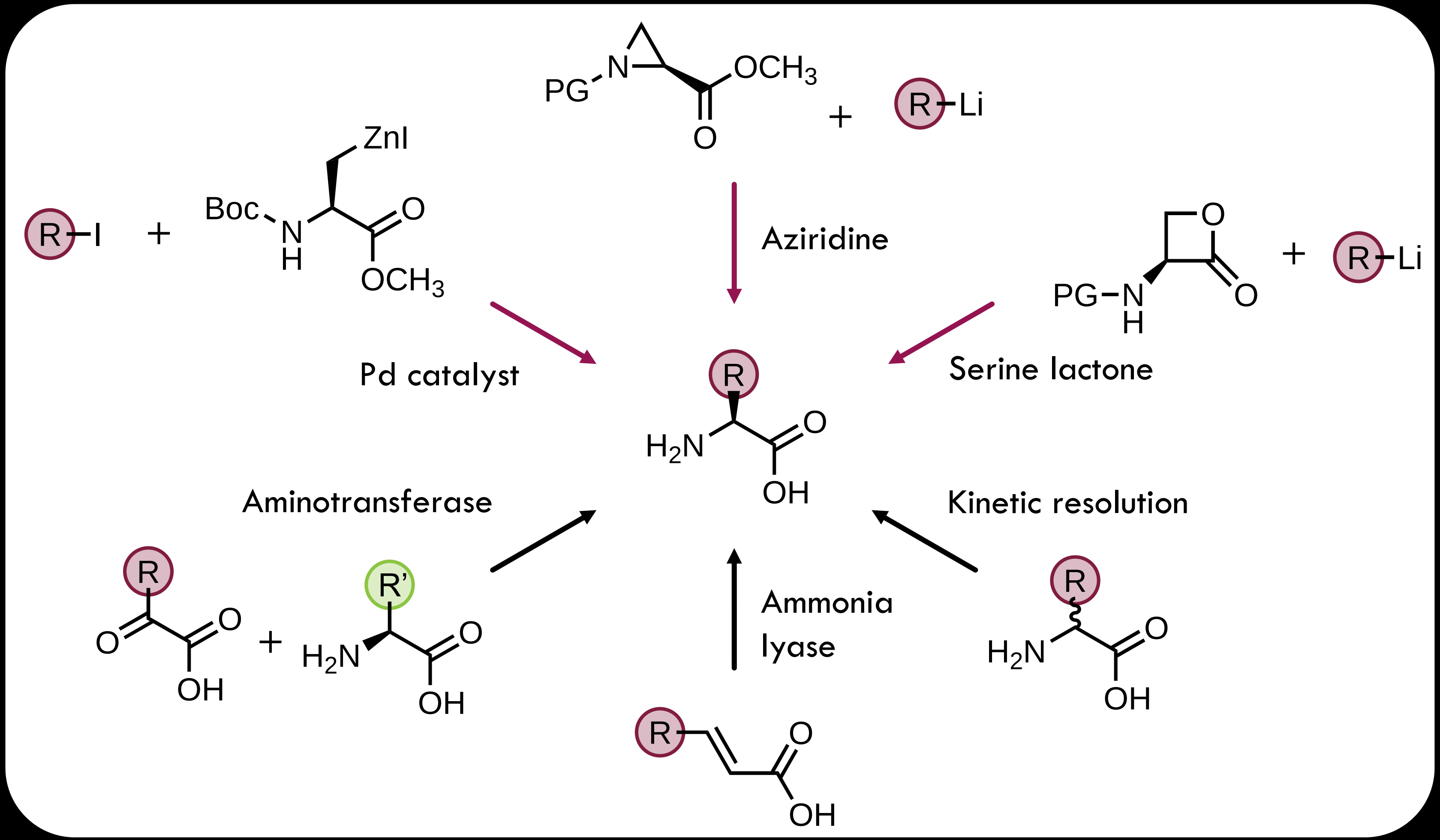


X-ray crystallography



How do we access non-canonical amino acids?

Challenges with ncAA synthesis



Chemical synthesis

- ✗ Requires protection
- ✗ Not selective
- ✗ Catalyst synthesis
- ✓ Broad scopes

Both:

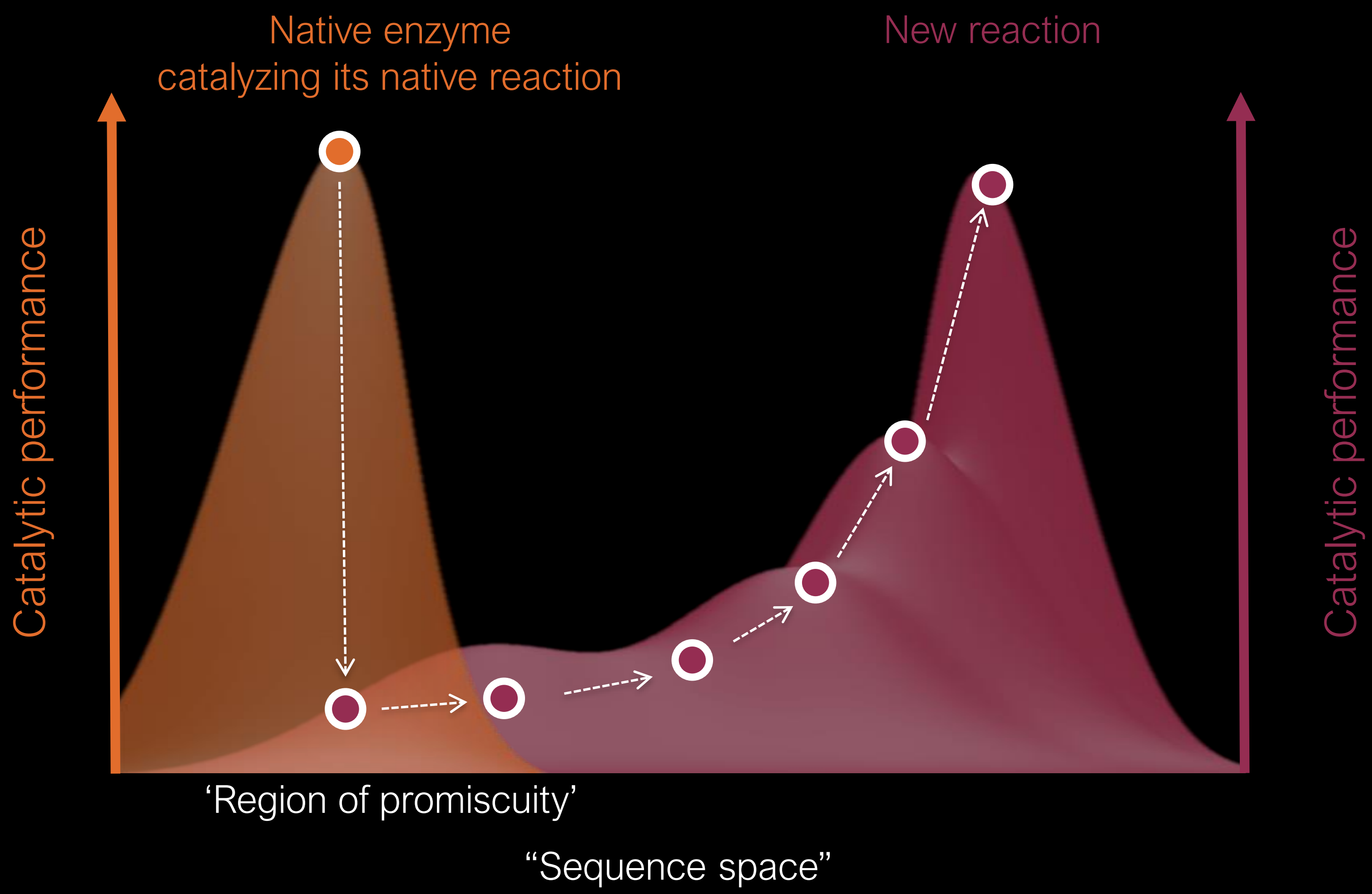
- ✗ Expensive starting materials

Biocatalysis

- ✓ Selective
- ✓ Easily produced
- ✗ Reversible
- ✗ Poor substrate scope

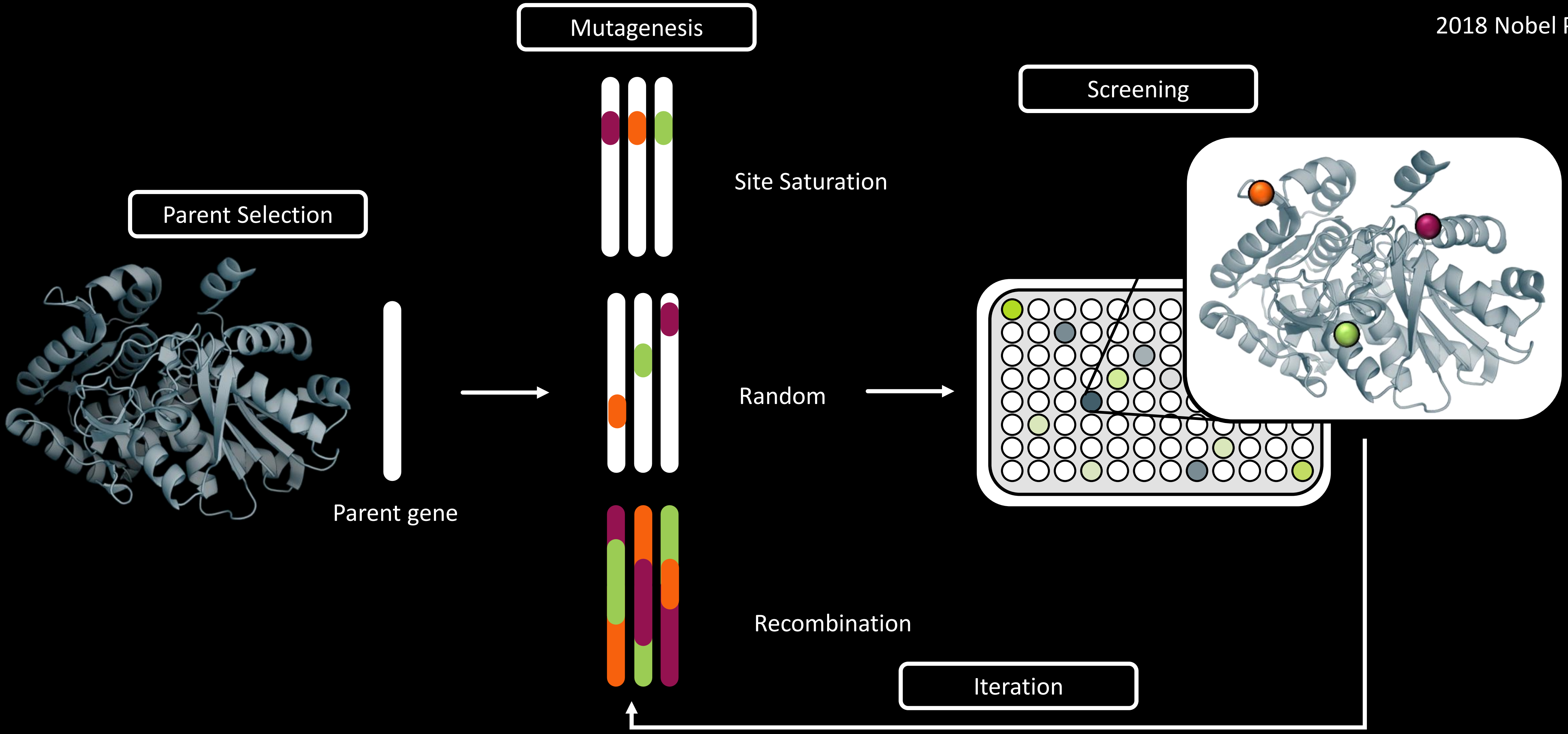
Almhjell PJ, Boville CE, Arnold FH. *Chem Soc Rev*. 2018;47(24):8980-8997

How do new functions arise?



Directed evolution

2018 Nobel Prize



Parent Selection

Parent gene

Mutagenesis

Site Saturation

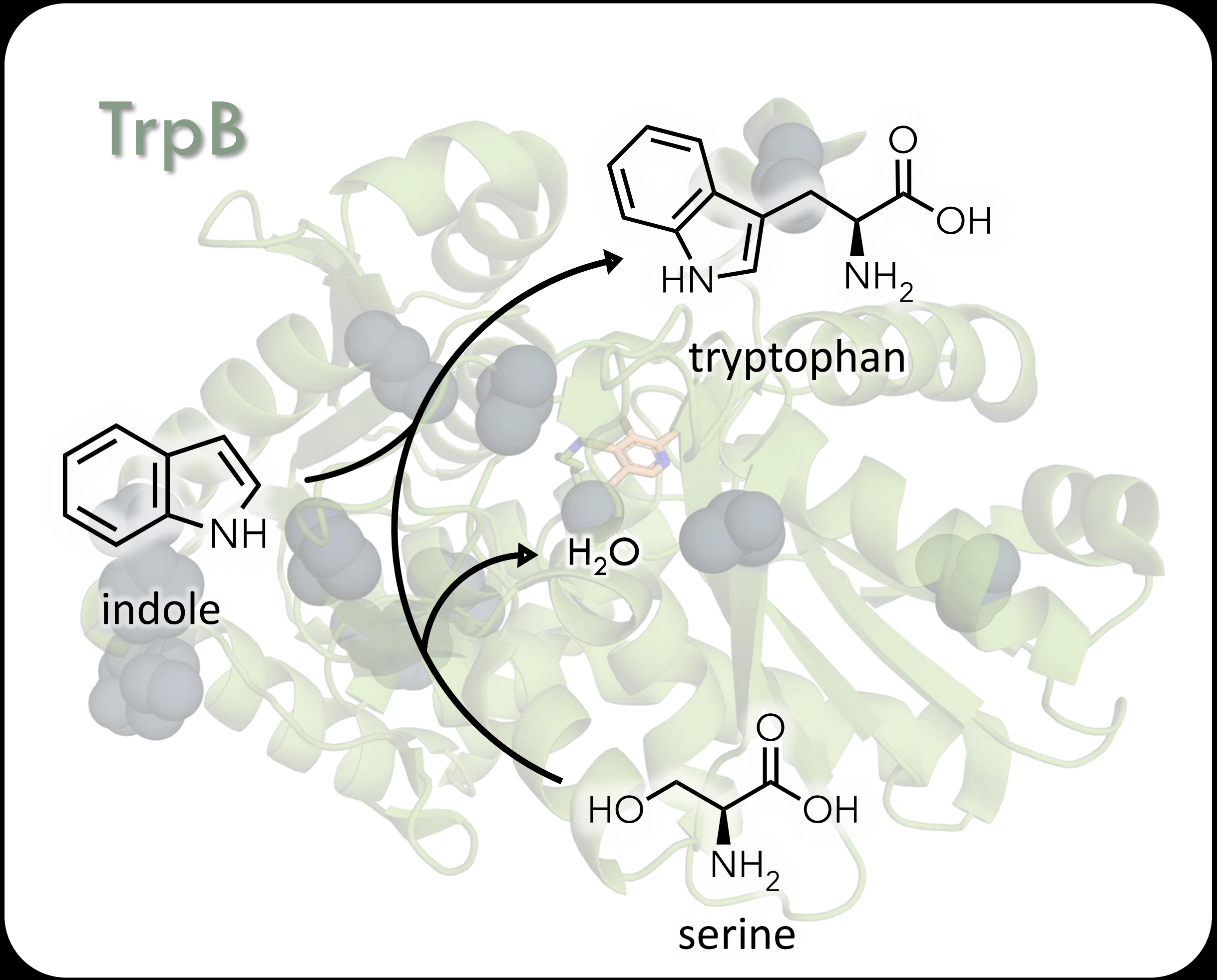
Random

Recombination

Screening

Iteration

Tryptophan synthase β-subunit



Pyrococcus furiosus

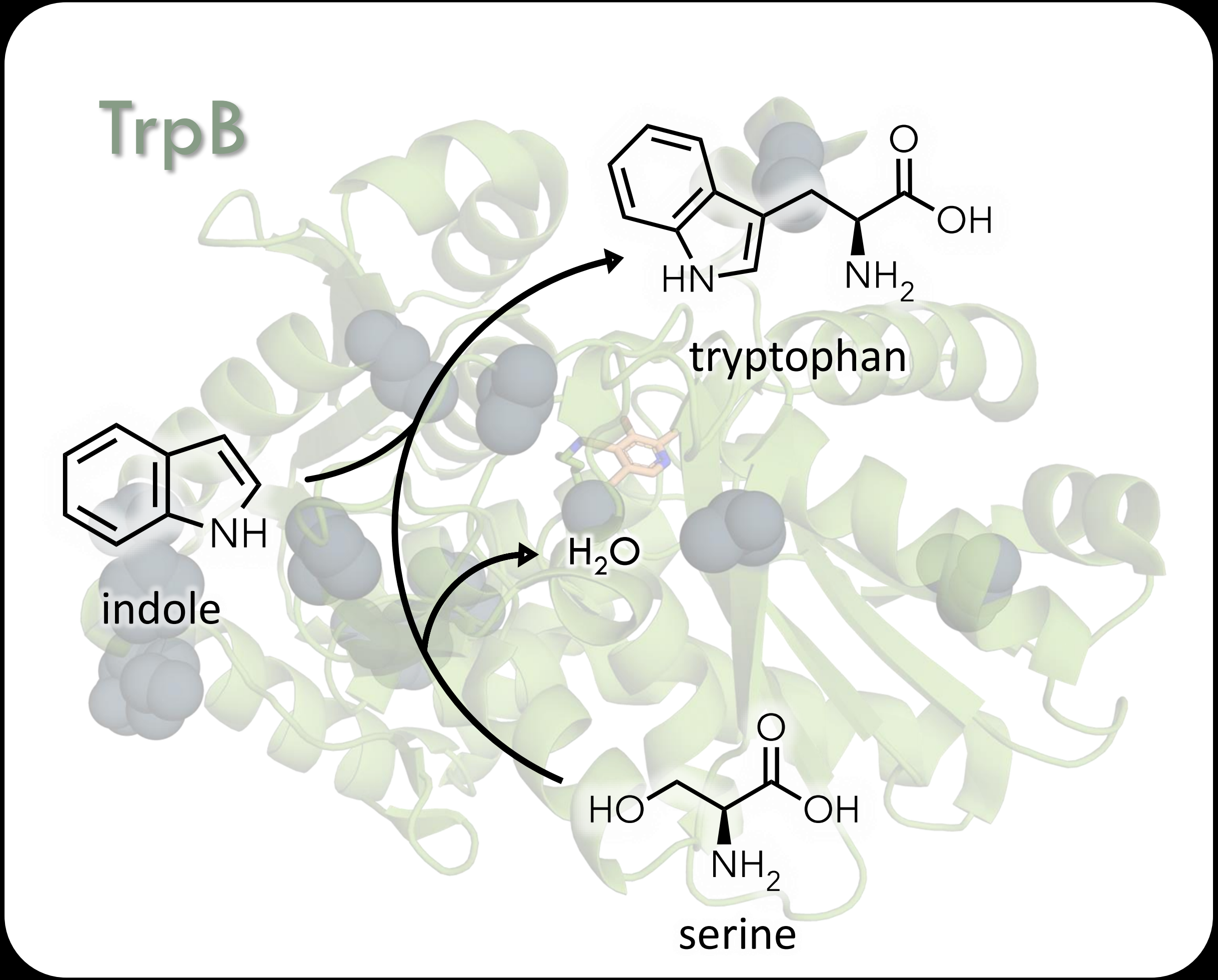
Name literally means
"Rushing fireball"

