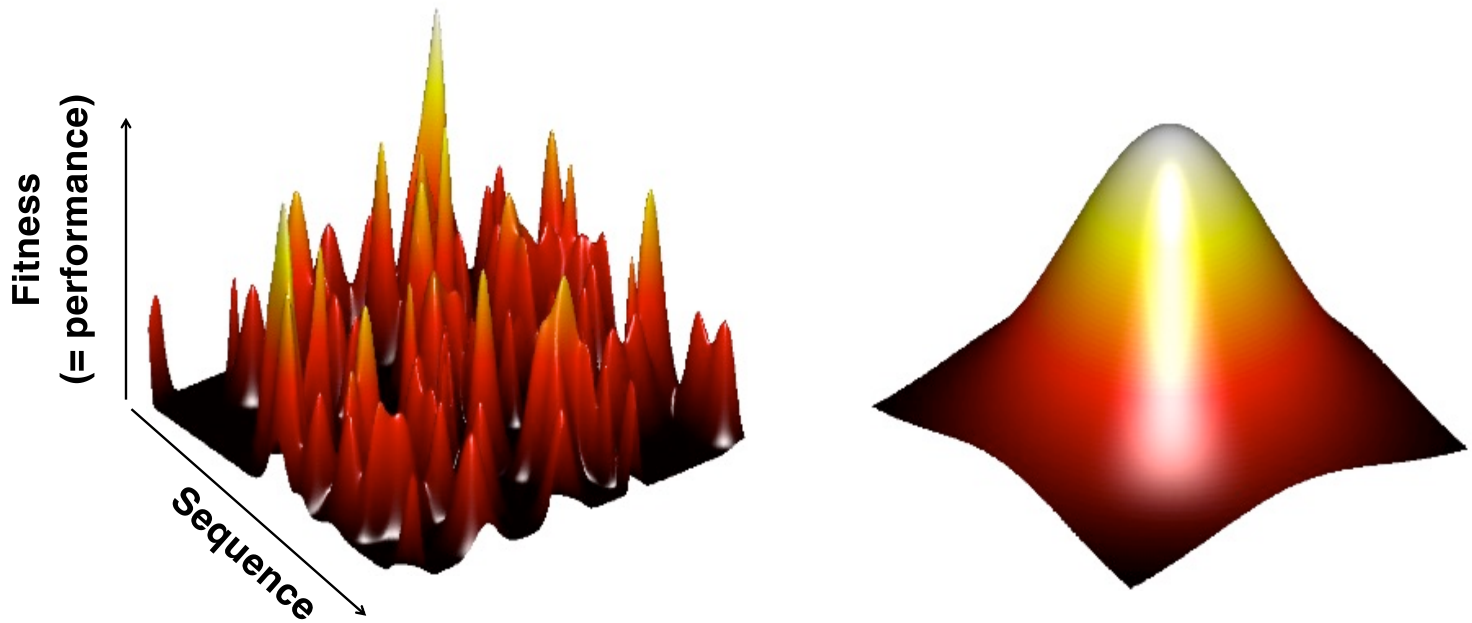
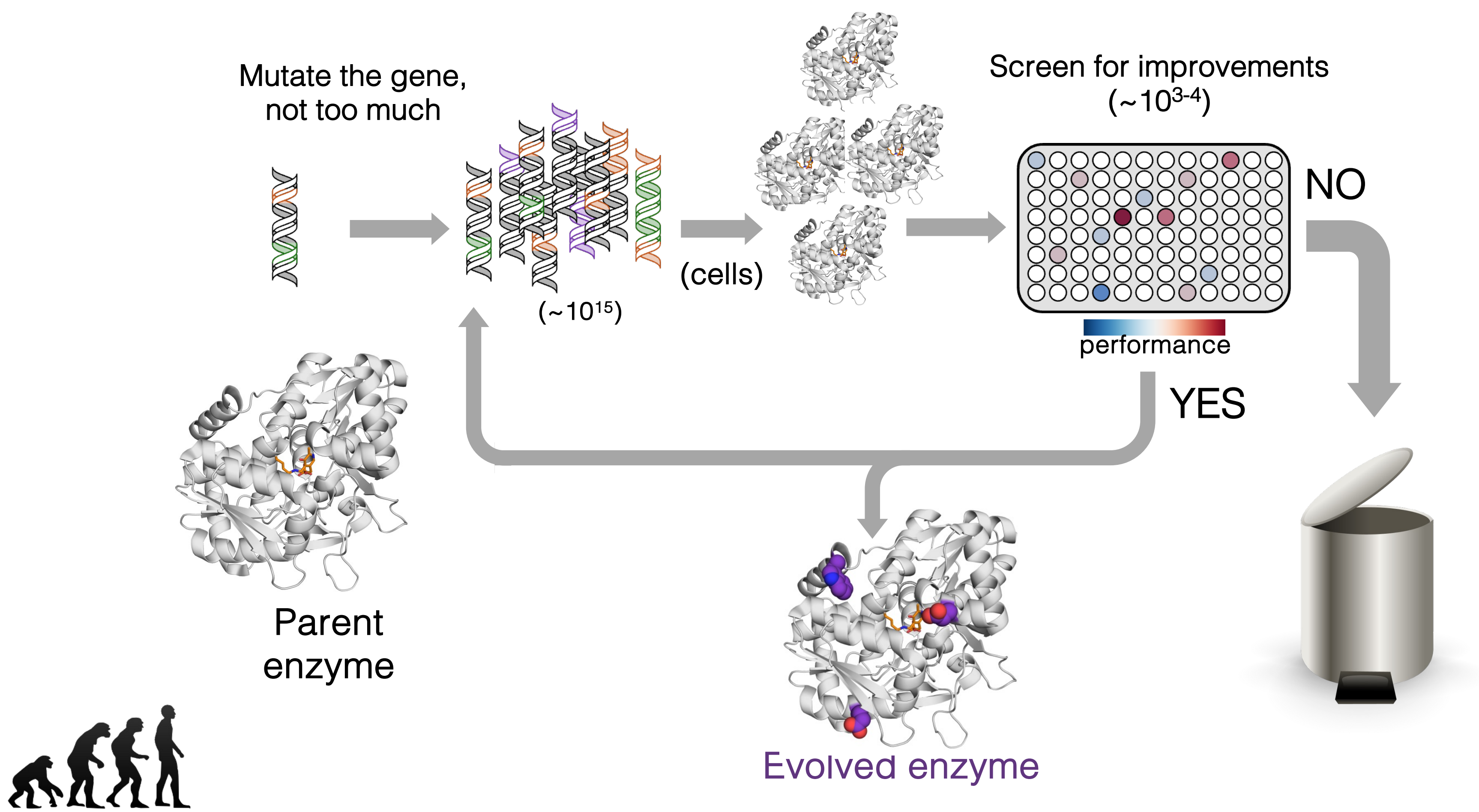
We want BETTER proteins for human applications.

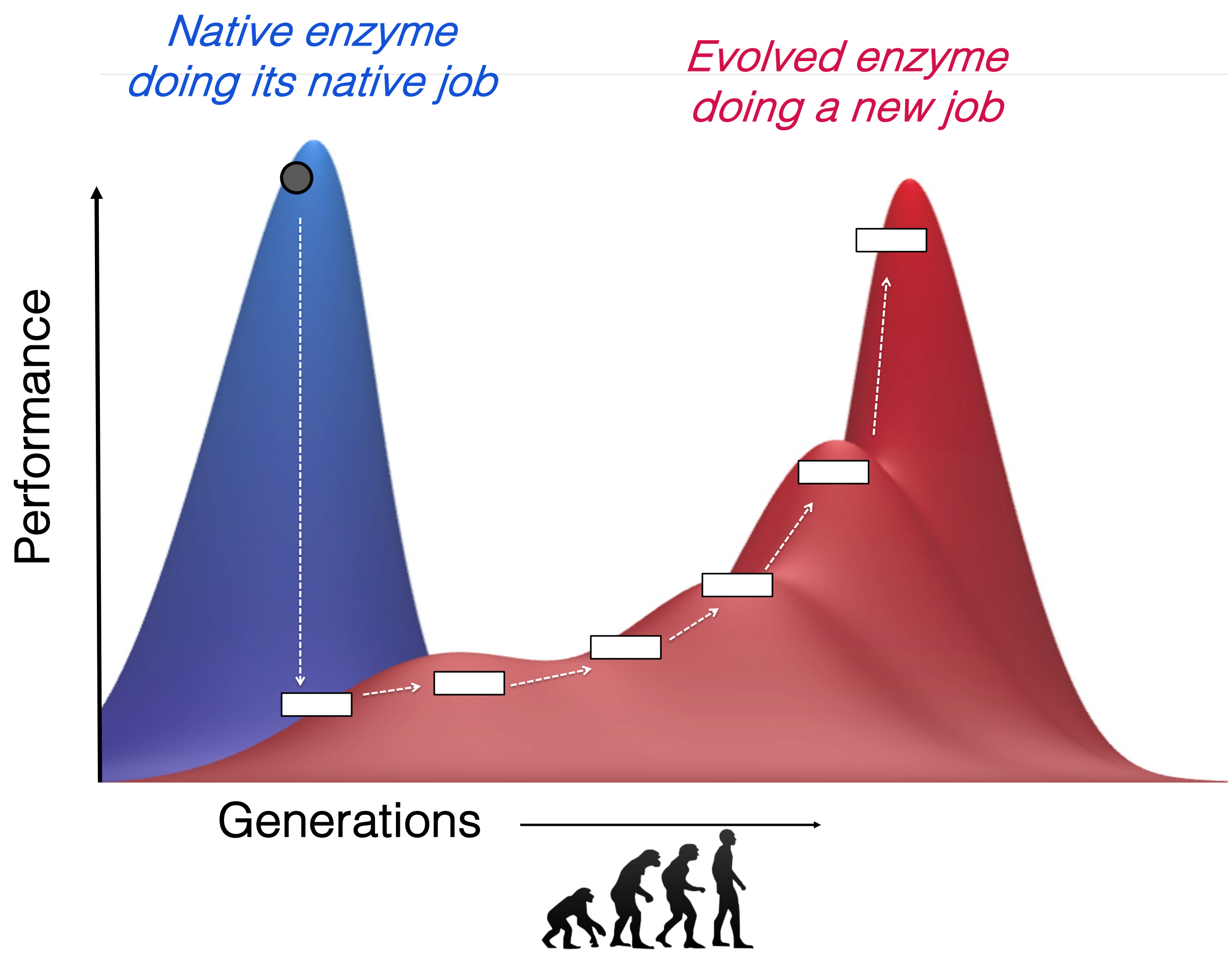
Directed evolution is a molecular optimization process on a multi-dimensional fitness landscape, where fitness is performance and is defined by me.



Directed enzyme evolution: making use of smooth paths in the fitness landscape



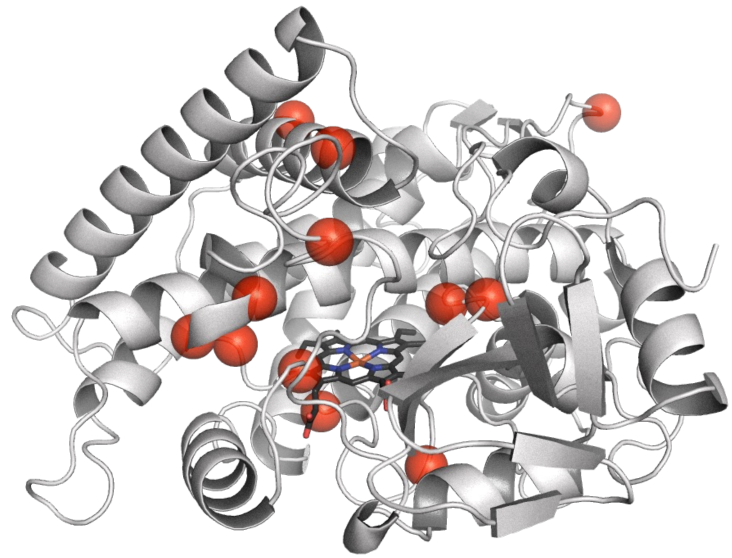
Directed evolution can optimize an enzyme for a new job




bio
Persil
small & mighty
25
Januvia
(sitagliptin, MSD)
MSD
Janumet
50 mg/500 mg
575

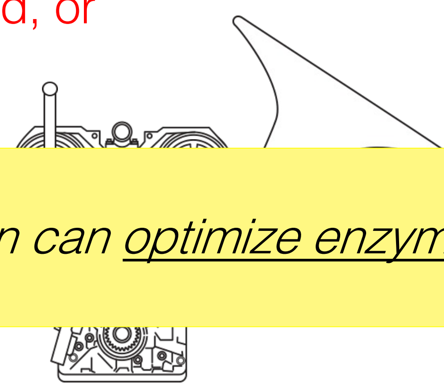
Enzymes (and other proteins) are highly evolvable

Proteins can adapt by accumulating beneficial mutations in a simple uphill walk.