&

Thermotoga maritima
From hydrothermal vents in Italy

Buller AR, Brinkmann-Chen S, Romney DK, Herger M, Murciano-Calles J, Arnold FH *Proc Natl Acad Sci*. 2015;112(47):14599-14604.

Tryptophan synthase β-subunit



The best of both worlds:

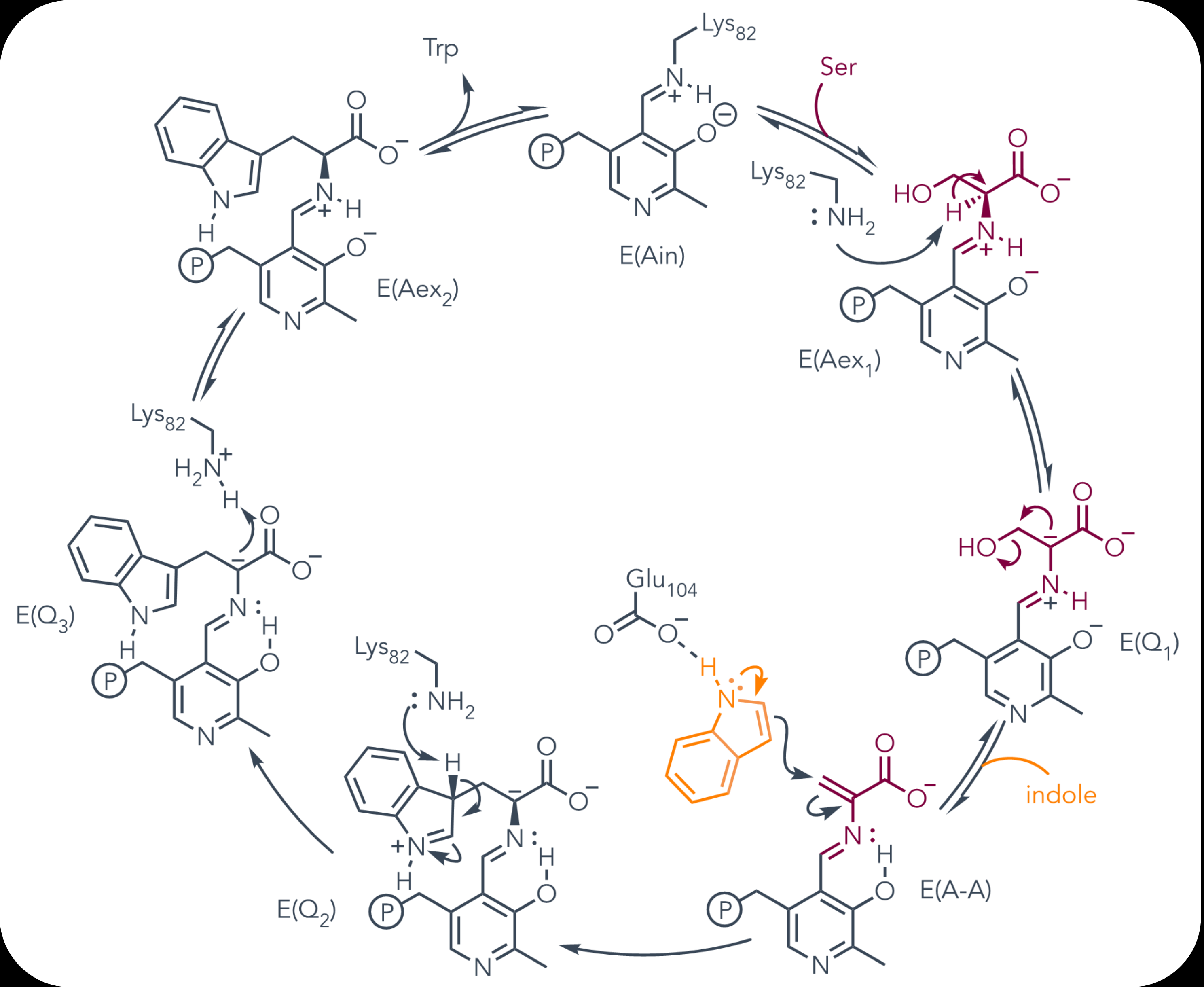
- ✓ Single step
- ✓ Irreversible
- ✓ Simple starting materials
- ✓ Catalytic
- ✓ Aqueous

Bonus!

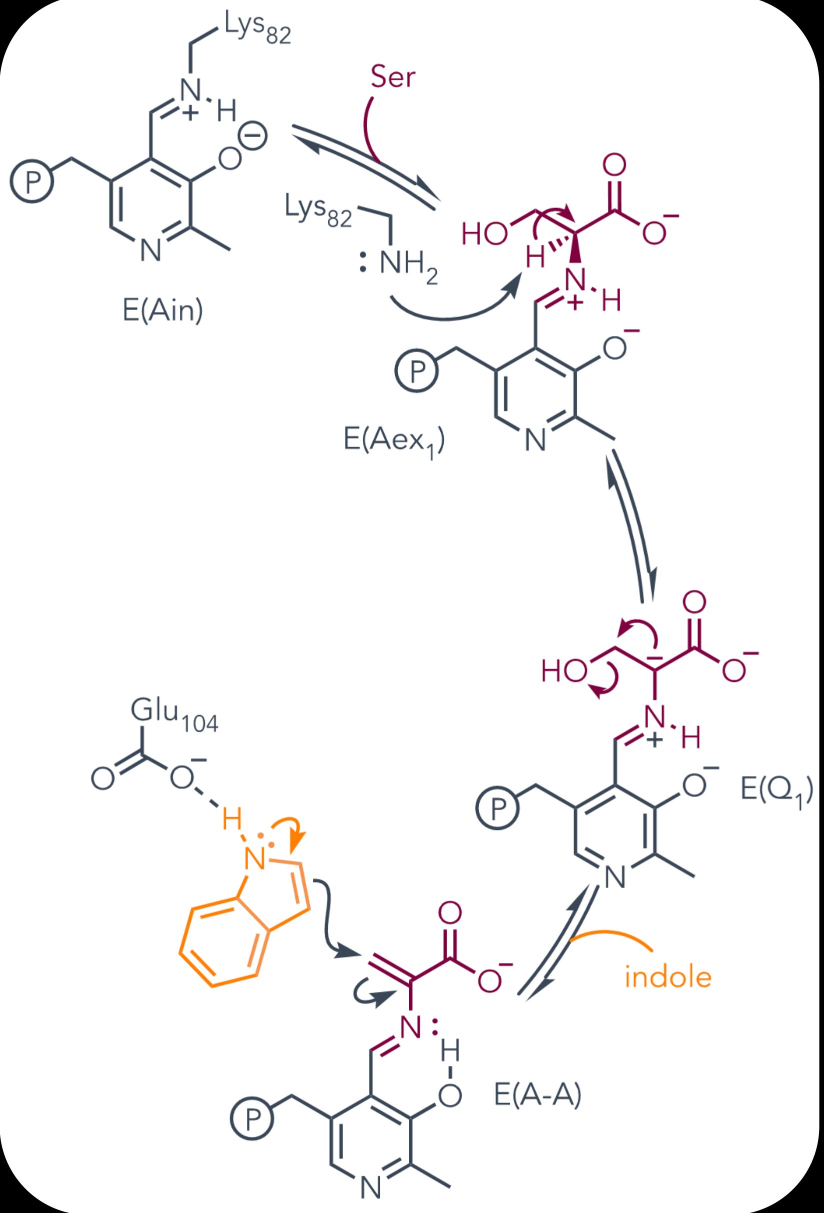
- ✓ ✓ Thermostable
- ✓ ✓ Highly expressing

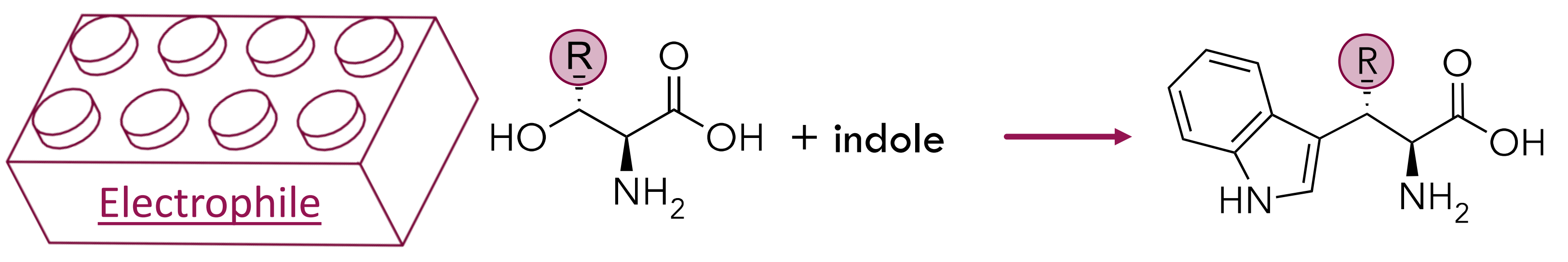
Buller AR, Brinkmann-Chen S, Romney DK, Herger M, Murciano-Calles J, Arnold FH *Proc Natl Acad Sci*. 2015;112(47):14599-14604.

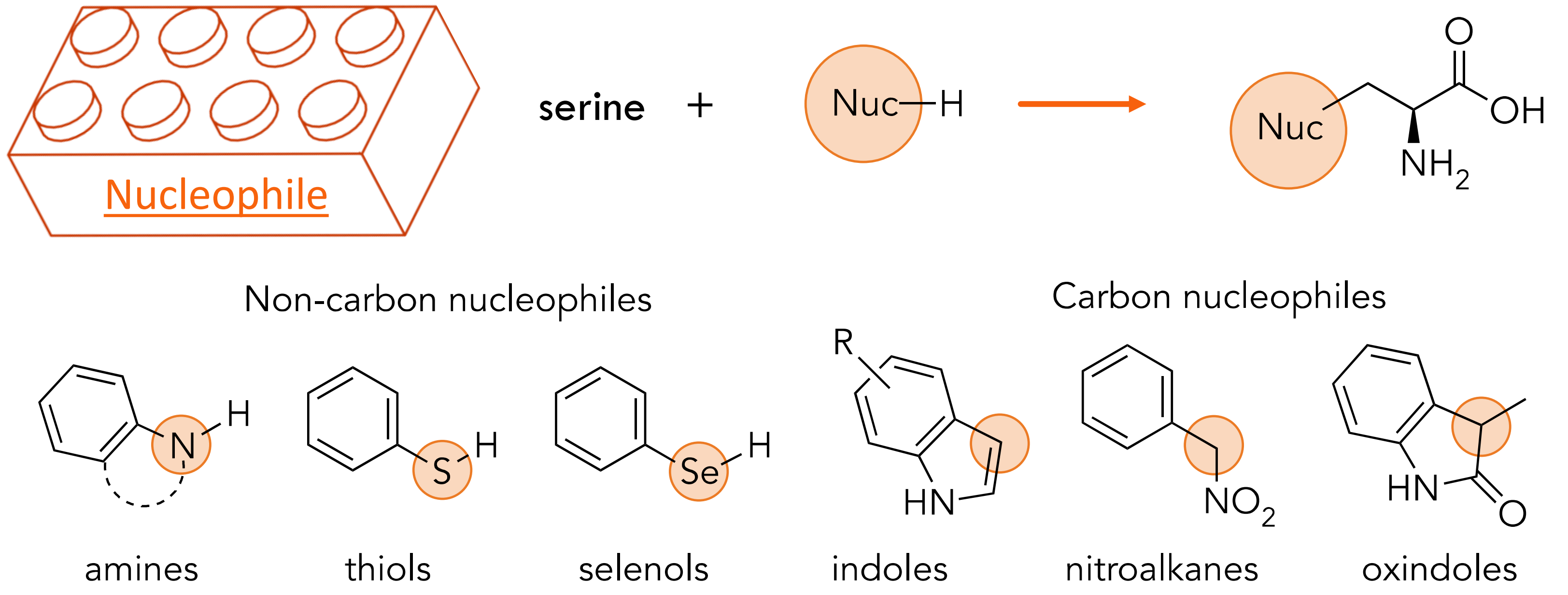
The TrpB catalytic cycle



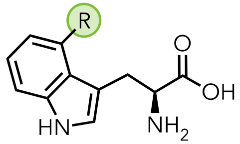
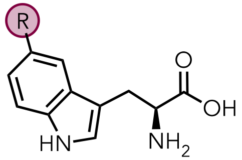
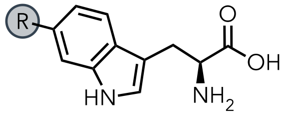
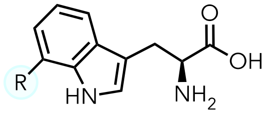
The TrpB reaction is modular

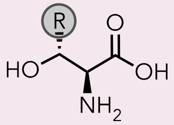






An impressive scope

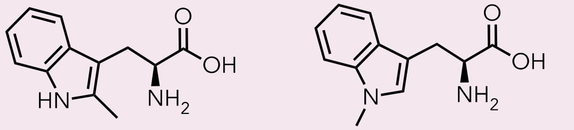
Substitution		EDG	Halogenated	EWG
4		CH_3 OCH_3 OH	F Cl Br	NO_2 CO_2H CN $CONH_2$
5		CH_3 OCH_3 OH	F Cl Br I	NO_2 CHO CN $CONH_2$ $B(OH)_2$
6		CH_3 OCH_3 OH	F Cl Br	NO_2 CN $B(OH)_2$
7		CH_3 OCH_3 OH	F Cl Br I	NO_2 CN



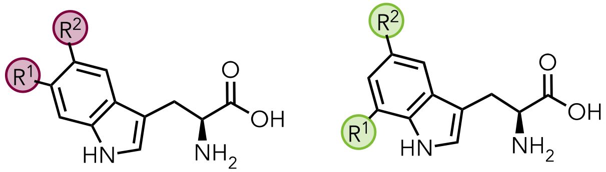
β-substituted electrophiles accepted

R = H, methyl, ethyl, propyl

1- and 2- substituted

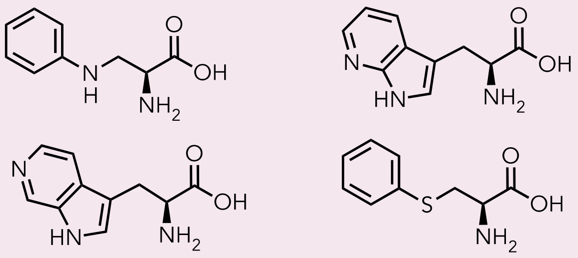


Disubstituted

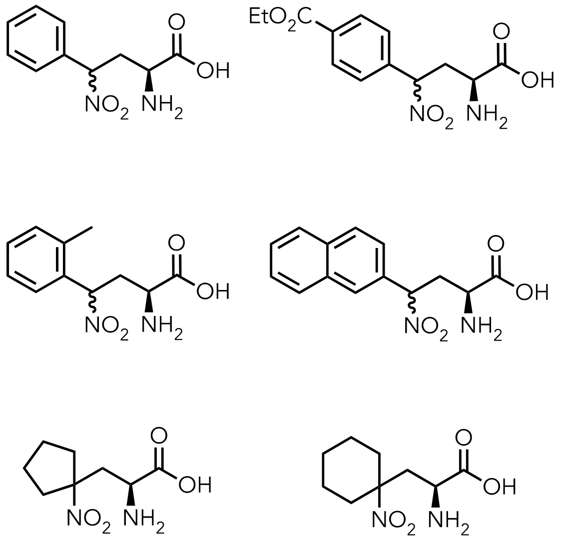


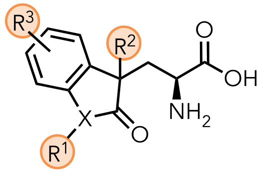
R^1, R^2 = F, Cl, Br, I

Other classes of α-amino acids:



Non-indole carbon nucleophiles

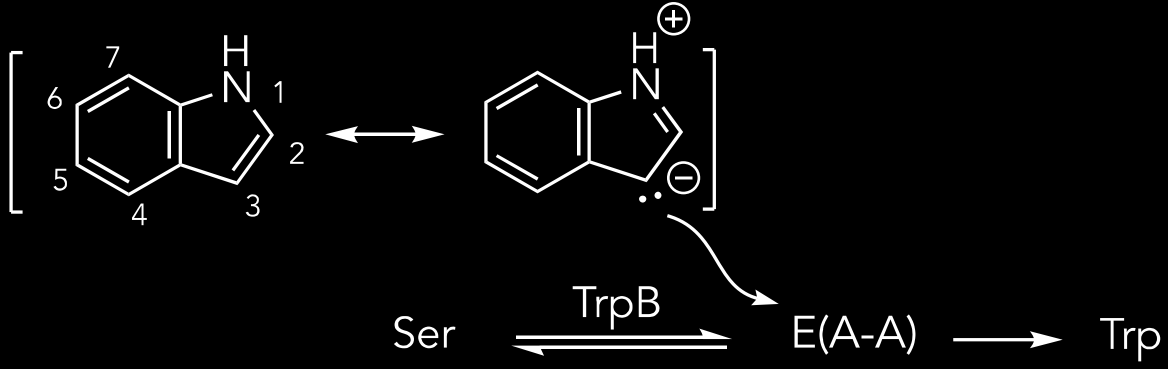




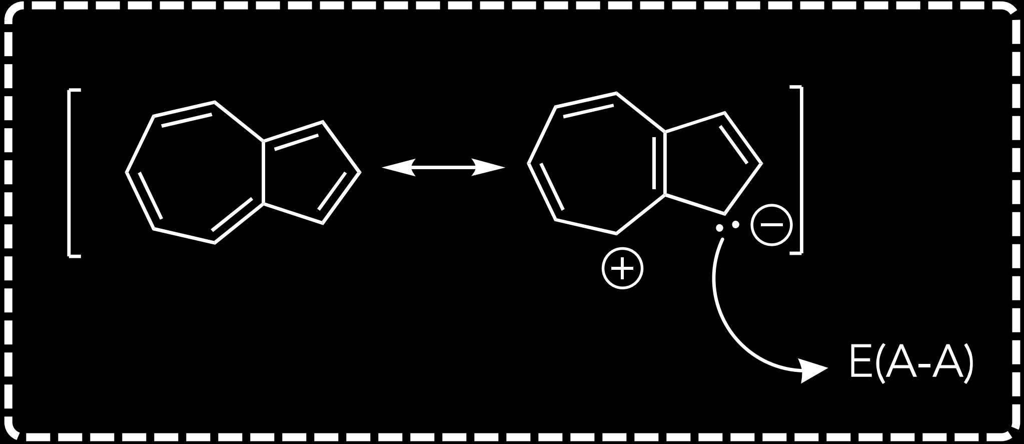
R^1 = CH_3, H ,
R^2 = methyl, ethyl, propyl, butyl, OH,
R^3 = Cl, Fl

Buller AR, Brinkmann-Chen S, Romney DK, Herger M, Murciano-Calles J, Arnold FH *Proc Natl Acad Sci*. 2015;112(47):14599-14604.
Romney DK, Murciano-Calles J, Wehrmüller JE, Arnold FH. *J Am Chem Soc*. 2017;139(31):10769-10776.
Herger M, van Roye P, Romney DK, Brinkmann-Chen S, Buller AR, Arnold FH. *J Am Chem Soc*. 2016:jacs.6b04836.
Boville CE, Scheele RA, Koch P, Brinkmann-Chen S, Buller AR, Arnold FH. *Angew Chemie Int Ed*. 2018.
Romney, David K., Nicholas S. Sarai, and Frances H. Arnold. *ACS Catalysis* 2019.
Dick, Markus, et al., *J Am Chem Soc*. 141.50 (2019): 19817-19822.

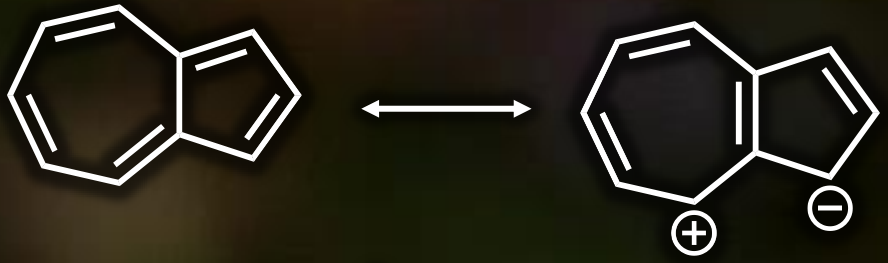
What non-indole carbon nucleophiles could TrpB react with?



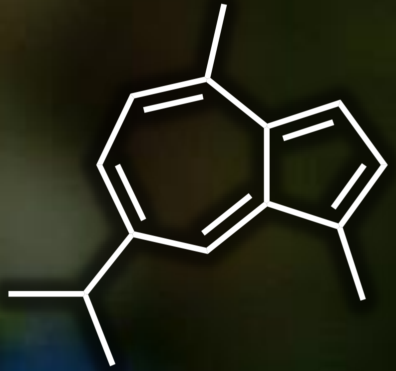
Azulene



The beauty and utility of azulene

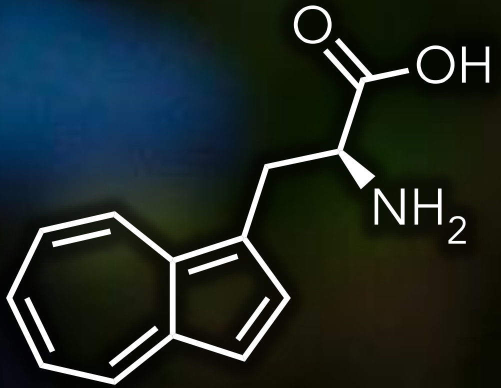


Azulene



Guaiazulene

Azulene derivative found in mushrooms



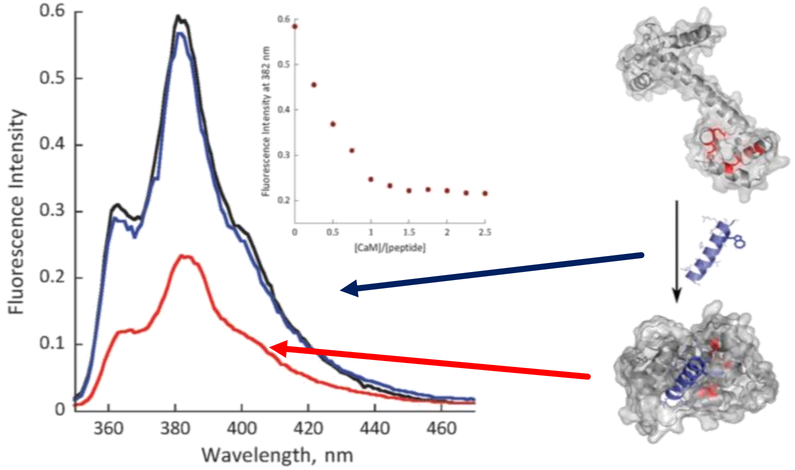
AzAla

Biological fluorescent probe
Tryptophan mimic

Loidl G, Musiol HJ, Budisa N, et al. *J Pept Sci*. 2000;6(3):139-144.
Moroz, Yurii S., et al. *Chemical communications* 49.5 (2013): 490-492.
Stempel E, Kaml RFX, Budisa N, Kalesse M*Bioorganic Med Chem*. 2018;26(19):5259-5269.

Applications of AzAla

Probe for protein-protein interactions

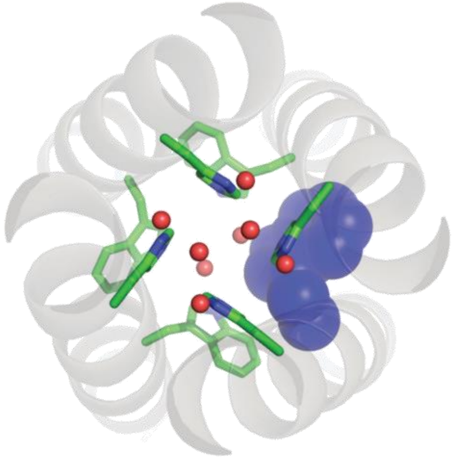


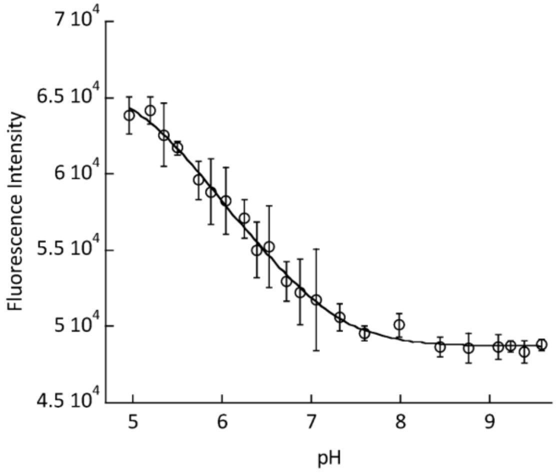
Calmodulin
(methionine residues)

Smooth muscle myosin light chain kinase with AzAla

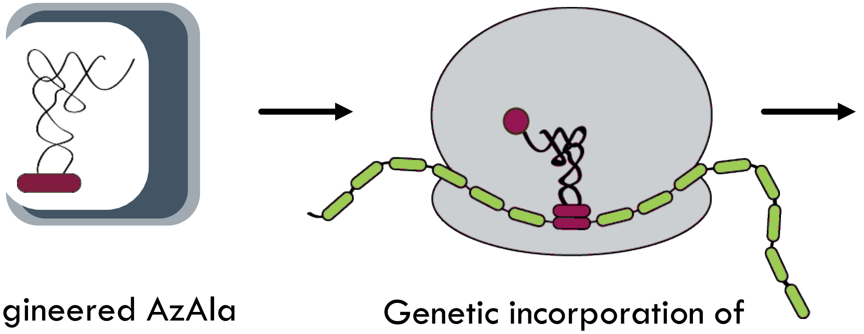
Methionine quenches AzAla signal

Probe for determination of pK_a of histidine residues





Probe for vibrational energy transfer



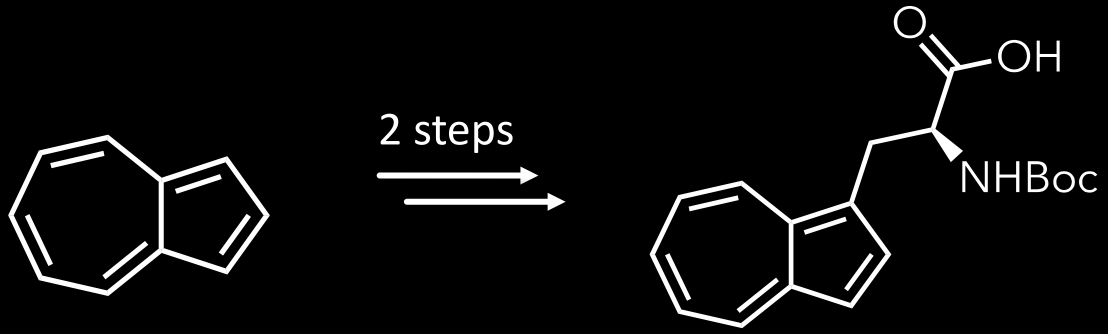
Engineered AzAla synthetase/tRNA pair

Genetic incorporation of AzAla

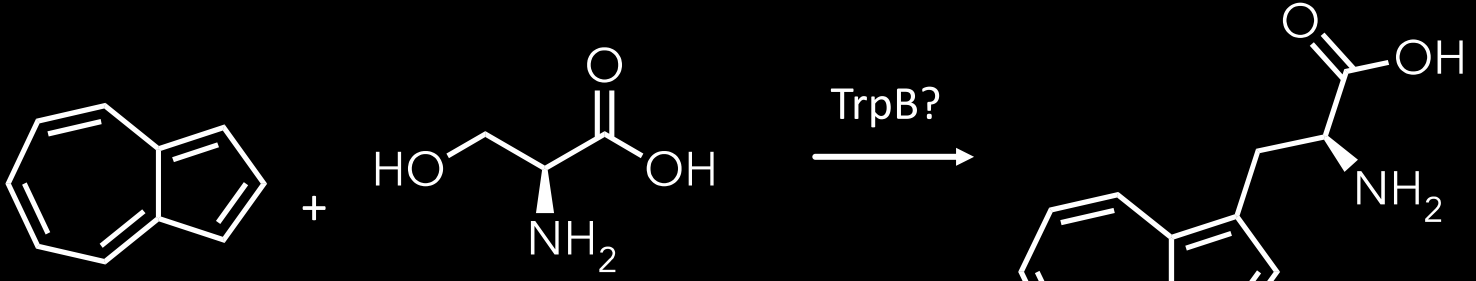
VET studies

Moroz YS, Binder W, Nygren P, Caputo GA, Korendovych I V. *Chem Commun*. 2013;49(5):490-492.
Gosavi PM, Moroz YS, Korendovych I V. *Chem Commun*. 2015;51(25):5347-5350.
Jaric J, Acevedo-Rocha CG, Dobbek H, et al. *Angew Chemie Int Ed*. 2019.

Synthesis of AzAla



- +2 steps: synthesis of protected amine
- +1 step: deprotection
- Air/water sensitive
- Intermediates unstable at RT