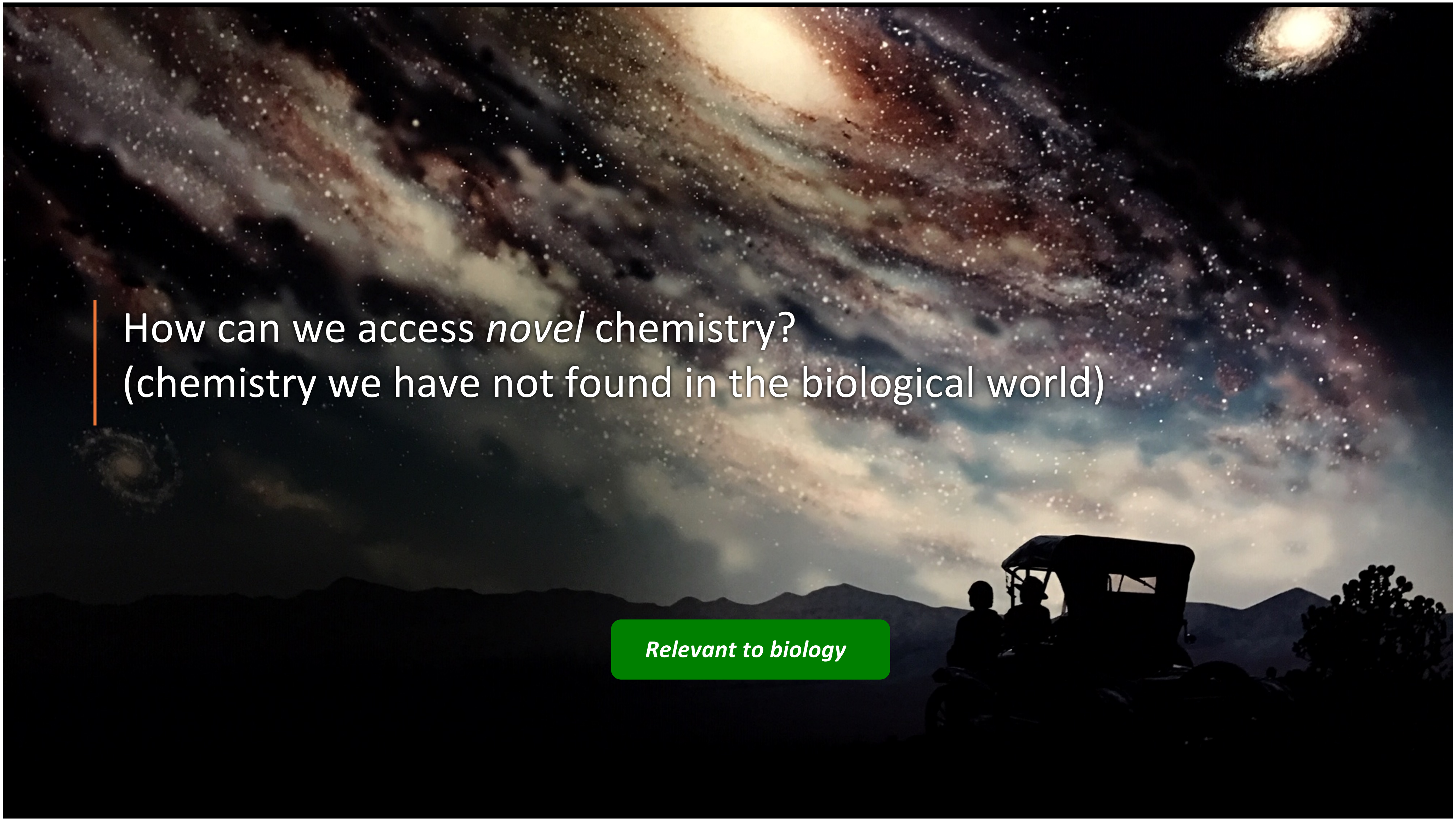


New functions by changing tiny fraction (<2 %) of sequence.

Scary fact: many beneficial mutations are far from the active site and cannot be predicted, or even explained.

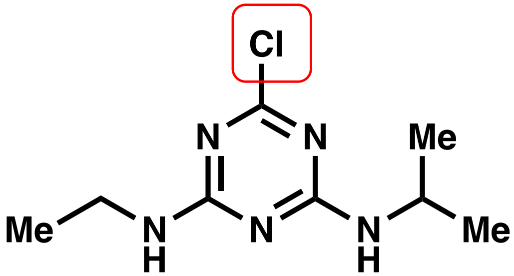


Directed evolution can <u>optimize enzyme function</u> in real time.


How can we access *novel* chemistry?
(chemistry we have not found in the biological world)
Relevant to biology

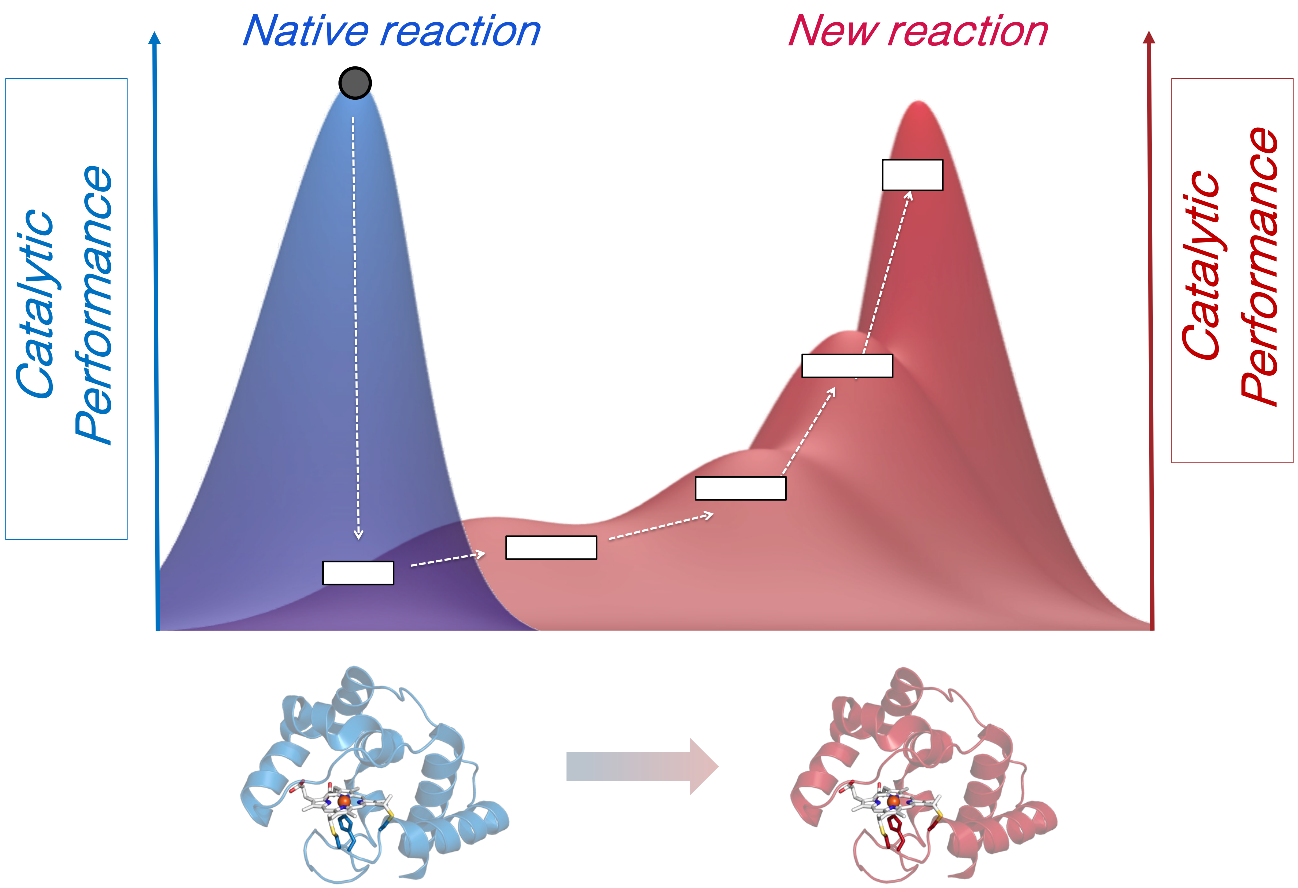
Nature innovates all the time…

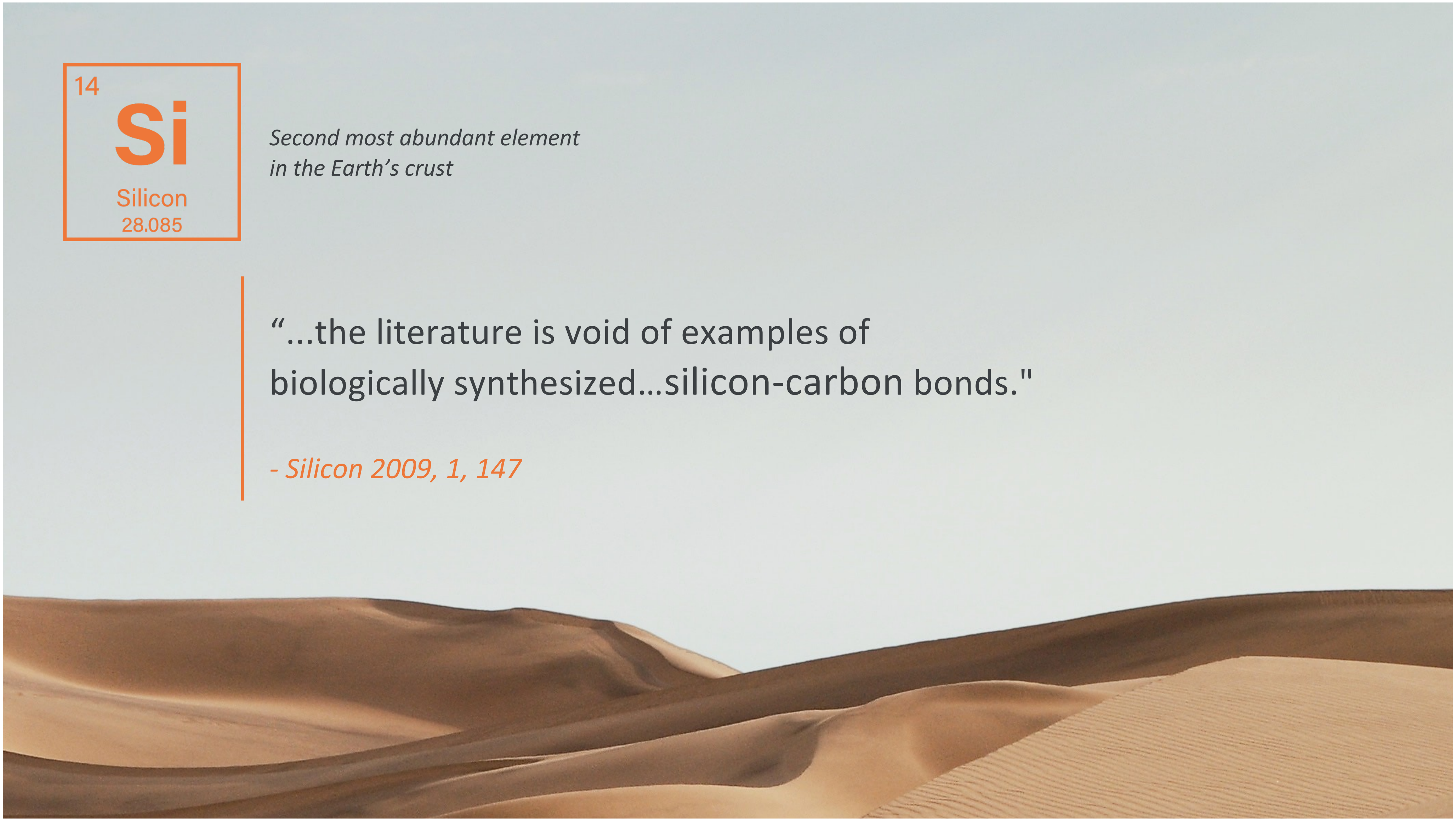
atrazine



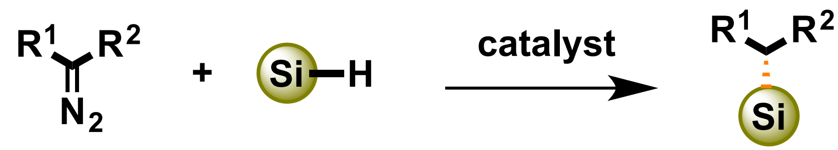
- 1950–1993 non-biodegradable; accumulated in soil
- From 1993 onwards, fast degradation observed