Azulene

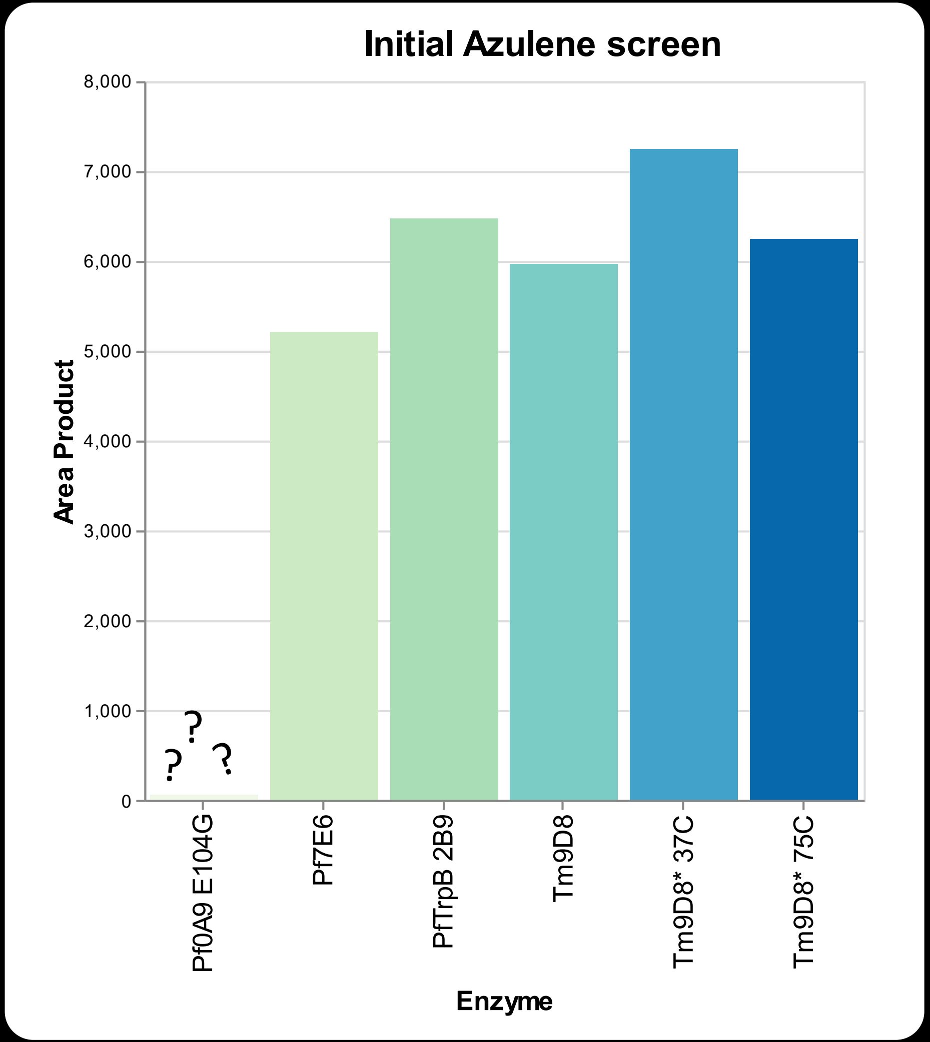
Serine

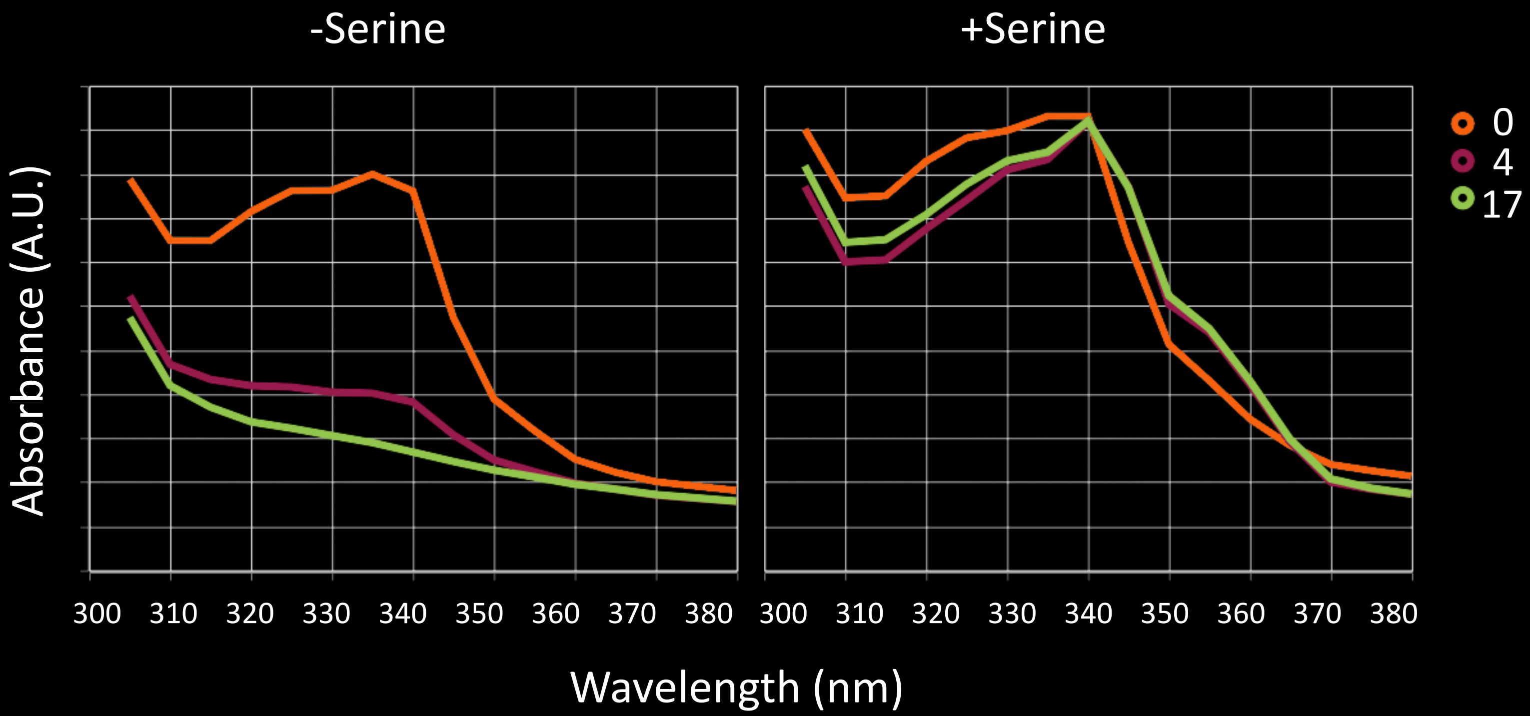
AzAla

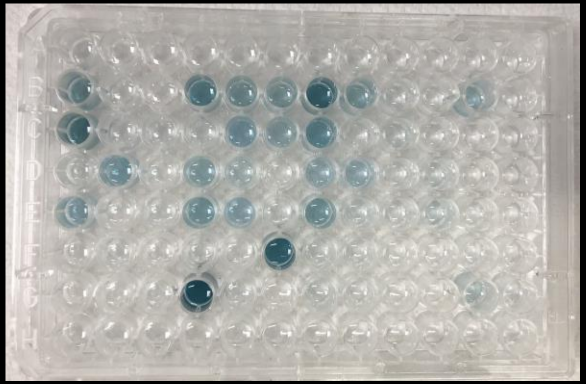
- 1 step
- No protection/deprotection
- Enzymatic
- Not air/water/time sensitive

Loidl G, Musiol HJ, Budisa N, et al. *J Pept Sci*. 2000;6(3):139-144.
Moroz, Yurii S., et al. *Chemical communications* 49.5 (2013): 490-492.
Stempel E, Kaml RFX, Budisa N, Kalesse M*Bioorganic Med Chem*. 2018;26(19):5259-5269.

Finding activity and developing a screen





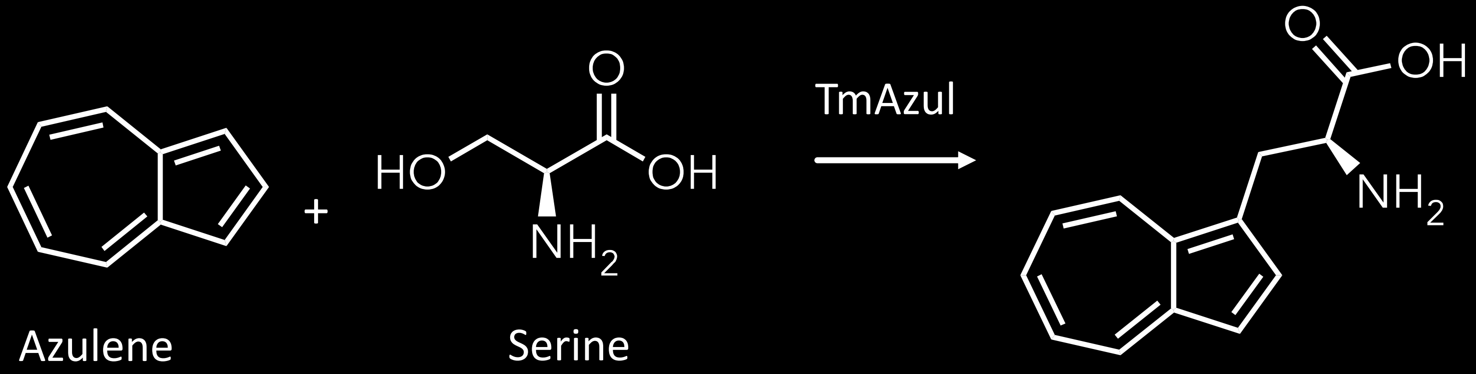


- Azulene evaporates off!
- More vibrant blue wells=better variants
- Only 1 round of evolution was needed
- Tm9D8* + W286R mutation = TmAzul

Watkins, Ella J., Patrick J. Almhjell, and Frances H. Arnold. *ChemBioChem* 21.1-2 (2020): 80-83.

Synthesis of AzAla

- Optimized scale up conditions
- Optimized purification conditions
    - Fun to do, but lots of work!
    - Easy to track product through every step of purification
- Performed scale up:

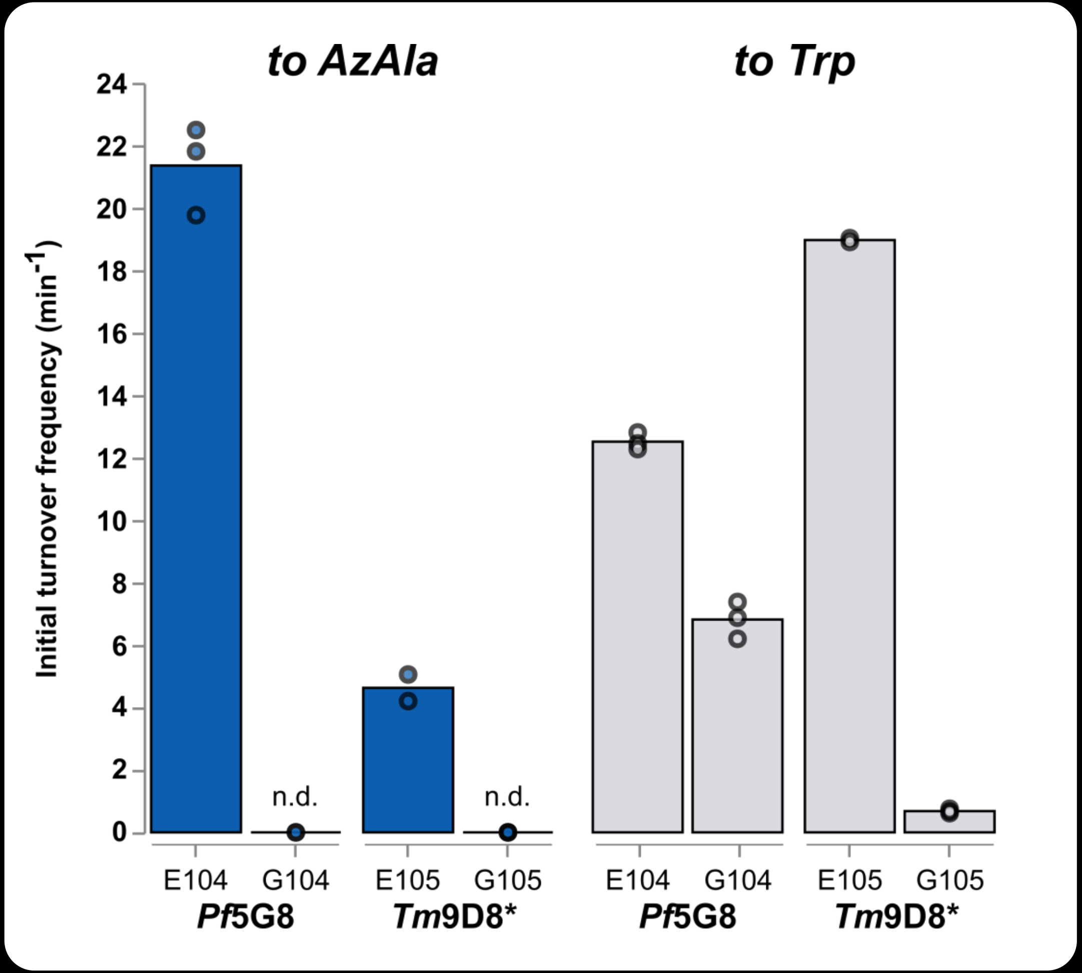


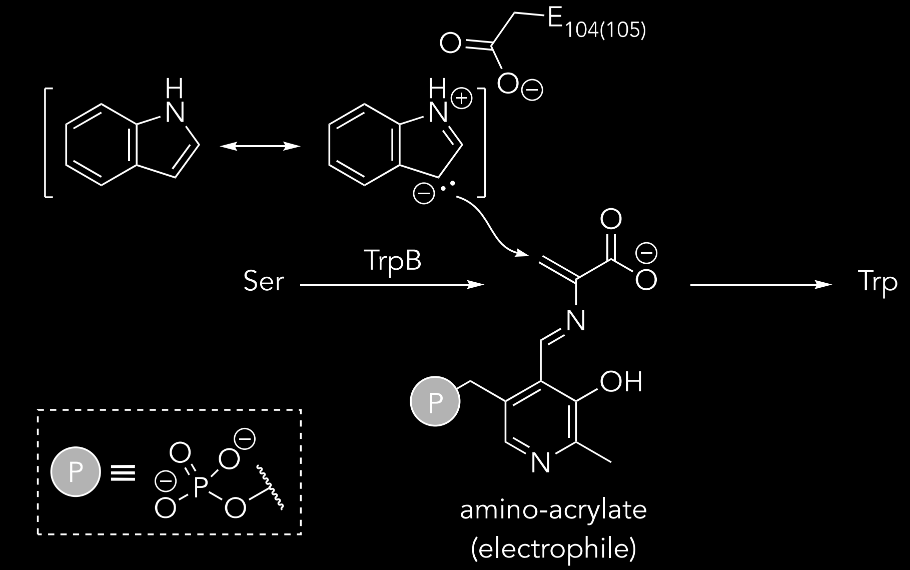
AzAla

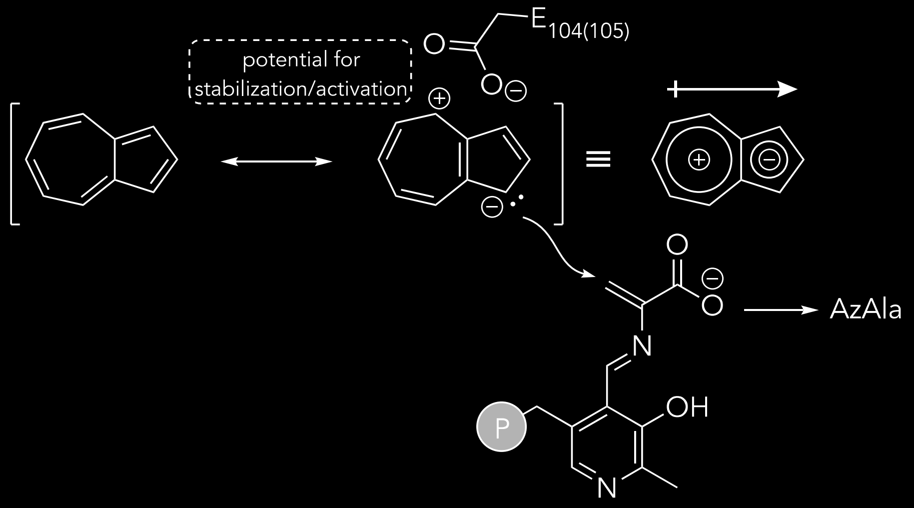
967 mg
57% isolated yield

Watkins, Ella J., Patrick J. Almhjell, and Frances H. Arnold. *ChemBioChem* 21.1-2 (2020): 80-83.

Why does E104(5)G destroy activity?

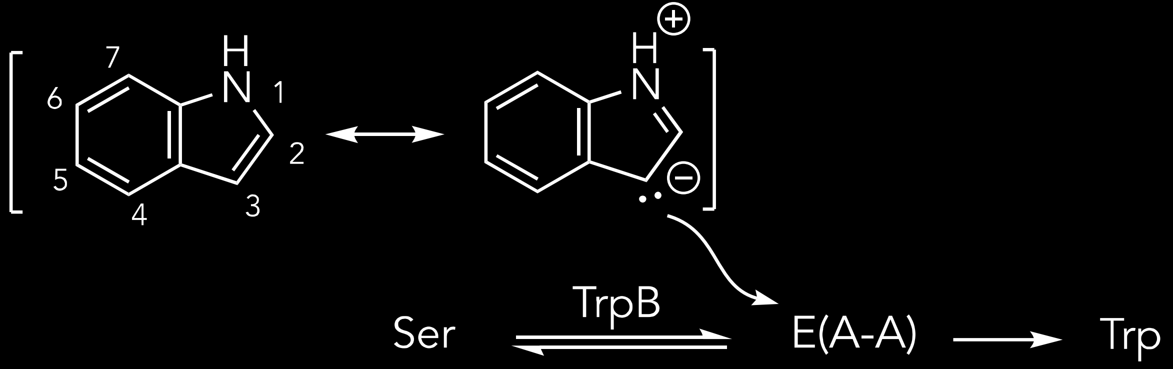




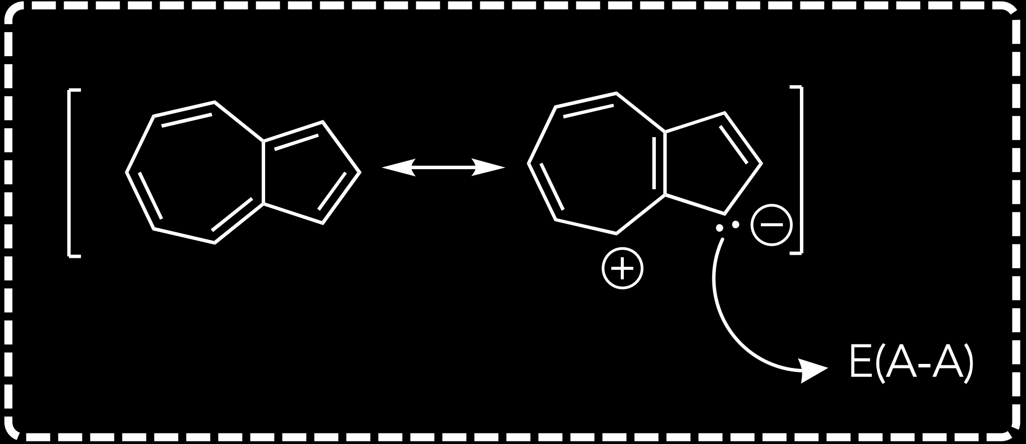


Watkins, Ella J., Patrick J. Almhjell, and Frances H. Arnold. *ChemBioChem* 21.1-2 (2020): 80-83.

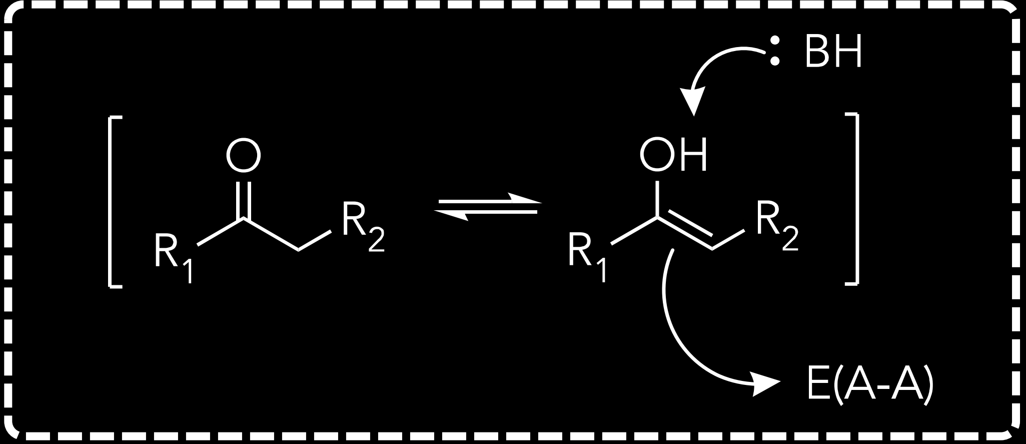
What non-indole carbon nucleophiles could TrpB react with?



Azulene



Ketones



Could TrpB react with *ANY* carbon nucleophile?

Some traditional carbon nucleophiles used in synthetic chemistry are really strong…

Let's steer clear of N-butyl lithium.

$$n\text{-BuLi} + O_2 \longrightarrow n\text{-BuOLi}$$

$$n\text{-BuLi} + HOH \longrightarrow \mathbf{n\text{-BuH}} + LiOH$$

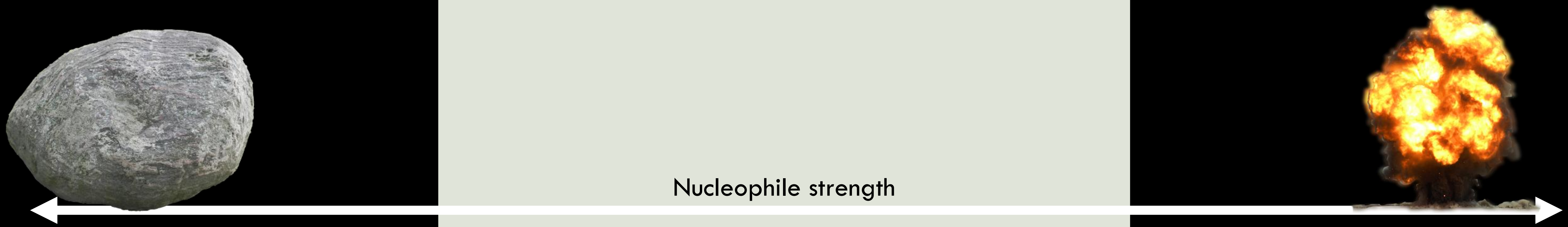
Not *too* nucleophilic that it reacts with water (or air)

What kind of carbon nucleophiles are possible?

Unreactive

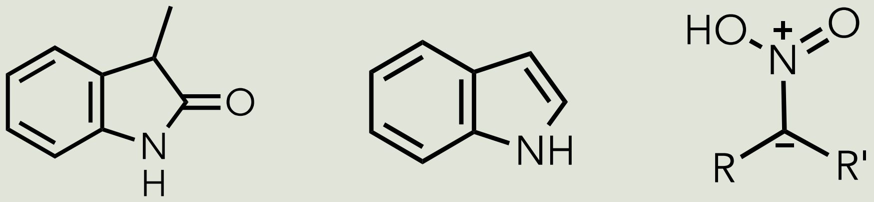
Sweet spot

Reactive



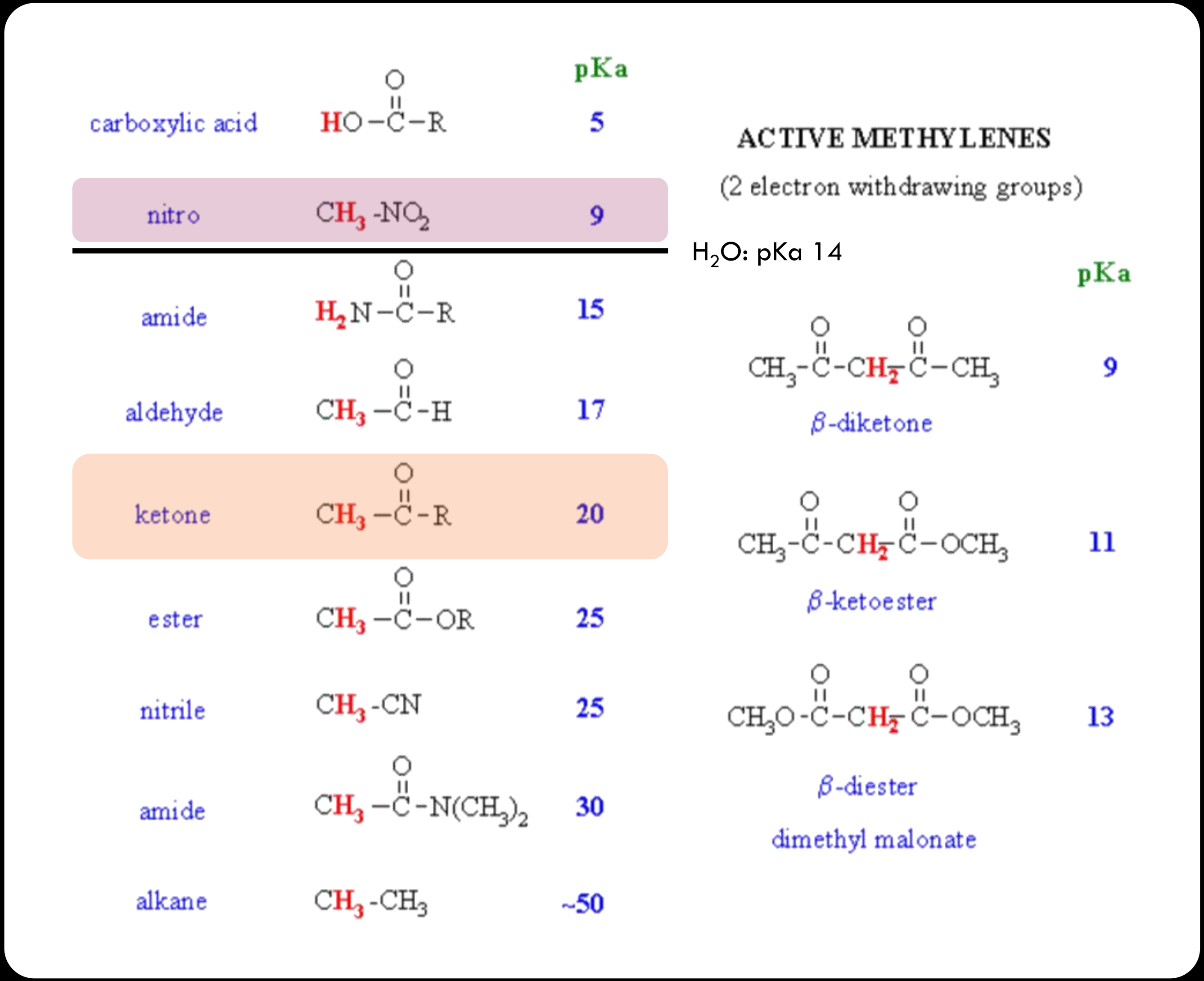
- Neutral arenes
- Olefins

Not too nucleophilic that it reacts with water



Ease of deprotonation

- Ability of carbonyl to stabilize adjacent electron density reflected in pKa values
- $pKa_{ketones} >> pKa_{water}$
- Would we be able to reach a **reactive** enolate form in water?



		pKa
carboxylic acid	$\mathrm{HO{-}\overset{\overset{O}{\|}}{C}{-}R}$	5
nitro	$\mathrm{CH_3{-}NO_2}$	9
amide	$\mathrm{H_2N{-}\overset{\overset{O}{\|}}{C}{-}R}$	15
aldehyde	$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}H}$	17
ketone	$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}R}$	20
ester	$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}OR}$	25
nitrile	$\mathrm{CH_3{-}CN}$	25
amide	$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}N(CH_3)_2}$	30
alkane	$\mathrm{CH_3{-}CH_3}$	~50

H_2O: pKa 14

ACTIVE METHYLENES

(2 electron withdrawing groups)

	pKa
$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}CH_3}$ β-diketone	9
$\mathrm{CH_3{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}OCH_3}$ β-ketoester	11
$\mathrm{CH_3O{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}OCH_3}$ β-diester dimethyl malonate	13

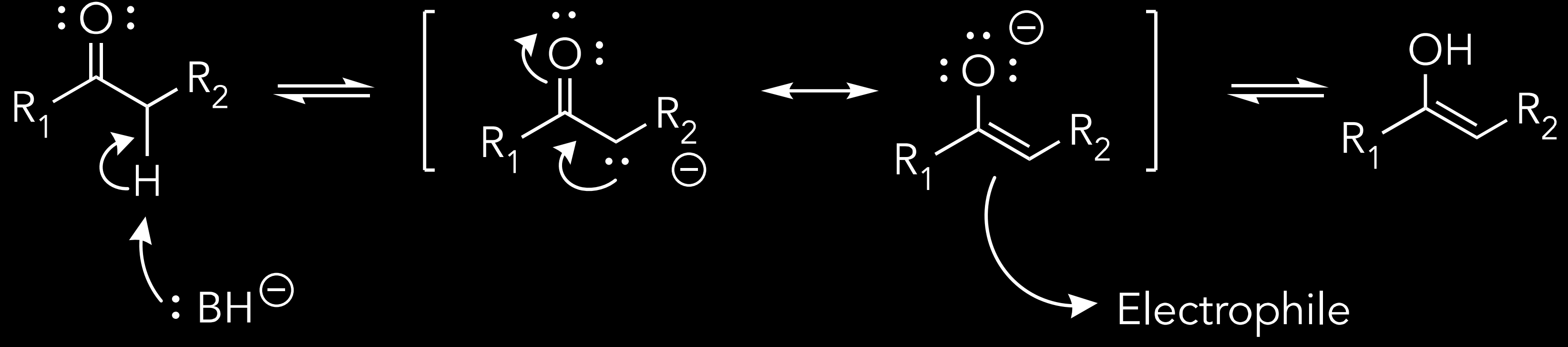
Ketone derived enolates are *classic* carbon nucleophiles

Ketone

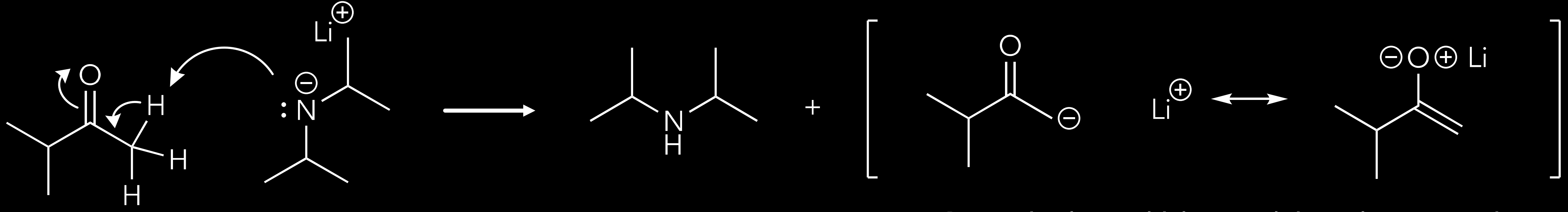
Enolate

(Very fleeting intermediate)

Enol



Example: Lithium diisopropylamide (LDA)



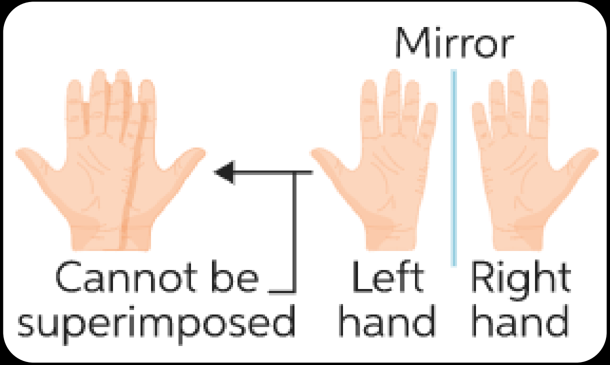
Positively charged lithium stabilizes the negatively charged enolate, making it a persistent species

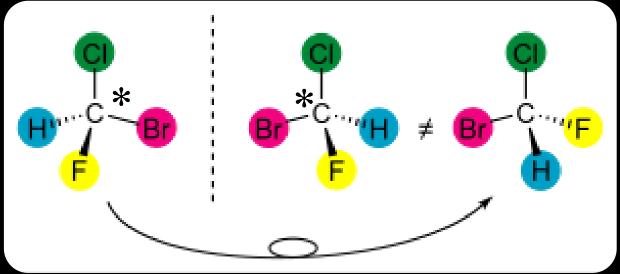
Easy for chemists to make, difficult to control…

“Direct asymmetric α-C–H alkylation of ketones still unconquered”

“Asymmetry”

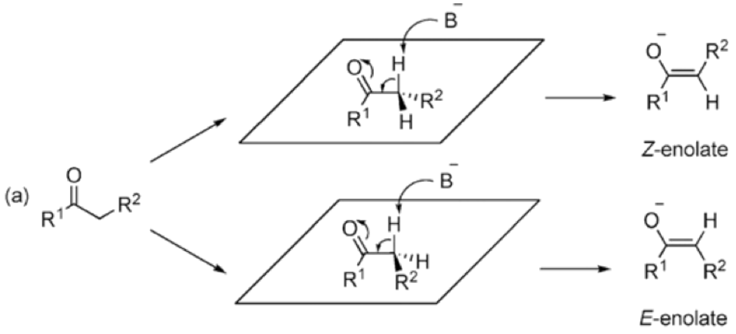
Molecules can be chiral, meaning that they are mirror images that can’t be superimposed.



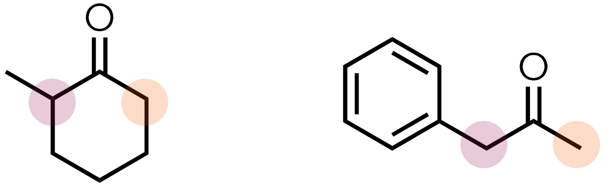


- If a molecule is chiral, it has a “stereocenter” (denoted by a star: *)
- The two mirror images of the molecules are called “stereoisomers”
- Selectivity is crucial: different stereoisomers interact with the body differently

E/*Z*-enolate selectivity

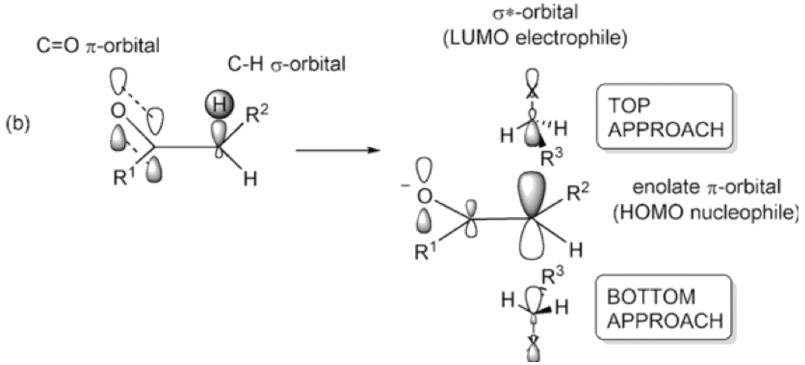


Regioselective enolate formation

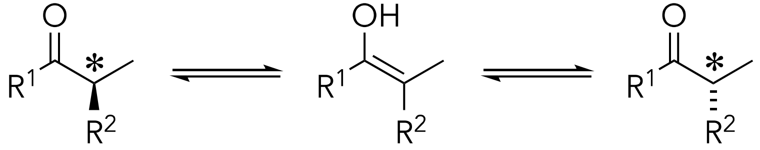


Kinetically vs. thermodynamically favorable enolate

Electrophile orbital approach



Racemization of new chiral center

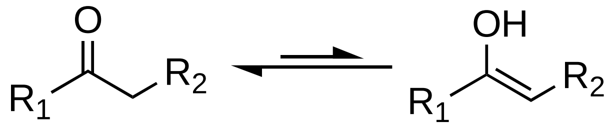


Cano, Rafael, Armen Zakarian, and Gerard P. McGlacken. "Direct asymmetric alkylation of ketones: still unconquered." *Angewandte Chemie International Edition* 56.32 (2017): 9278-9290.

Could TrpB accept ketones as nucleophiles?