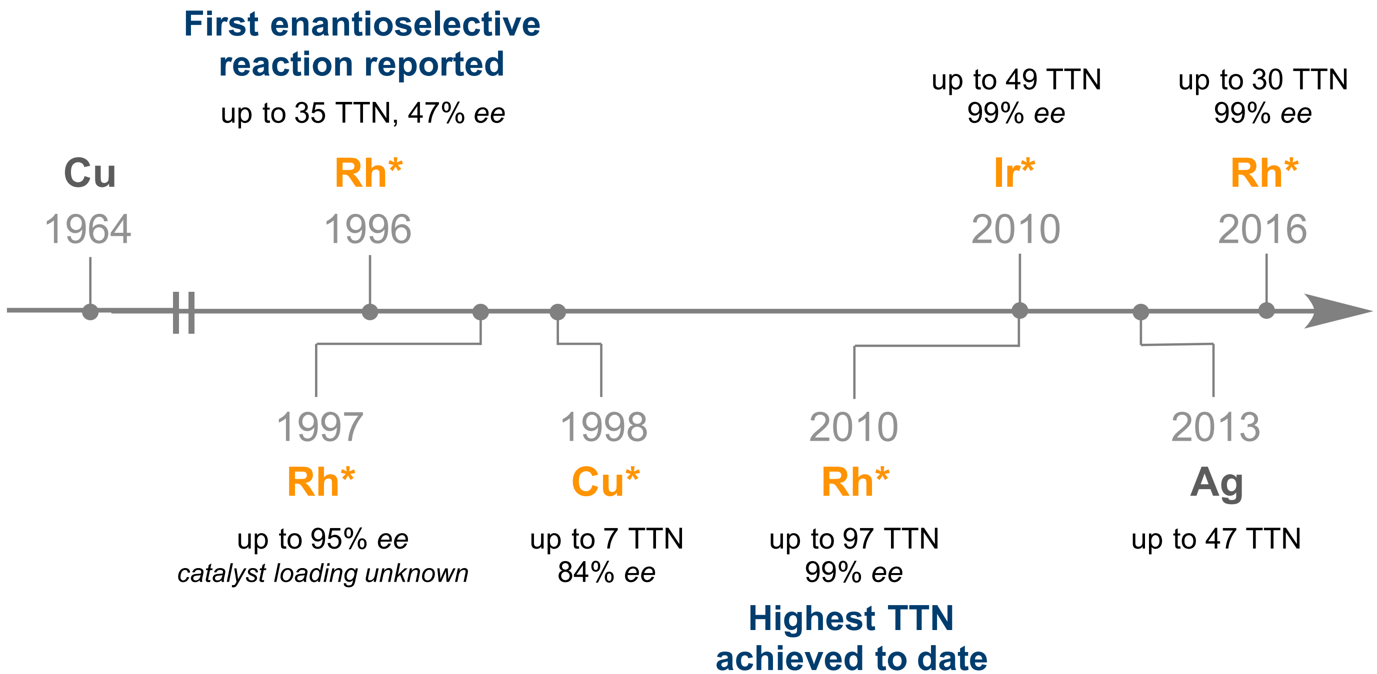
Promiscuous activities can be a starting point for evolution of new functions




14
Si
Silicon
28.085
Second most abundant element in the Earth’s crust
“...the literature is void of examples of biologically synthesized…silicon-carbon bonds."
- Silicon 2009, 1, 147

Inspiration from human chemistry? Brief history of transition metal catalysis for enantioselective carbene insertion into Si-H bonds





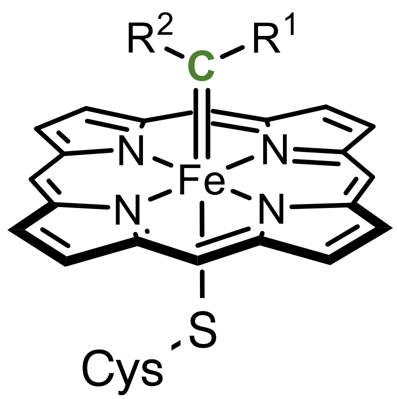
TTN = total turnover number

*** = enantioselective catalyst**

Evolution to explore/create the future…

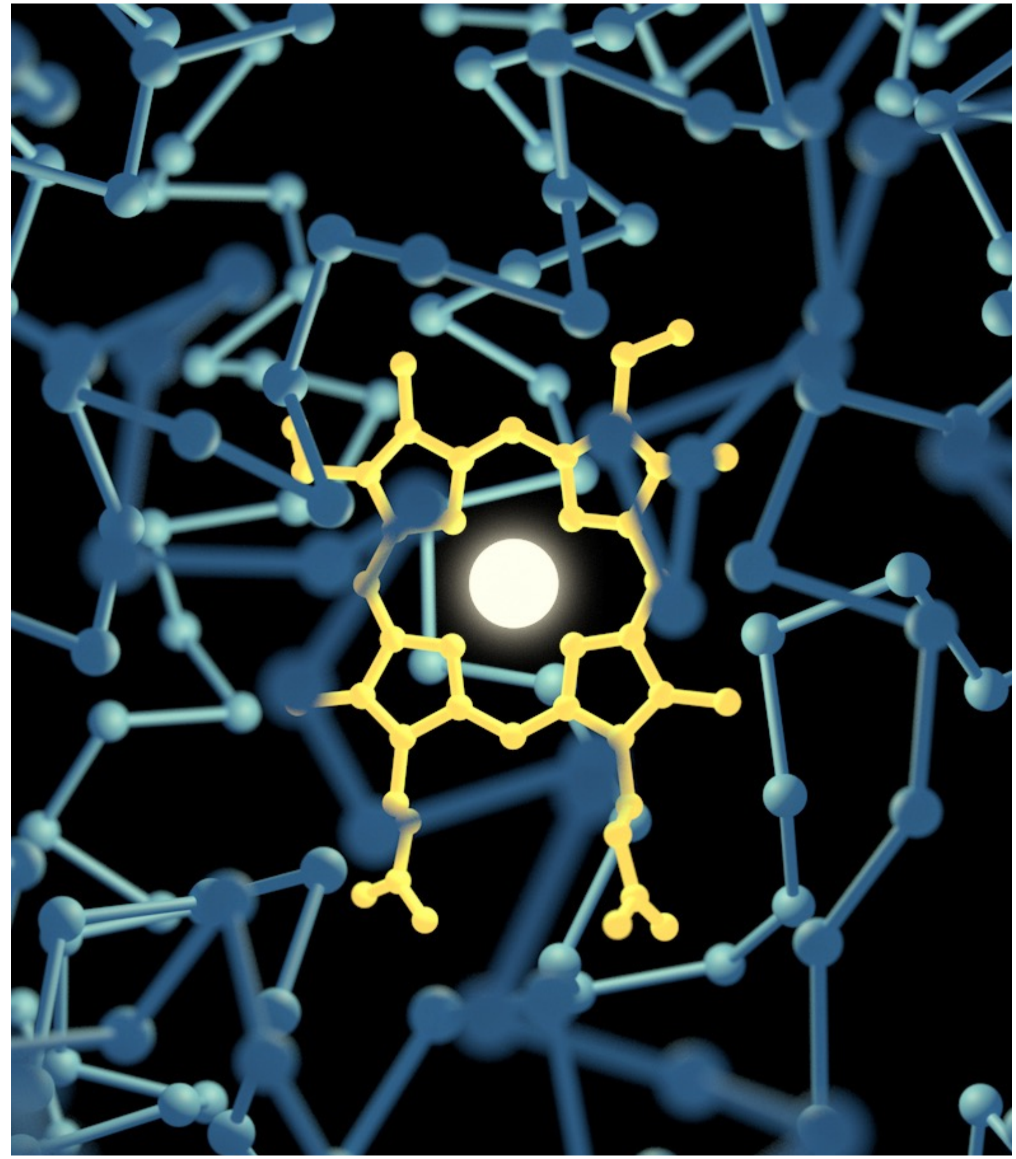
Can an iron-heme protein do this?

Form a reactive Fe-carbenoid Intermediate

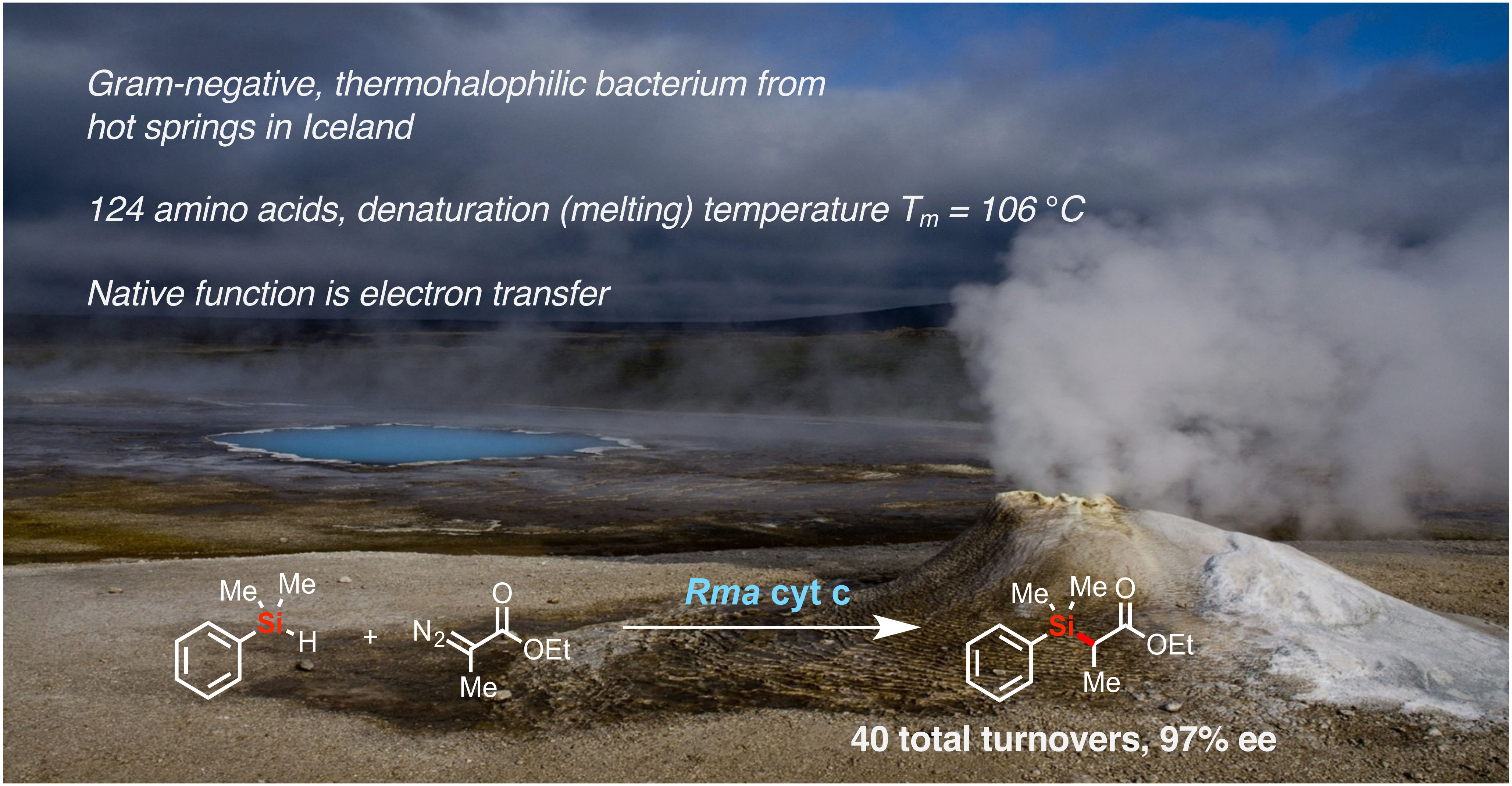


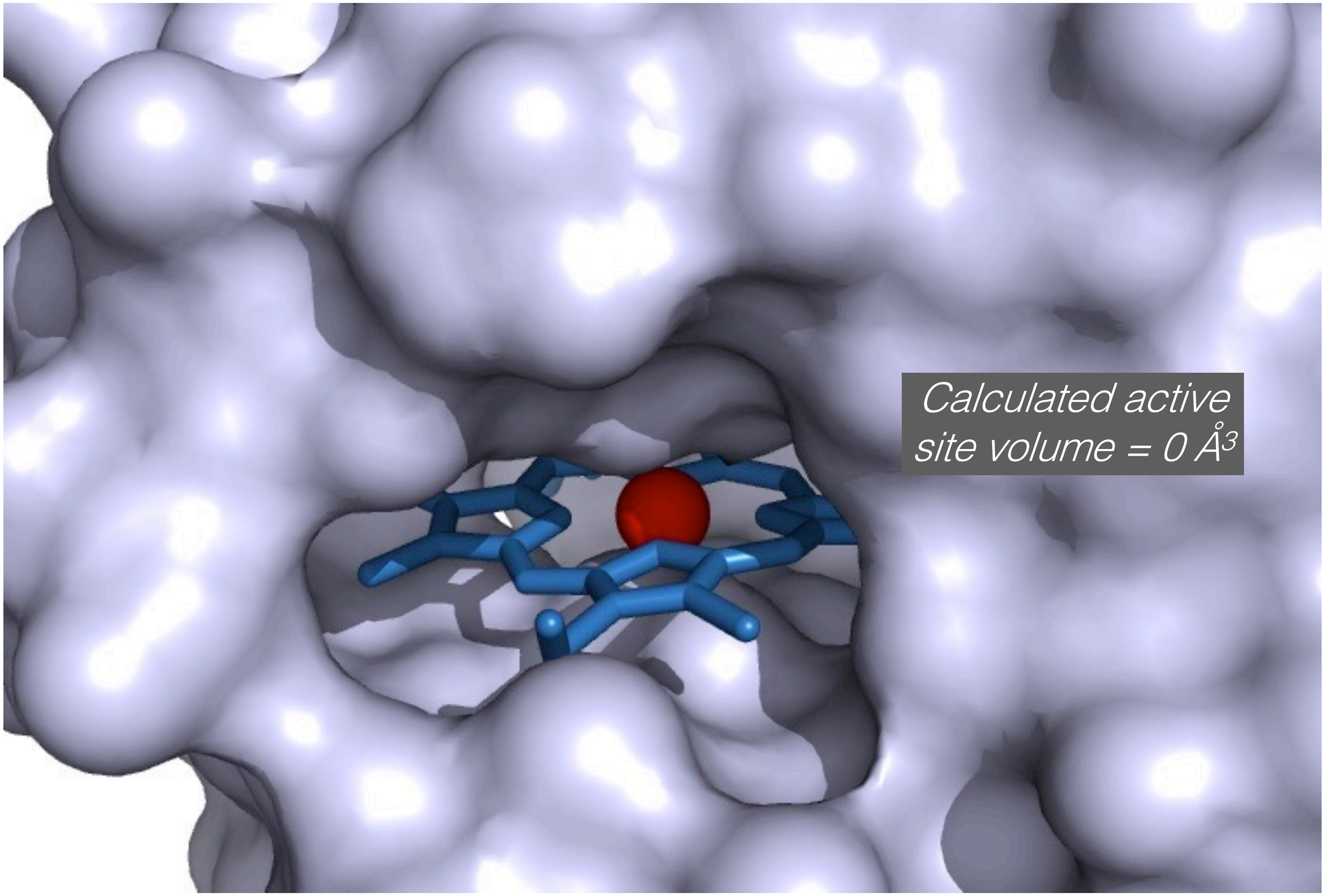
Transfer the carbene to a second substrate

Does it evolve?



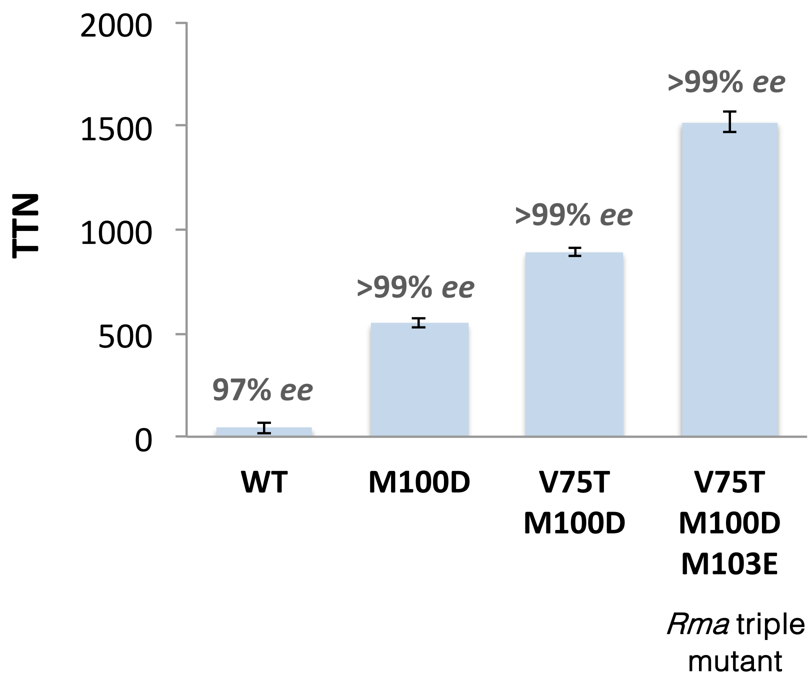
Rhodothermus marinus cytochrome *c*

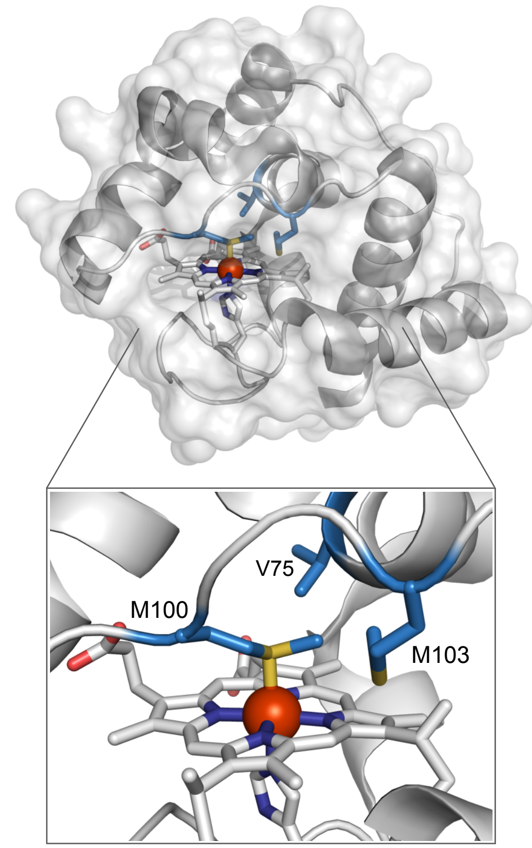




1.23 Å X ray crystal structure of *Rma* cyt *c*

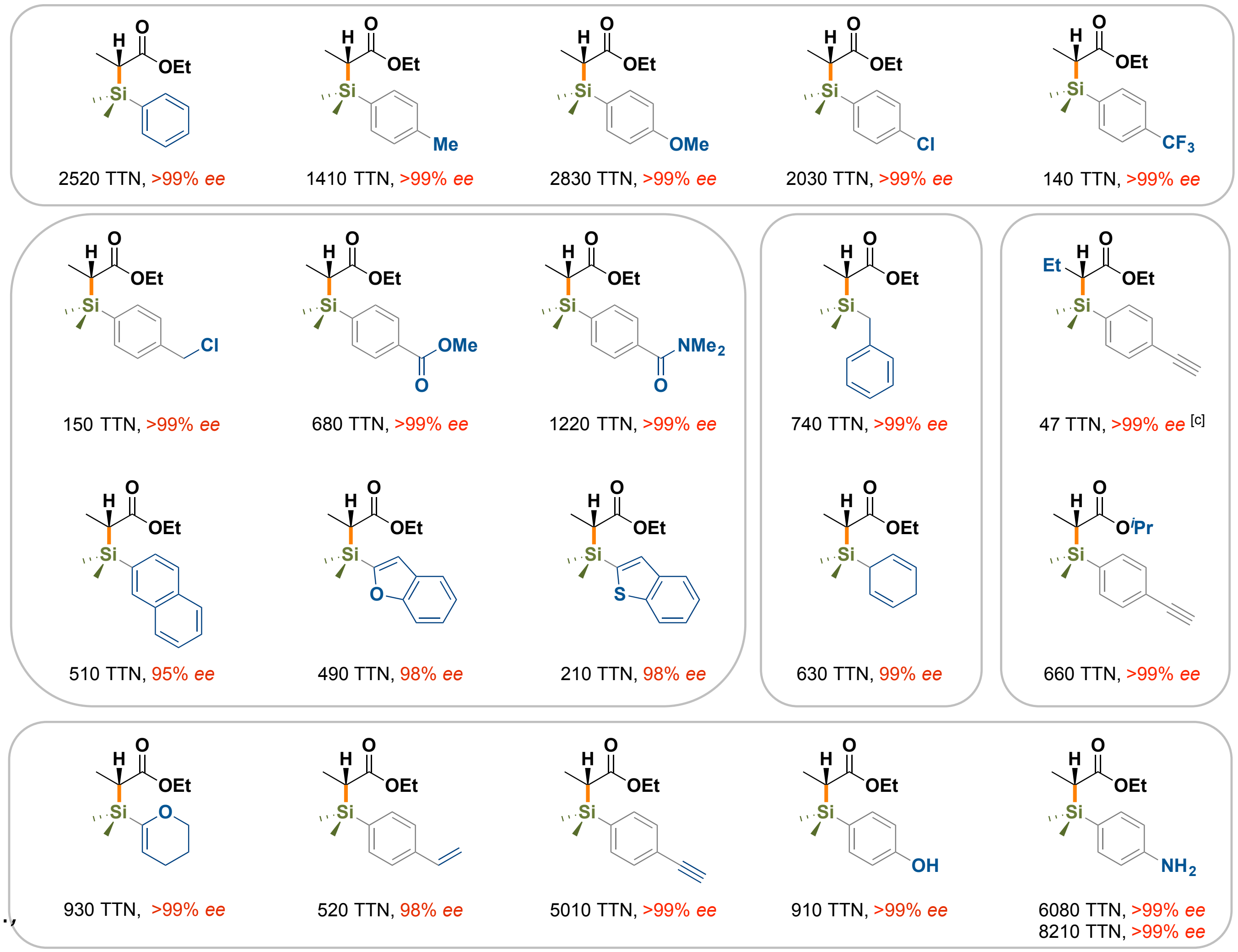
40-fold increased activity in just three generations