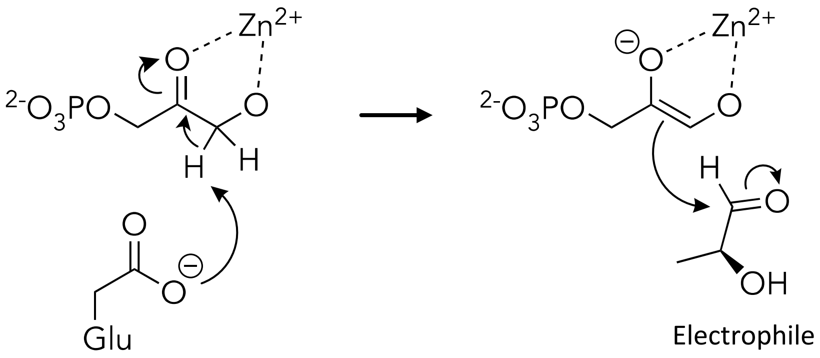
- Enols/enolates are **thermodynamically unfavorable** in water

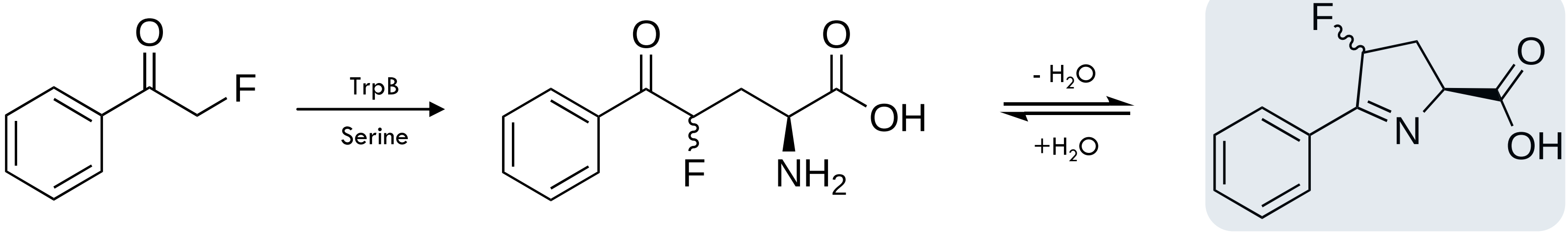
Ketone in aqueous solution



- Very few enzymes that use enolates. Those that do are **very specialized**.

Type II aldolase



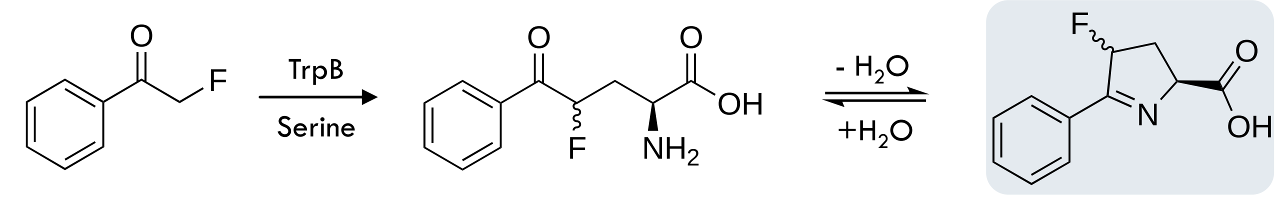


2-fluoroacetophenone (2F)

linear amino acid

cyclized imine

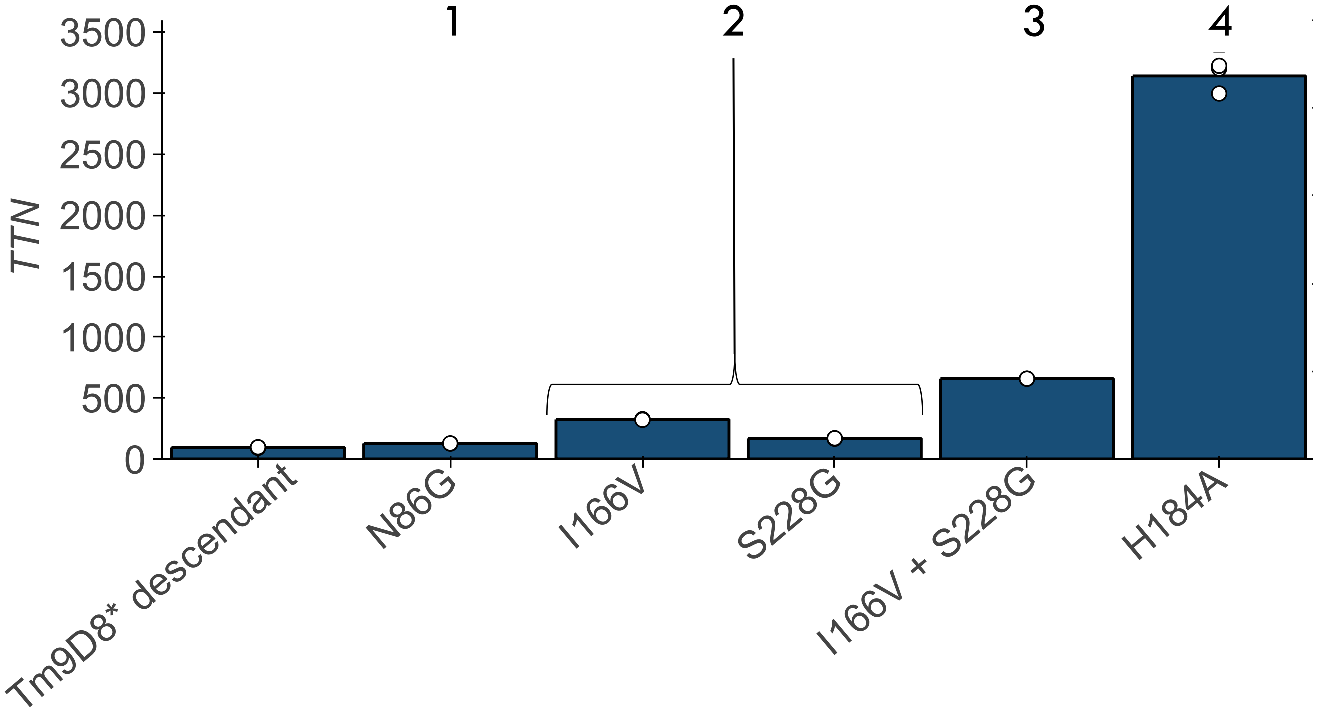
Directed evolution for ketone alkylation



2-fluoroacetophenone (2F)

linear amino acid

cyclized imine



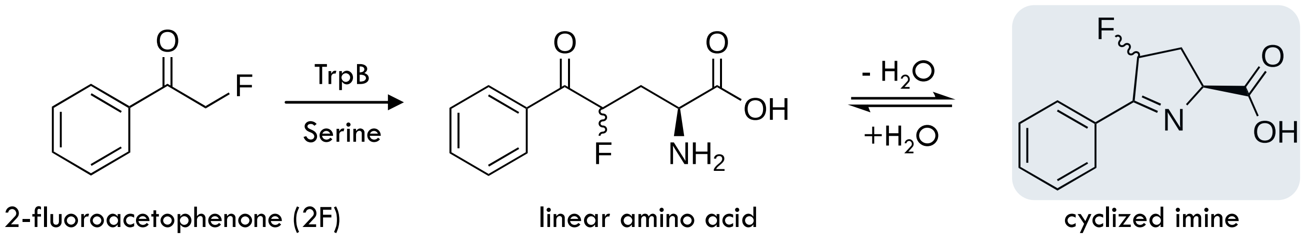
Round 1: Site saturation mutagenesis

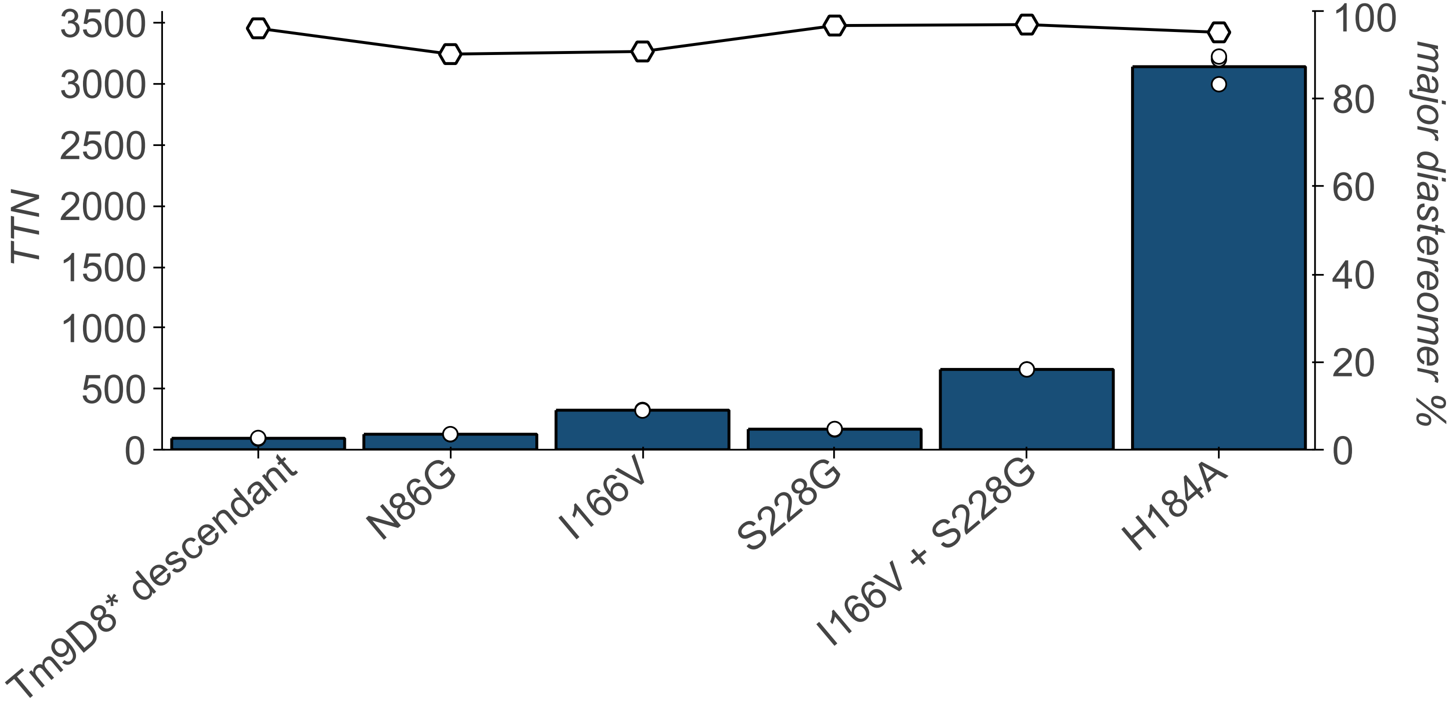
Round 2: Random mutagenesis

Round 3: Recombination

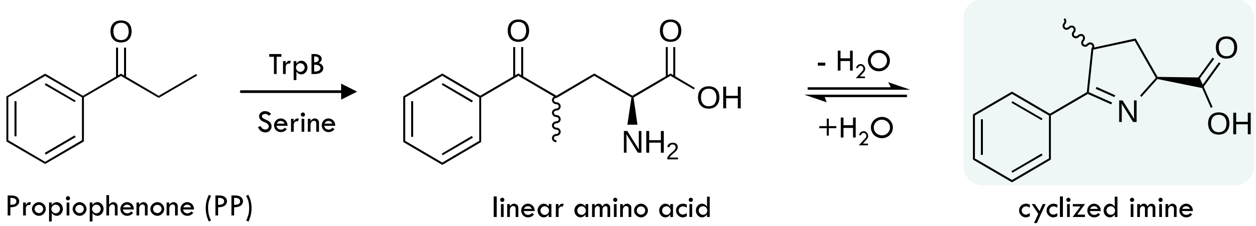
Round 4: Site directed mutagenesis

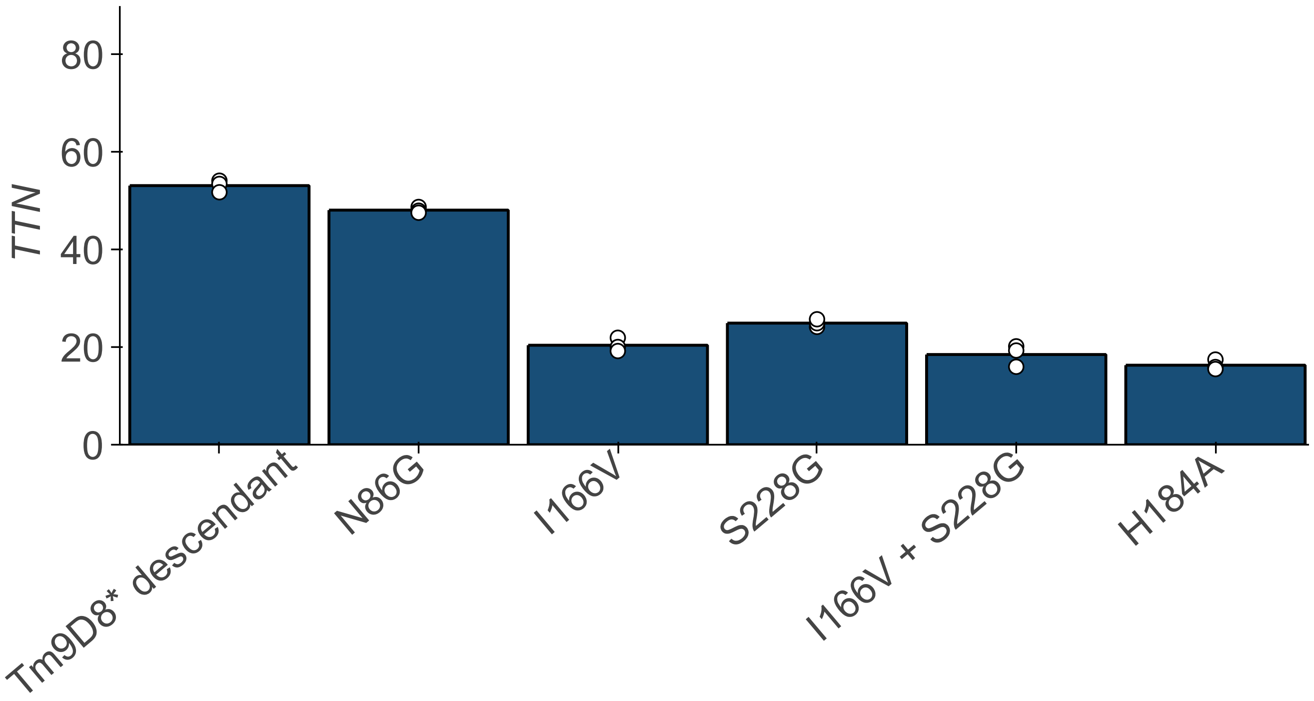
TrpB catalyzes asymmetric alkylation!