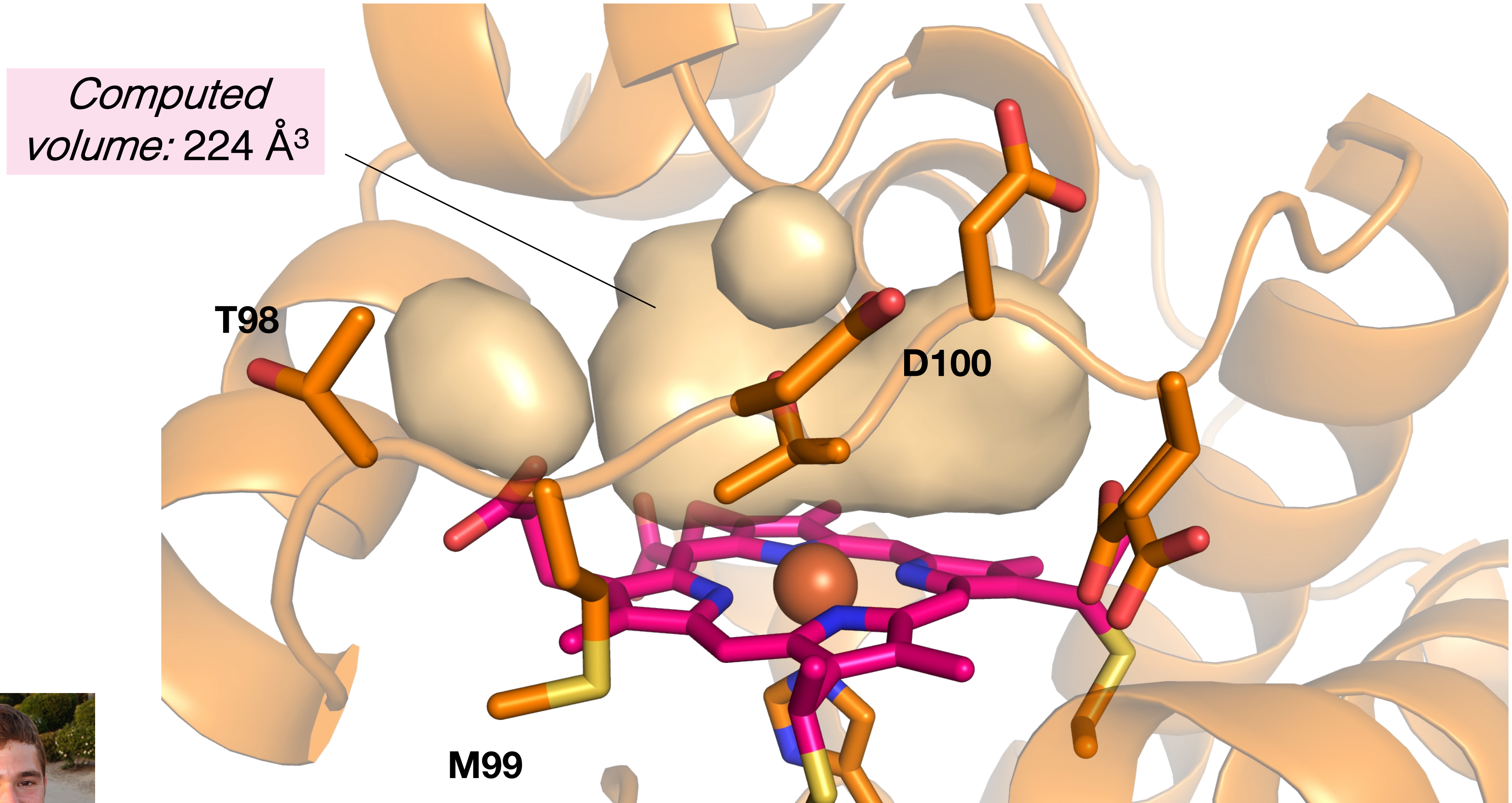
Jennifer Kan, Kai Chen et al. *Science* 354, 1048 (2016)

One enzyme: 20 example products, most are enantiopure



Jennifer Kan, Kai Chen et al., *Science* 354, 1048 (2016)

High resolution crystal structure shows a loop over the heme 'flipped' to form a new active site pocket

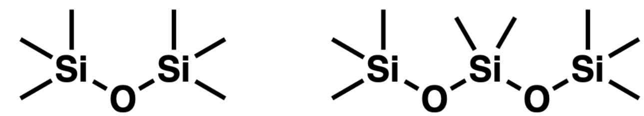


Rusty Lewis et al., PNAS 115, 7308 (2018)

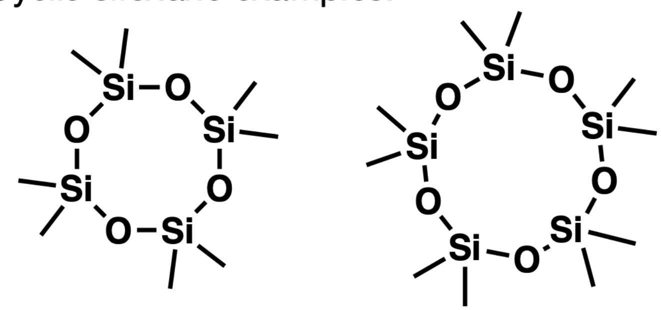
We can now MAKE carbon-silicon bonds with biology…can we BREAK them?

- Volatile methylsiloxanes (VMS) are the building blocks of silicone polymers
- Not biodegradable, megatons/year production
- Accumulating in the environment
- Some are banned in the EU
- **No enzyme known to break Si-C bonds**

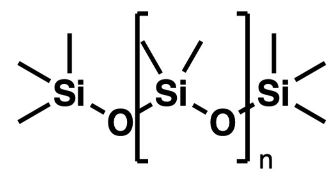
Linear siloxane examples:



Cyclic siloxane examples:



Polydimethylsiloxane (PDMS):



Science **2024**, *383*, 438–443

RESEARCH

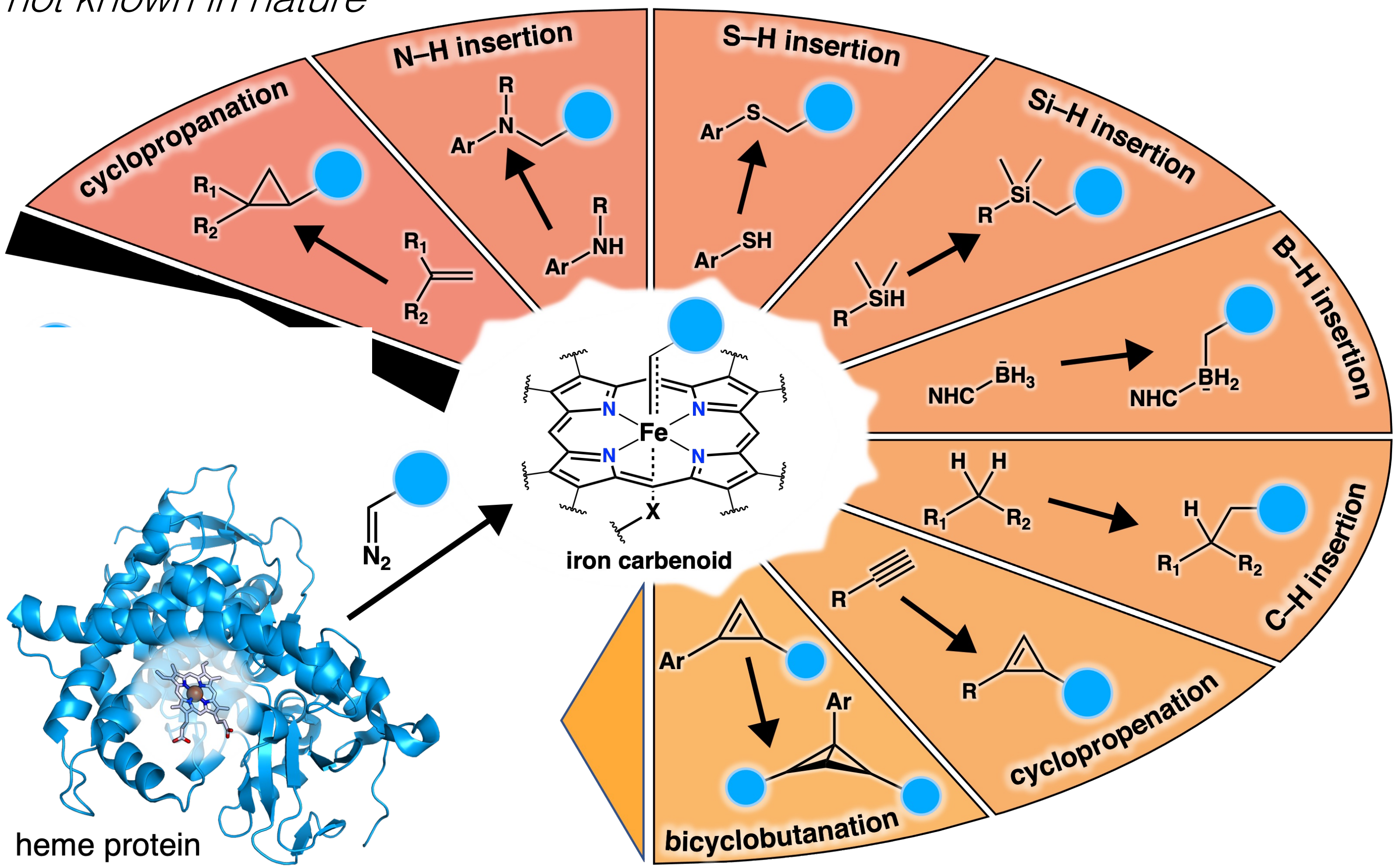
BIOCATALYSIS

Directed evolution of enzymatic silicon-carbon bond cleavage in siloxanes

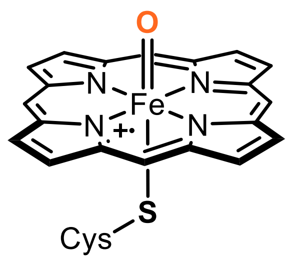
Nicholas S. Sarai[1]†‡, Tyler J. Fulton[1]†, Ryen L. O'Meara[1]†, Kadina E. Johnston[2]§, Sabine Brinkmann-Chen[1], Ryan R. Maar[3], Ron E. Tecklenburg[3], John M. Roberts[3], Jordan C. T. Reddel[3], Dimitris E. Katsoulis[4]*, Frances H. Arnold[1]*

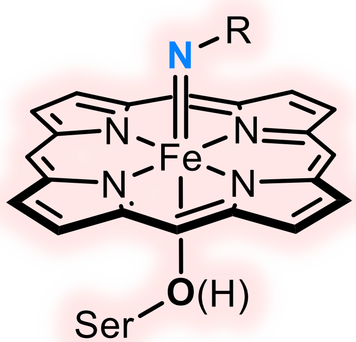
Volatile methylsiloxanes (VMS) are man-made, nonbiodegradable chemicals produced at a megaton-per-year scale, which leads to concern over their potential for environmental persistence, long-range transport, and bioaccumulation. We used directed evolution to engineer a variant of bacterial cytochrome $P450_{BM3}$ to break silicon-carbon bonds in linear and cyclic VMS. To accomplish silicon-carbon bond cleavage, the enzyme catalyzes two tandem oxidations of a siloxane methyl group, which is followed by putative [1,2]-Brook rearrangement and hydrolysis. Discovery of this so-called siloxane oxidase opens possibilities for the eventual biodegradation of VMS.

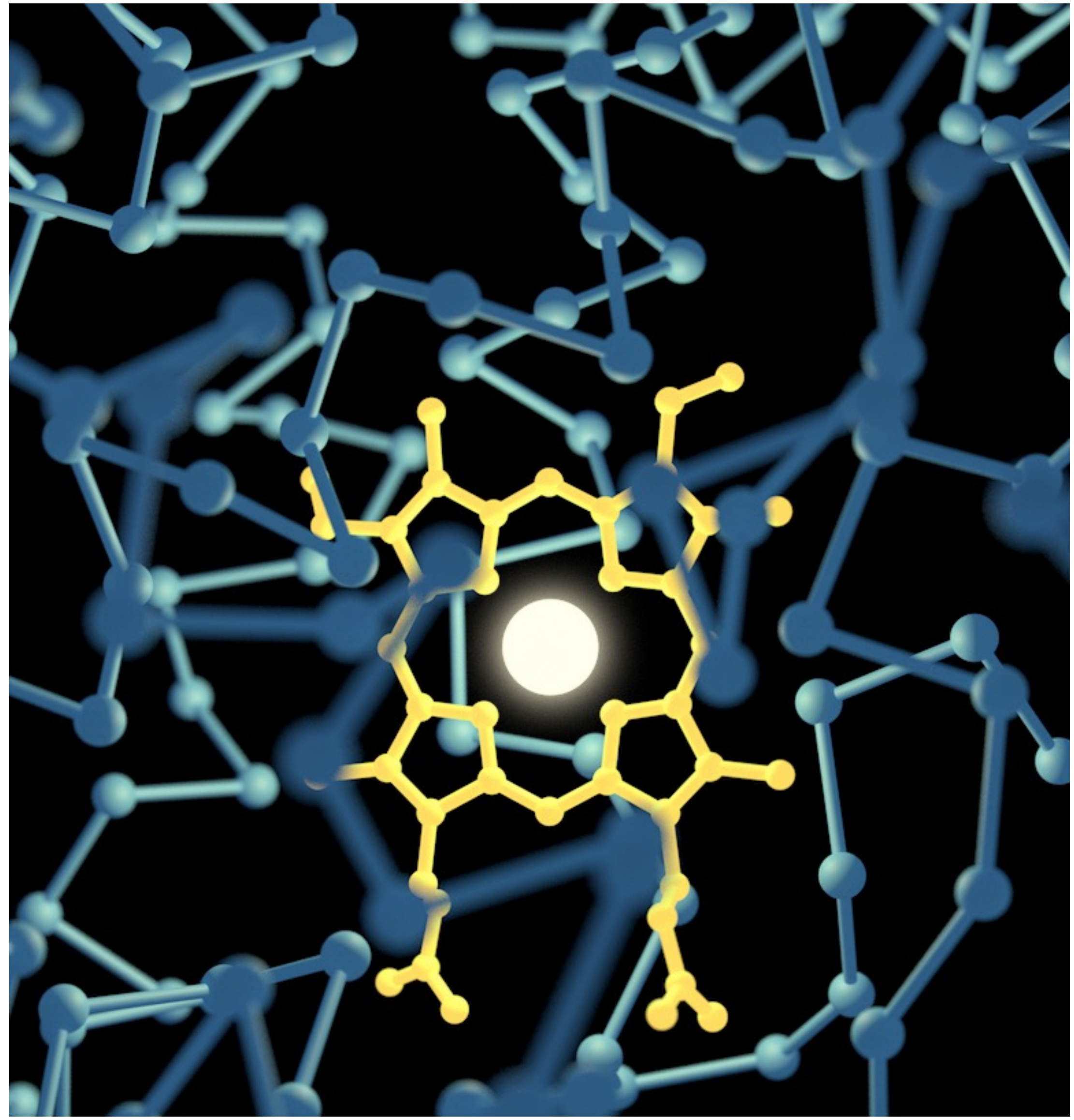
Since 2013, we have generated whole families of 'carbene transferases' with many activities not known in nature

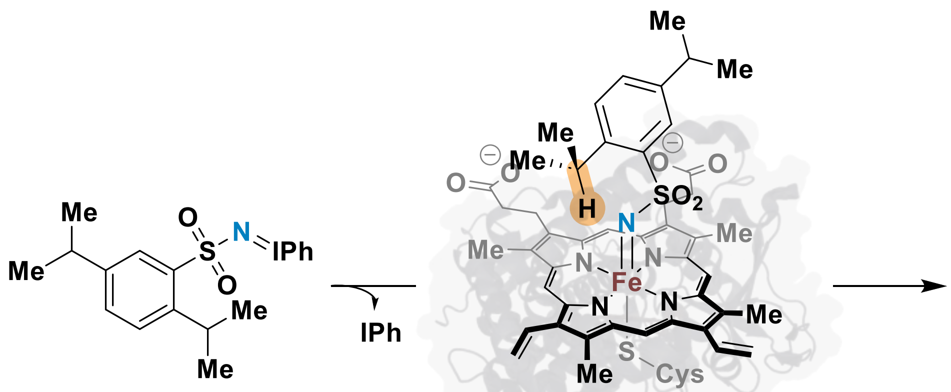


Heme proteins can also generate and transfer nitrenes….





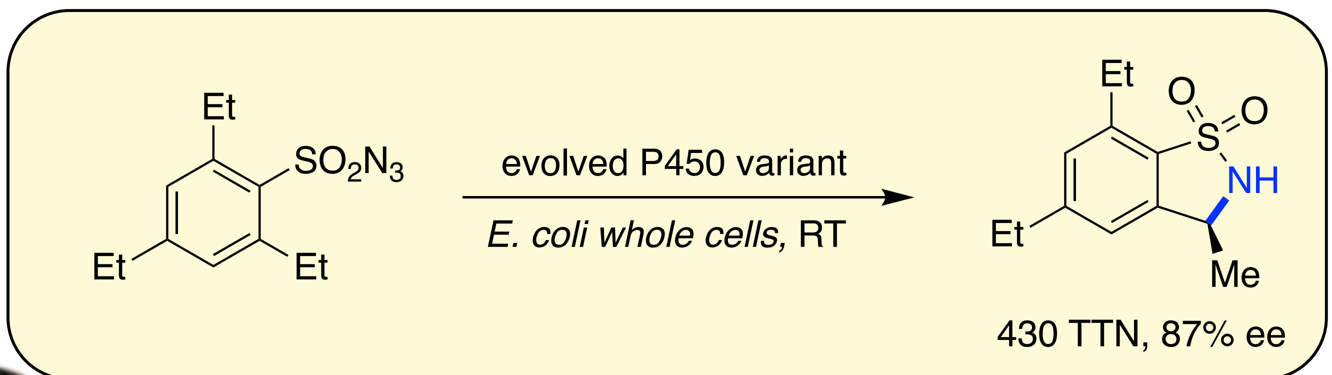


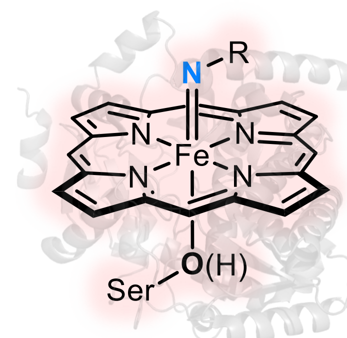


2.2 TTN

Rabbitt P450 LM3,4

Svastits, Breslow et al. *JACS* **1985**, *107*, 6427-6428.

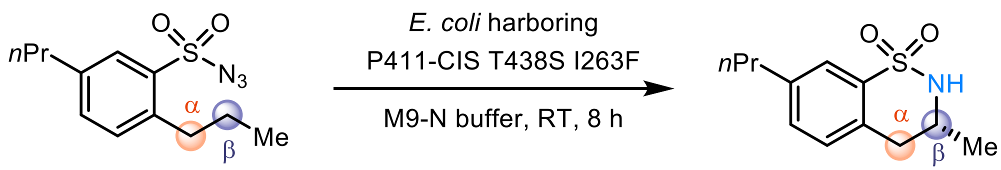




Cytochrome P411
nitrene transferases

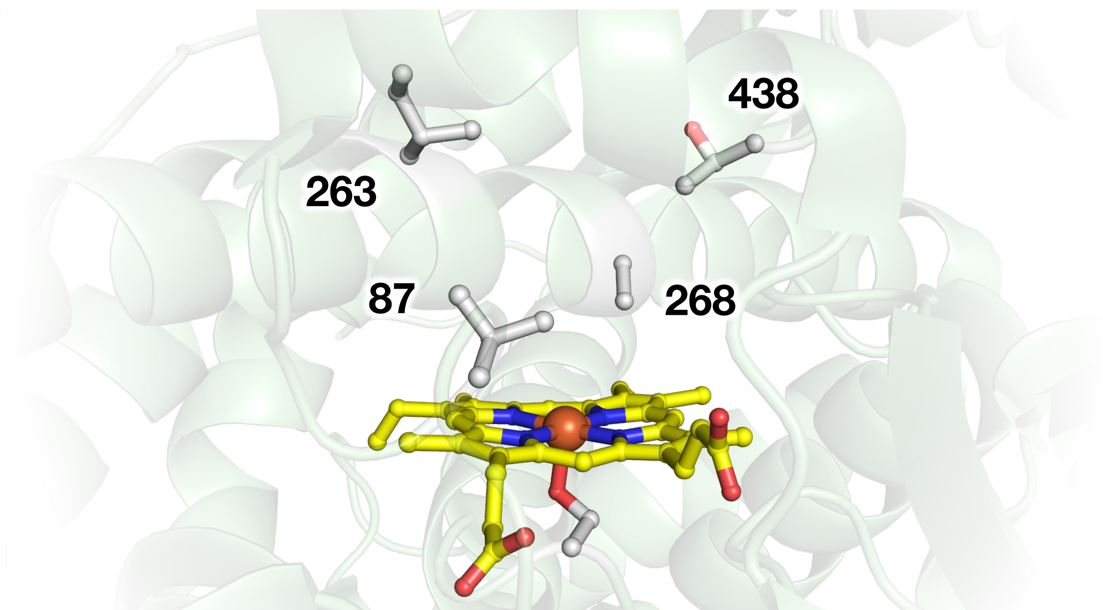
John McIntosh, Pedro Coelho *et al.*, *Angew. Chem. Int. Ed.*, **2013**, *52,* 9309.

The enzyme can also selectively target the less reactive homobenzylic C–H bond



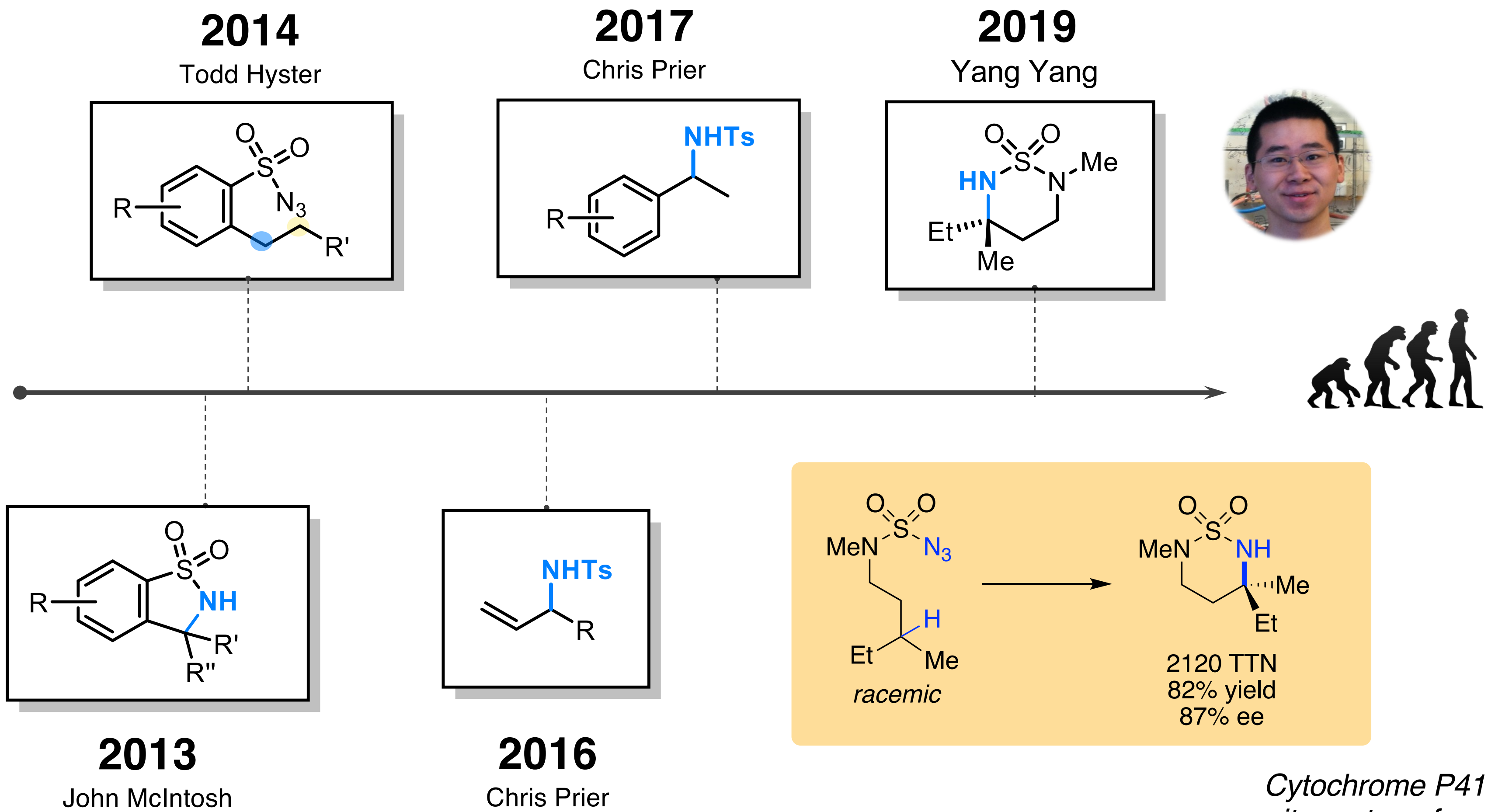
BDE **-C H : 86 kcal/mol**
-C H : 98 kcal/mol