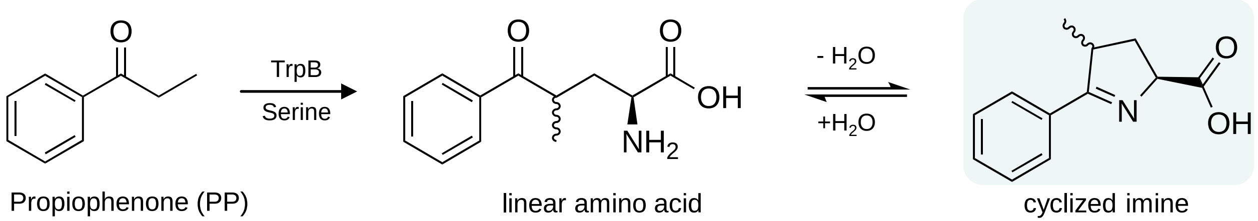


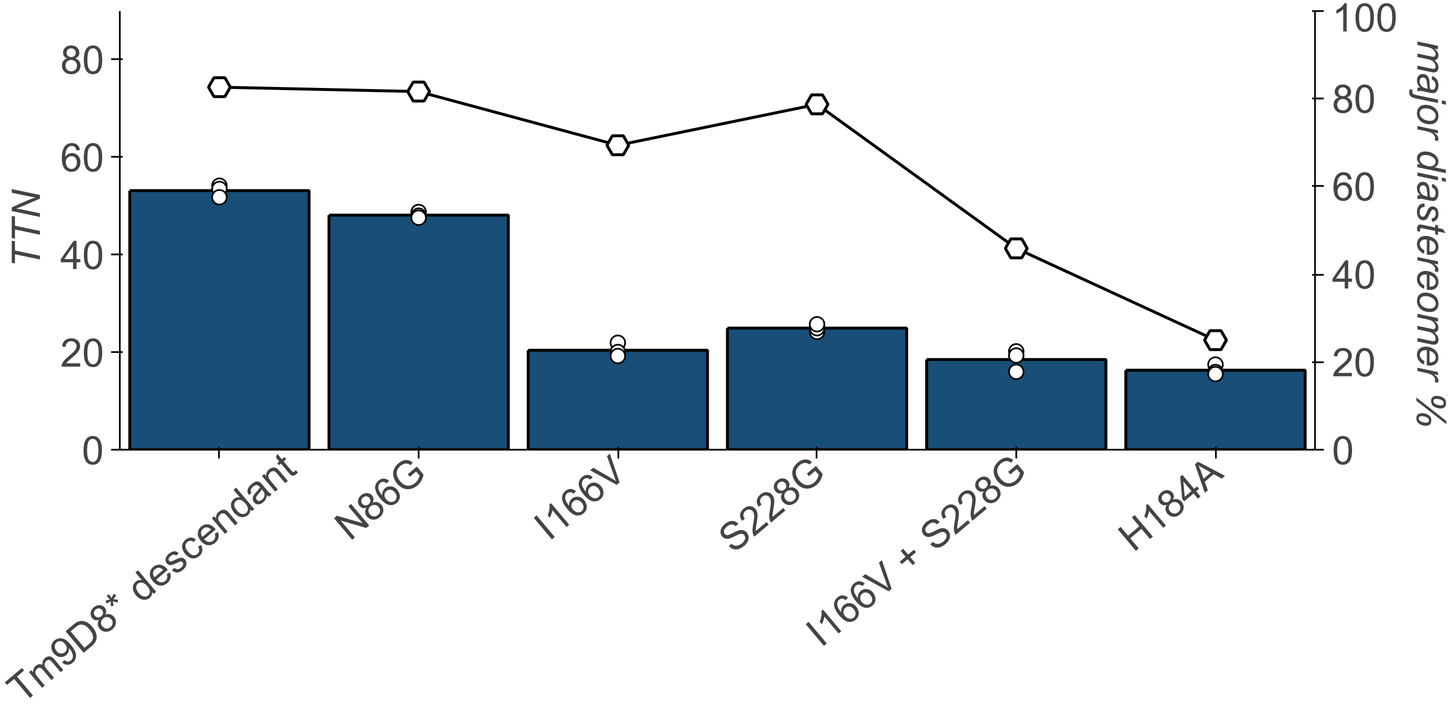
You get what you screen for





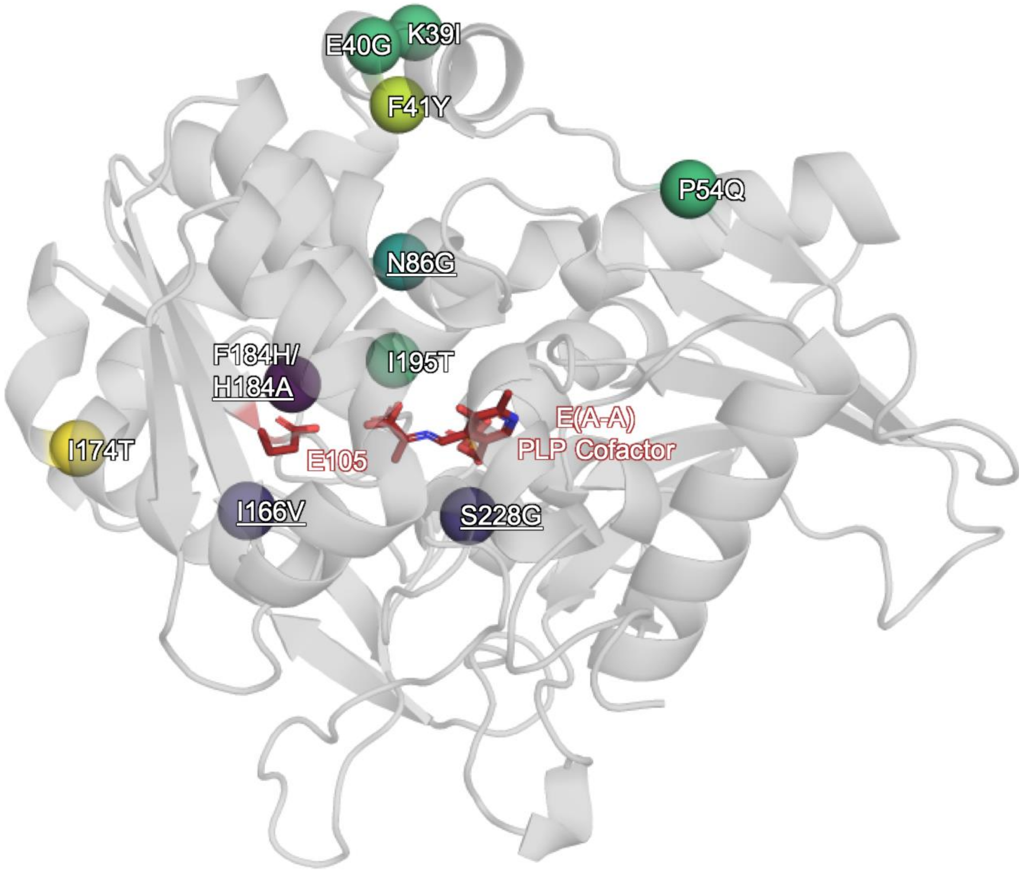
But if you try sometimes, well you might find, you get what you need!

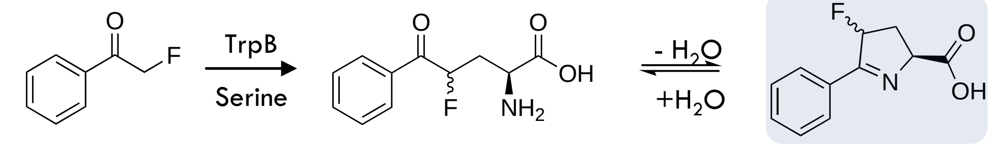




Selectivity was entirely inverted! TrpB can make both diastereomers.

Characterization of TmEnolate



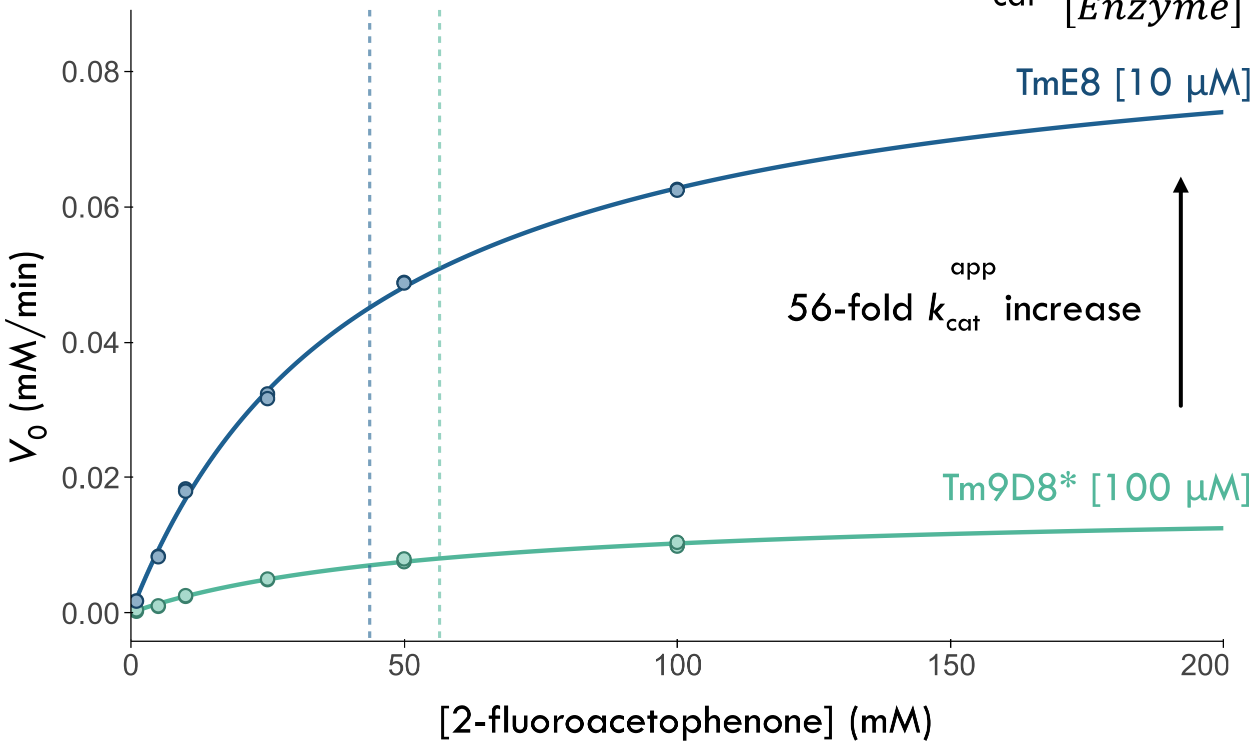


2-fluoroacetophenone (2F)

linear amino acid

cyclized imine

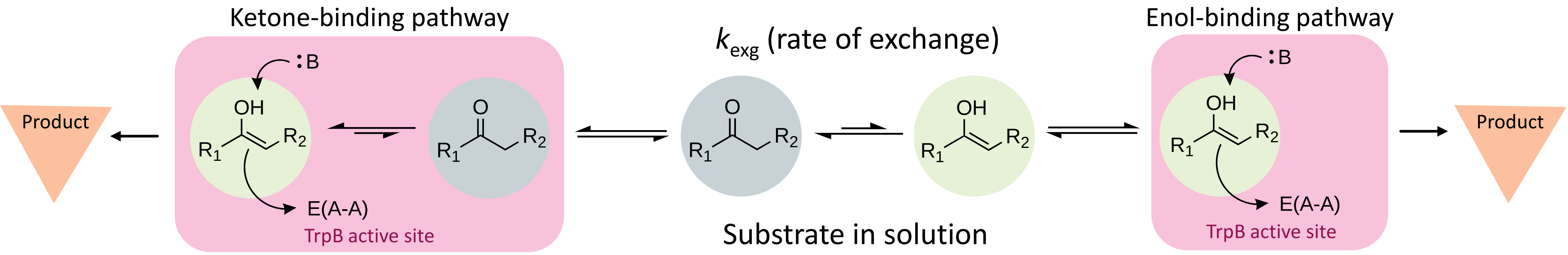
k_{cat}: $\frac{V_{max}}{[Enzyme]}$



56-fold k_{cat}^{app} increase

What did evolution do to speed up the reaction?

Does TrpB bind the ketone or enol?



Ketone-binding pathway

k_{exg} (rate of exchange)

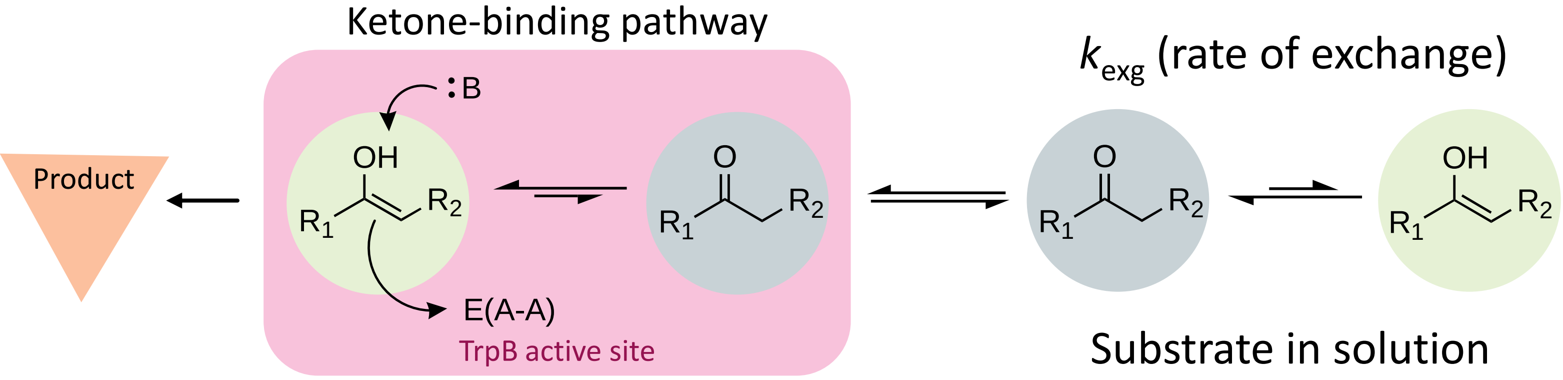
Enol-binding pathway

Substrate in solution

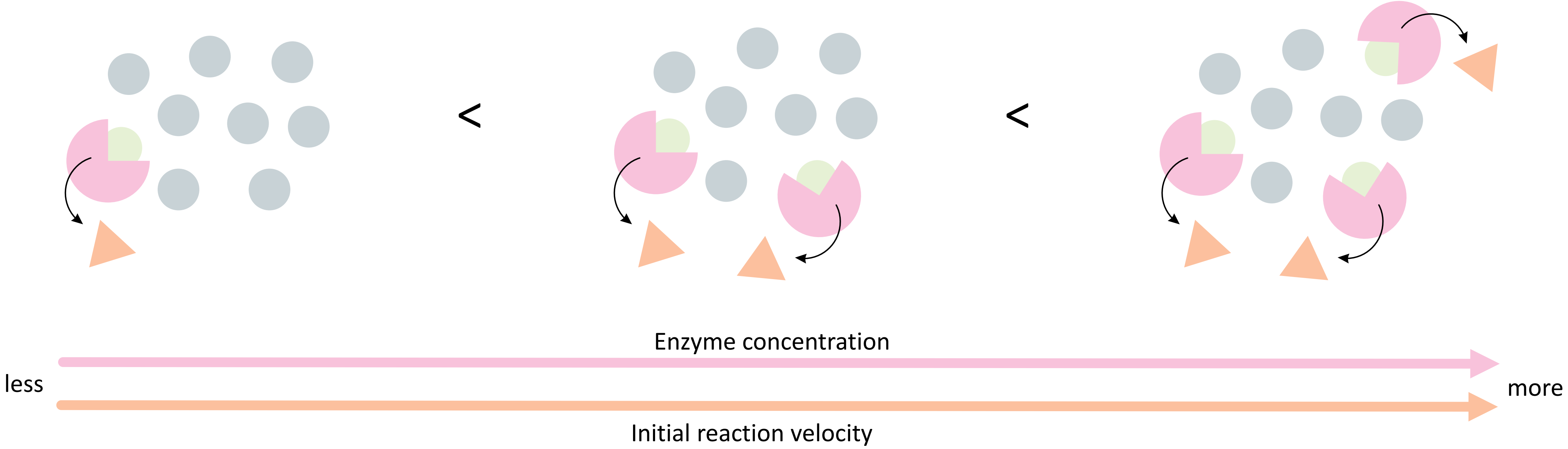
TrpB facilitates tautomerization, increasing availability of enolate.

TrpB reacts with small population of enol tautomer when it forms in solution.

Does TrpB bind the ketone or enol?

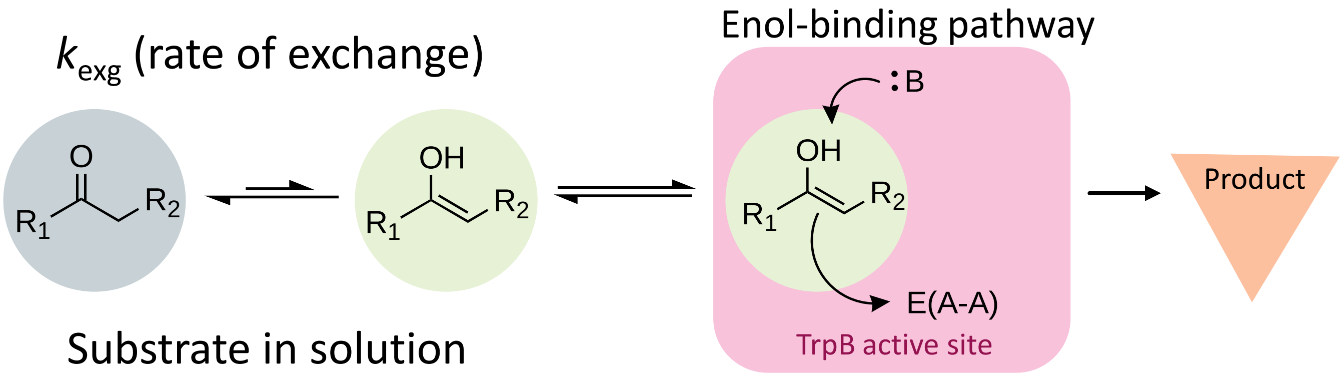


TrpB facilitates tautomerization, increasing availability of enolate.



Does TrpB bind the ketone or enol?