361 TTN
3:97 **:** , 99% *ee*



Todd Hyster, John McIntosh *et al.*, *J. Am. Chem. Soc.* 136, 15505 (2014).

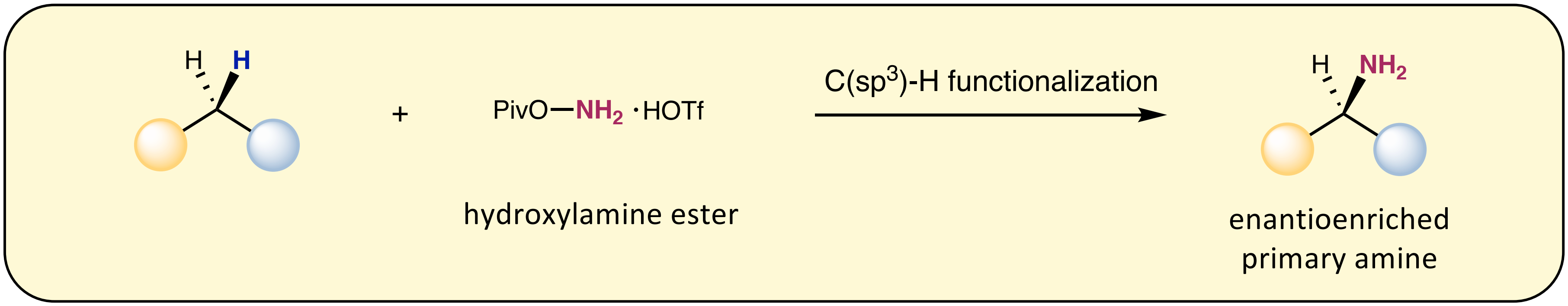
Cytochrome P411$_{BM3}$ evolution for C-H insertion of nitrenes using <u>azide</u> precursors

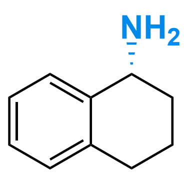


Cytochrome P411
nitrene transferases

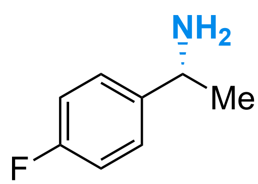
see review Yang, Y.; Arnold, F. H. *Acc. Chem. Res.* 2021, *54*, 1209-1225.

Intermolecular C(sp³)-H amination: unprotected primary amines using a hydroxylamine ester nitrene precursor

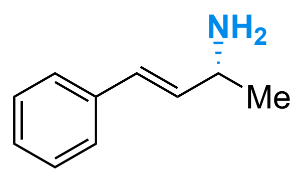




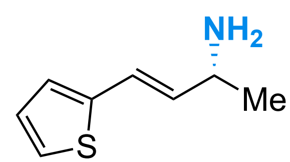
3a, 85% yield
580 TTN, 93% *ee*



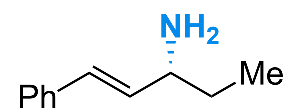
3g, 44% yield
300 TTN, 89% *ee*



6a, 93% yield
3900 TTN, 94% *ee*



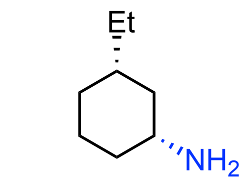
6h, 62% yield
600 TTN, 89% *ee*



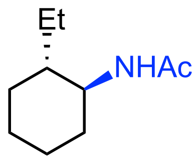
6i, 35% yield
1420 TTN, 85% *ee*


Zhijun Jia, Shilong Gao *JACS 142,* 10279 (2020)

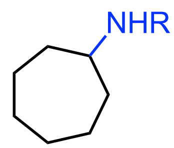
The enzymes can aminate unactivated C-H bonds!



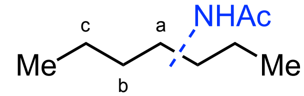
100 TTN
> 99% *ss*, 10.5:1 *dr*
98:2 *er*



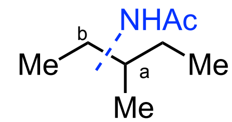
205 TTN
78% *ss*, 1:1 *dr*
cis diast. - 98:2 *er*
trans diast. (S,S)- 75:25 er



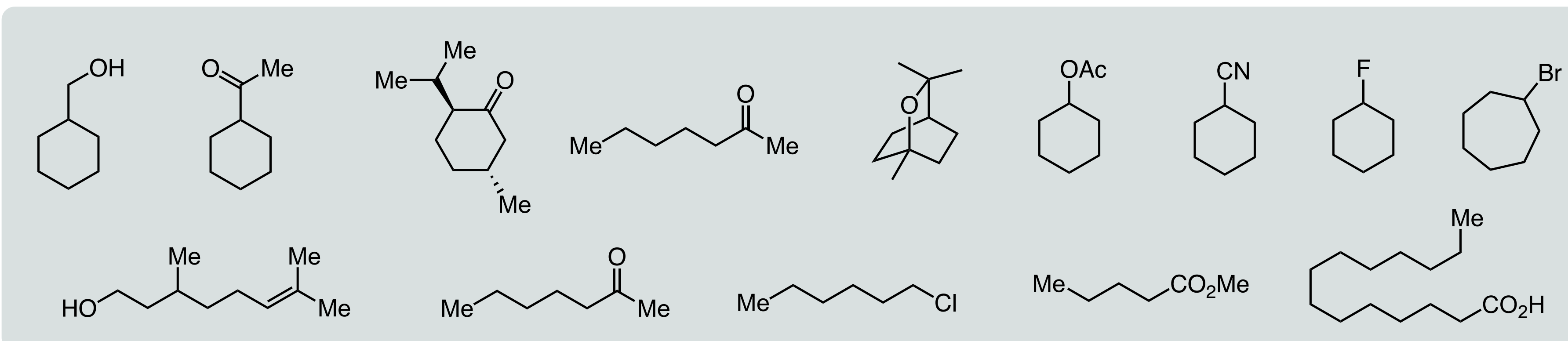
R=H:
125 TTN
R=Ac:
25 TTN



45 TTN
ss for a:b:c = 1.3: 3.2:1
er (for b) = 63:37
er (for b) = 53:47

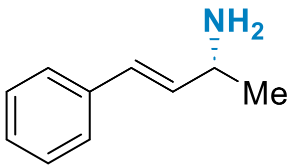


70 TTN
ss for a:b = 1:8
dr (for b) = 1:1.7
er (minor diast.) = 85:15
er (major diast.) = 58:42



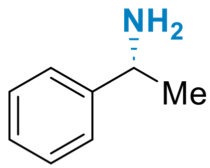
Soumitra Athavale, Shilong Gao, Anuvab *Das* *JACS* 2022

Evolution continues: P450s and hydroxylamine esters to access primary amines



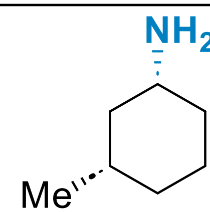
93% yield, er = 97:3

P411



30% yield, er = 95:5

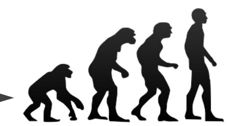
P411

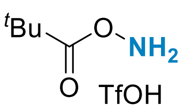


dr = 8:1, er = 93:7

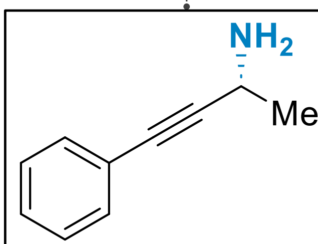
P411

Athavale, Gao, Das
(*JACS* 2022)



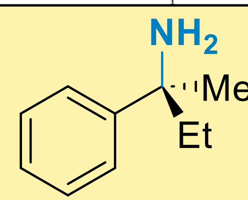


Liu, Qin
(*JACS* 2022)



79% yield, er = 94:6

P411



17% yield,er = 96:4

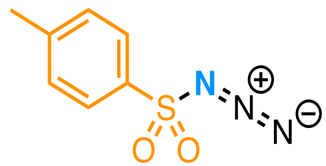
P411

(benzylic) enantioselective tertiary amination

Runze Mao
(*Nat. Chem.* 2024)

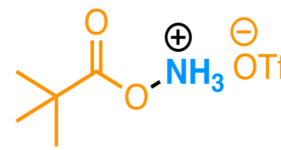
Mao R. et. al. *Nat. Catal.* 2024
Athavale et. al. *JACS* 2022, *144*, 19097.
Liu et. al. *JACS* 2022, *144*, 80.

Amination enzymes use azides and hydroxylamine-derived reagents.



Tosyl azide

- Explosive

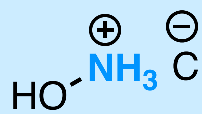


PivONH$_3$OTf

- Electrophilic group required
- Stoichiometric waste

Can enzymes use simple hydroxylamine?