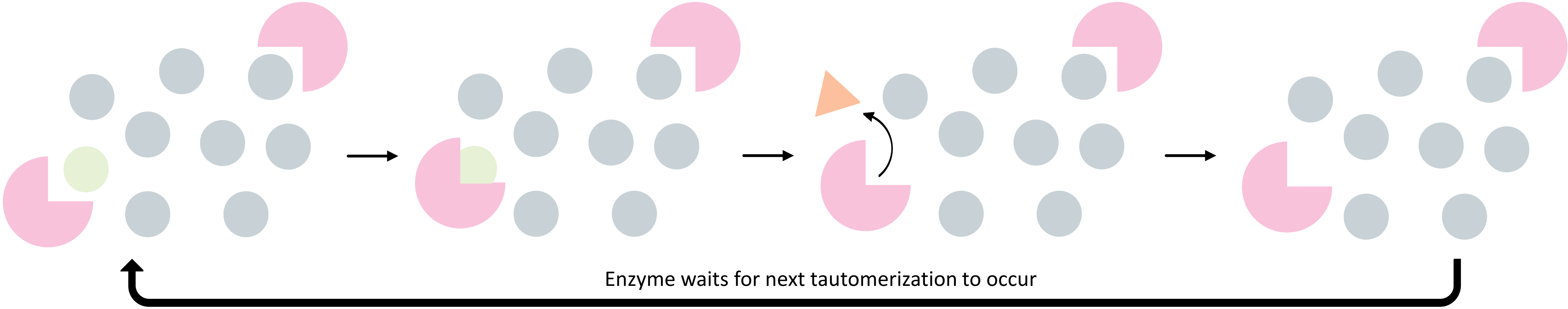
TrpB reacts with small population of enol tautomer when it forms in solution



k_{exg} (rate of exchange)

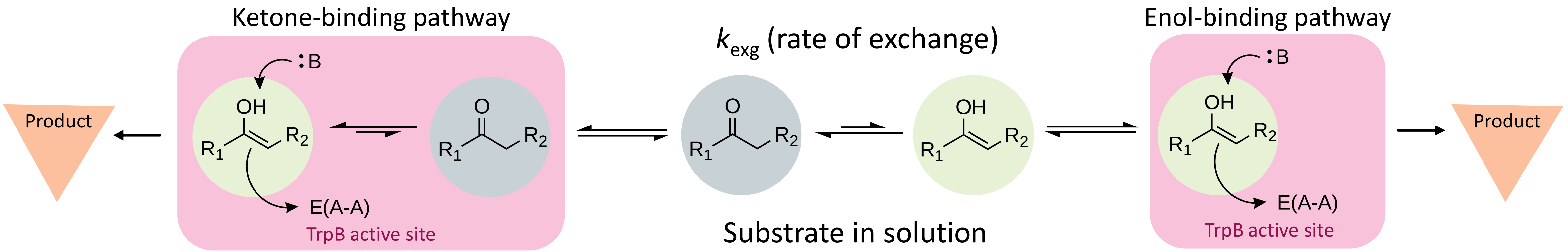
Enol-binding pathway

Substrate in solution



Enzyme waits for next tautomerization to occur

Does TrpB bind the ketone or enol?

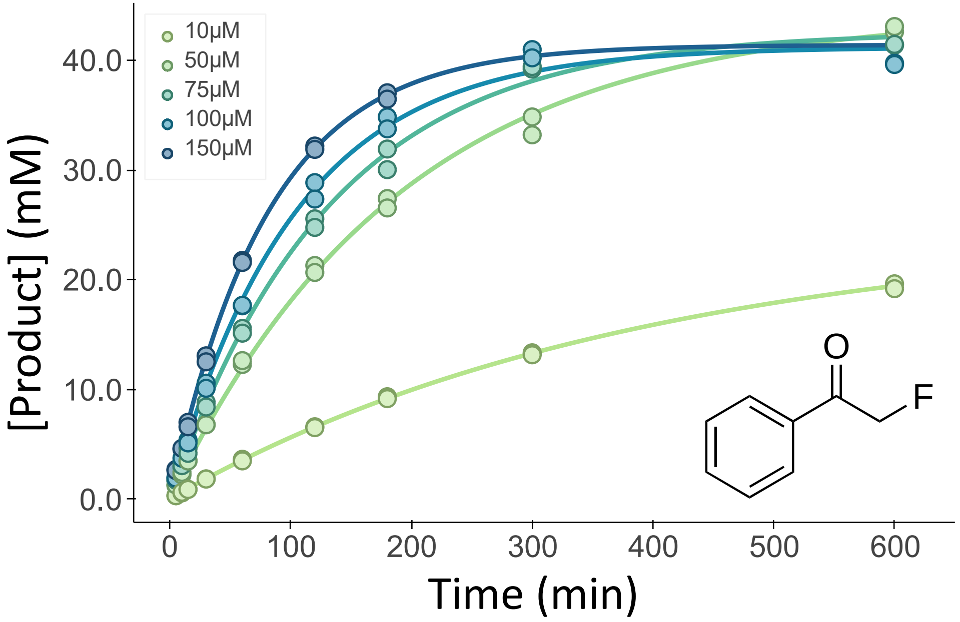


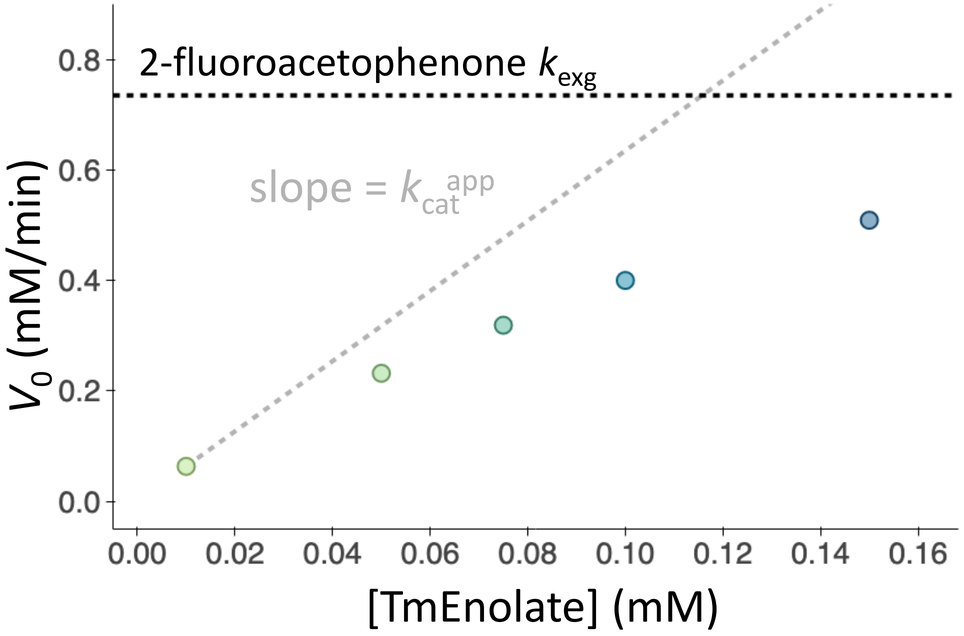
Ketone-binding pathway

k_{exg} (rate of exchange)

Enol-binding pathway

Substrate in solution

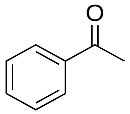
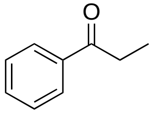
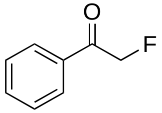
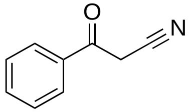
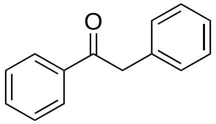
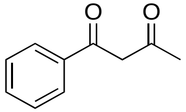




[Enzyme] is not proportional to V_0 ∴ TrpB binds the enol

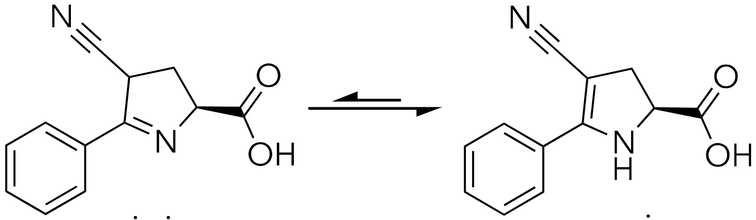
Product scope

k_{exg} is not the only determining factor for activity

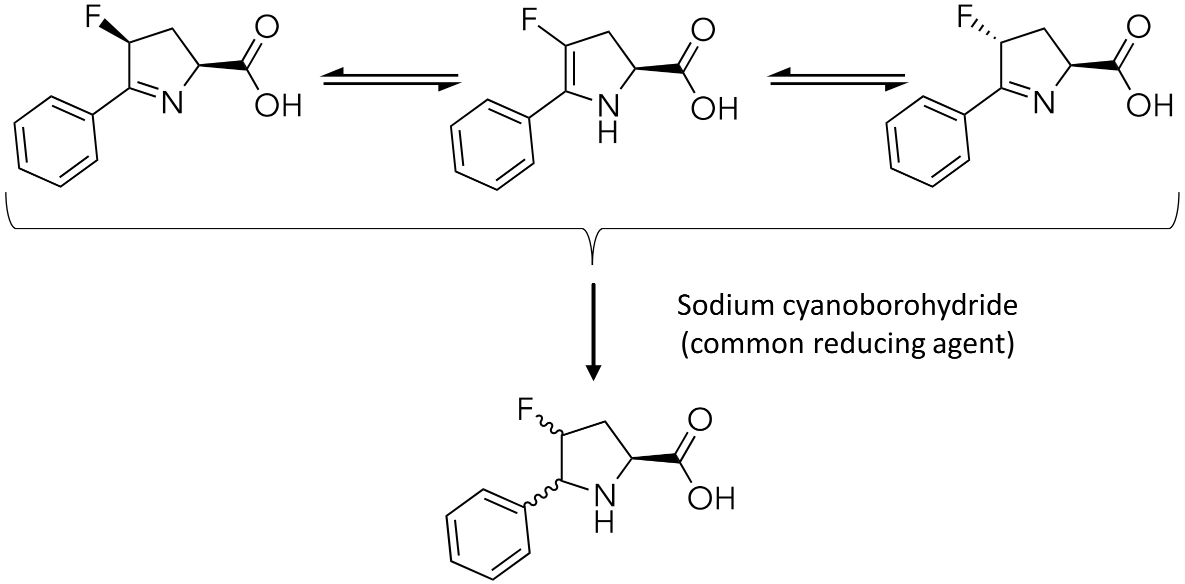
Entry	Substrate	TTN in 24 h	k_{exg} (min^{-1})
1			
2			
3			
4			
5			
6			

[a]2-CN-acetophenone is insoluble in water [b] Benzoylacetone hydrolyzes

Some products preferred the enamine state



Reducing the product makes proline analogs!



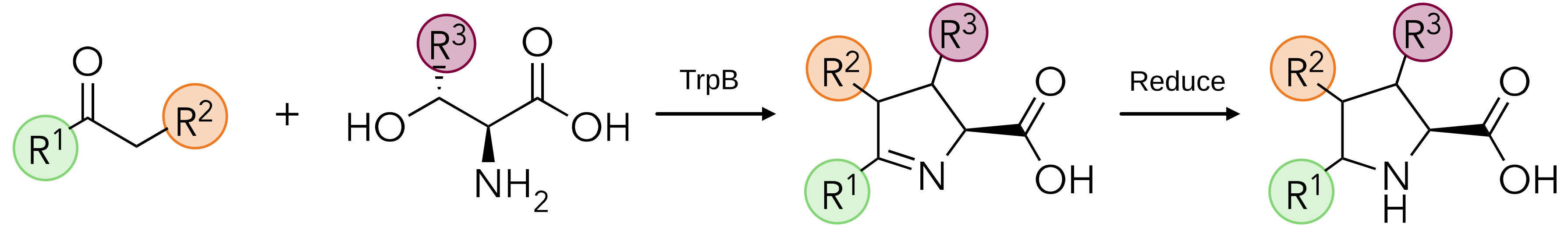
Sodium cyanoborohydride
(common reducing agent)

Summary and future directions

- Expanded the TrpBs nucleophile scope to include **ketones**.

- Demonstrated that **enzymes *can* intercept enolates**
  - No need for highly specific modes of activation
  - Kinetics, not thermodynamics, are what is important
  - Sets precedence for exploration of enolate chemistry in other enzymes

- In the future, proline analogs dense with chiral centers could be accessed using TrpB



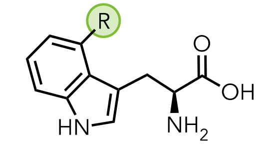
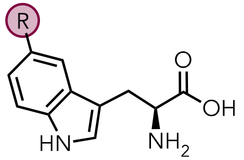
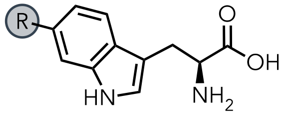
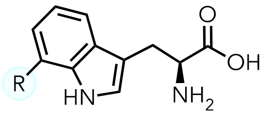
aldehydes, ketones

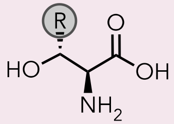
β-branched serines

Chemically,
enzyme cascade (imine reductase)

Watkins-Dulaney E.J., Dunham N.P., Straathof S., Turi S., Arnold F.H., Buller A.R. Asymmetric Alkylation of Ketones Catalyzed by Engineered TrpB. Manuscript in progress.

New kids on the block

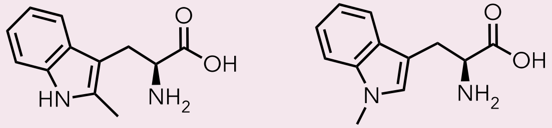
Substitution		EDG	Halogenated	EWG
4		CH_3 OCH_3 OH	F Cl Br	NO_2 CO_2H CN $CONH_2$
5		CH_3 OCH_3 OH	F Cl Br I	NO_2 CHO CN $CONH_2$ $B(OH)_2$
6		CH_3 OCH_3 OH	F Cl Br	NO_2 CN $B(OH)_2$
7		CH_3 OCH_3 OH	F Cl Br I	NO_2 CN



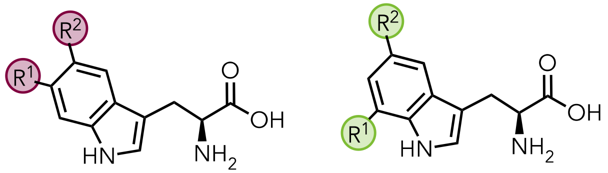
β-substituted electrophiles accepted

R = H, methyl, ethyl, propyl

1- and 2- substituted

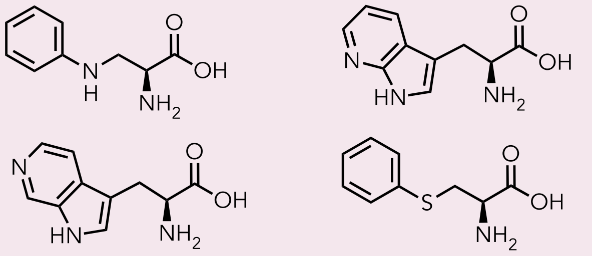


Disubstituted

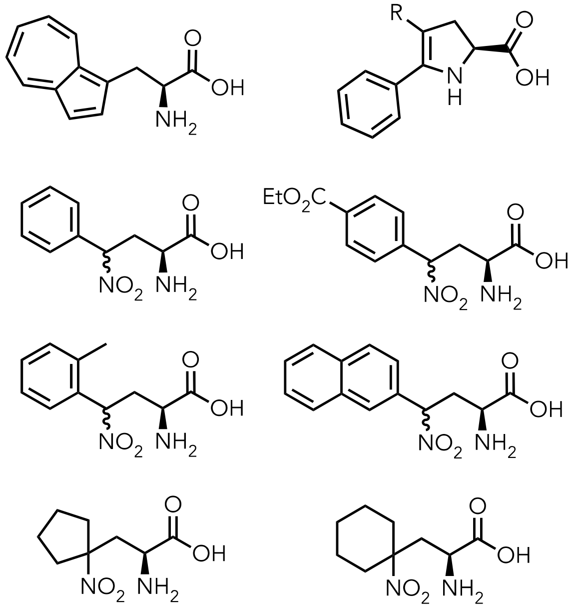


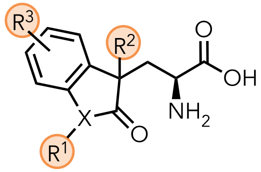
R^1, R^2 = F, Cl, Br, I

Other classes of α-amino acids:



Non-indole carbon nucleophiles





R^1 = CH_3, H ,
R^2 = methyl, ethyl, propyl, butyl, OH,
R^3 = Cl, Fl

Buller AR, Brinkmann-Chen S, Romney DK, Herger M, Murciano-Calles J, Arnold FH *Proc Natl Acad Sci*. 2015;112(47):14599-14604.
Romney DK, Murciano-Calles J, Wehrmüller JE, Arnold FH. *J Am Chem Soc*. 2017;139(31):10769-10776.
Herger M, van Roye P, Romney DK, Brinkmann-Chen S, Buller AR, Arnold FH. *J Am Chem Soc*. 2016:jacs.6b04836.
Boville CE, Scheele RA, Koch P, Brinkmann-Chen S, Buller AR, Arnold FH. *Angew Chemie Int Ed*. 2018.
Romney, David K., Nicholas S. Sarai, and Frances H. Arnold. *ACS Catalysis* 2019.
Dick, Markus, et al., *J Am Chem Soc*. 141.50 (2019): 19817-19822.

Watkins, Ella J., Patrick J. Almhjell, and Frances H. Arnold. *ChemBioChem* 2019.
Watkins-Dulaney E.J., Dunham N.P., Straathof S., Turi S., Arnold F.H., Buller A.R.(2021) Manuscript in progress.

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- Committee members
    - Mikhail Shapiro
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    - Andrew Buller
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    - Patrick Almhjell
    - Sabine Straathof
- Enolates
    - Noah Dunham
    - Nicholas Porter
    - David Miller
    - Sabine Straathof
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    - Anders Knight
- TrpB collaborators
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    - Gordon Rix

Frances Arnold

Sabine Brinkmann-Chen

EVOLVING A BETTER FUTURE.

Caltech

Thank you to the entire Arnold group! You made my time at Caltech very special.


RON JON
ONE OF A KIND
MOMENT