Selective binding at N
Higher N-O BDE

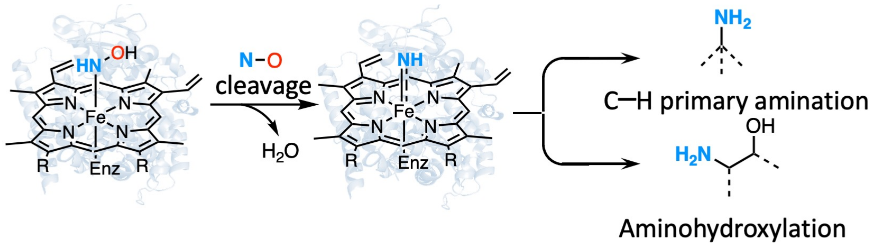


Hydroxylammonium chloride

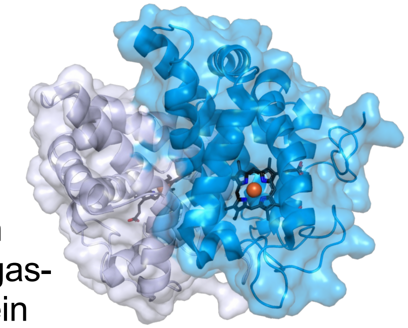
- Bench stable
- Cheap
- Water is sole byproduct

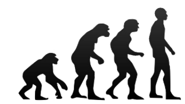
Shilong Gao *et al.*, JACS **145**, 20196 (2023)

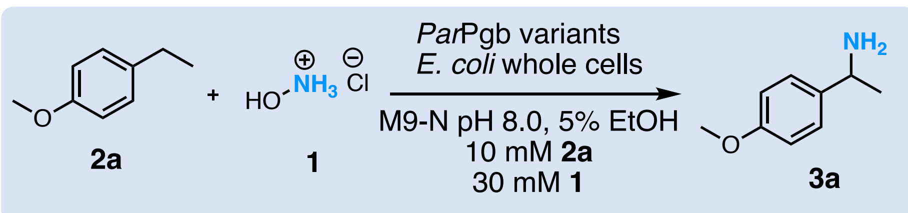
Enzymatic activation of hydroxylamine

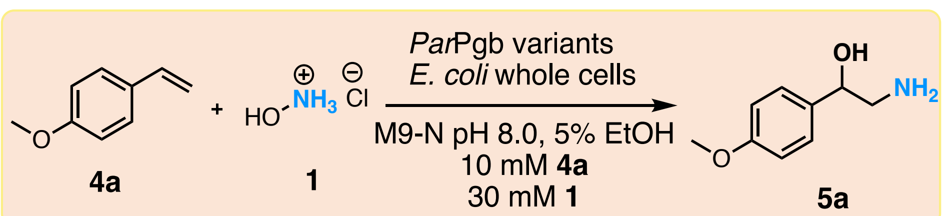


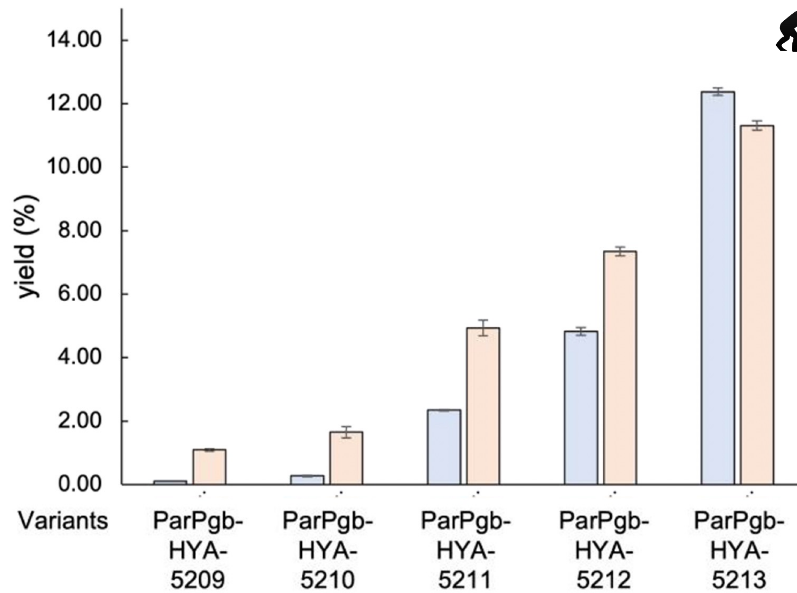
Protoglobin
Highly stable gas-binding protein









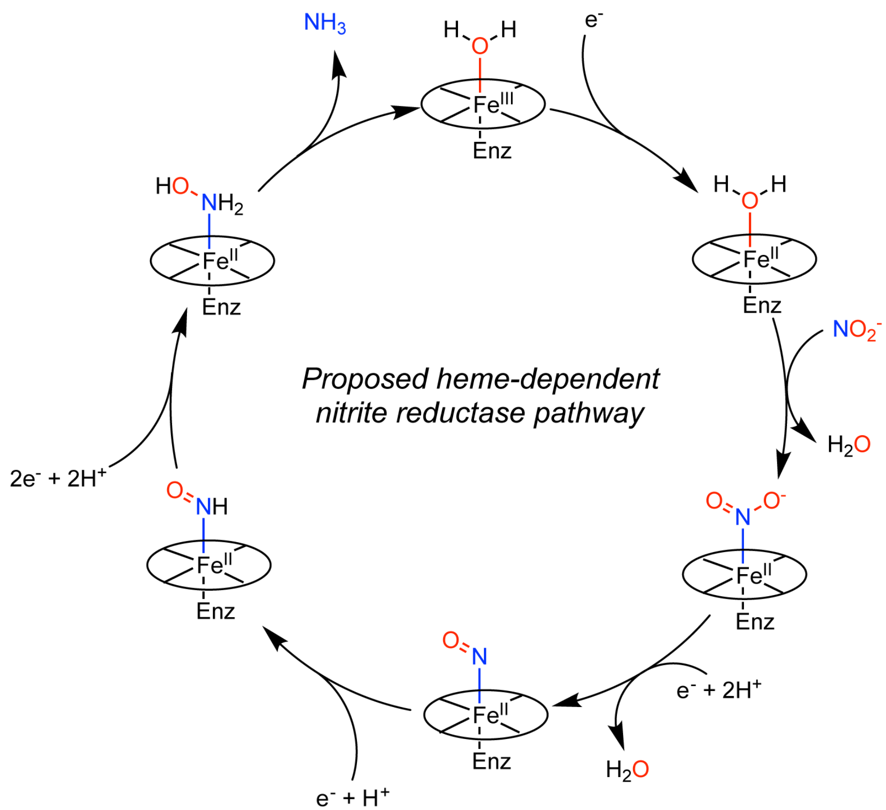


TTN (**3a**)	<5	15	70	180	320
TTN (**5a**)	25	100	150	280	300

Gao, S.; Das, A., *J. Am. Chem. Soc.* 2023, *145*, 20196.

Inspiration from nitrite reductase

$$NO_2^- + 6e^- + 8H^+ \xrightarrow{\textit{Nitrite Reductase}} NH_4^+ + 2H_2O$$

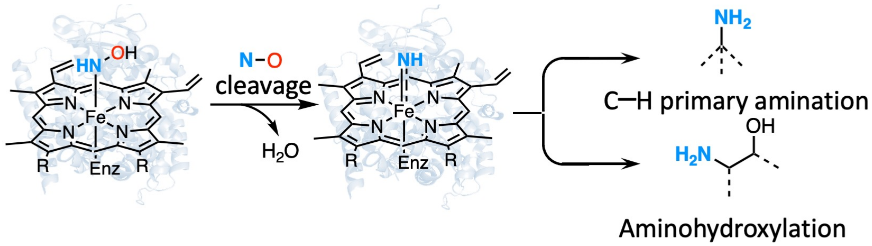


Einsle, Neese *et al.*
J. Am. Chem. Soc. 2002, *124, 11737.*

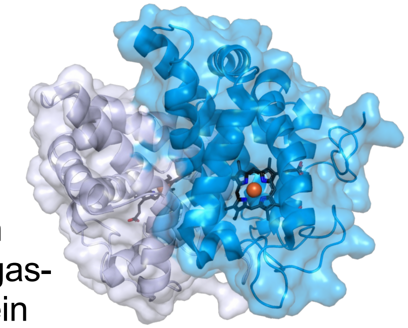
- *Nitrite coordinates via nitrogen to the reduced but not oxidized iron.*
- *Heterolytic cleavage of the N-O bond with release of water followed by two rapid one-electron reductions and a protonation give Fe(II)HNO nitroxyl adduct.*
- *Hydroxylamine, formed by a consecutive two-electron two-proton step, is dehydrated in the final two-electron reduction step to give ammonia and an additional water.*

Das, A.; Gao, S. et al. *J. Am. Chem. Soc.* 2024, *in press*

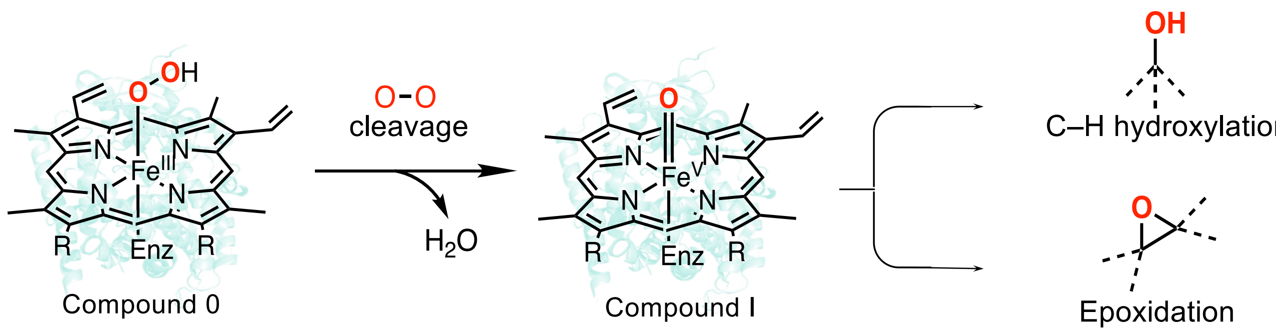
Enzymatic activation of hydroxylamine



Protoglobin
Highly stable gas-binding protein



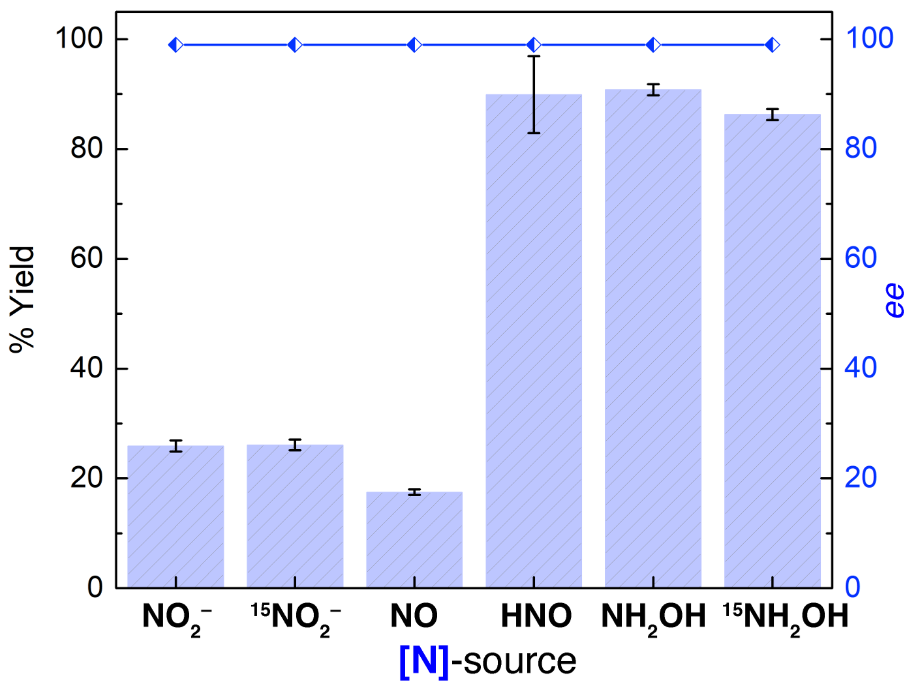
Analogous to peroxygenase?
Activation of hydrogen peroxide by peroxygenase:



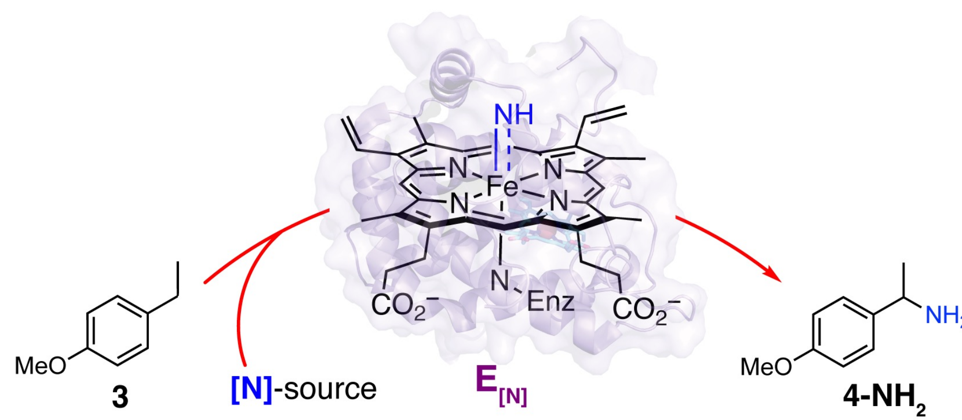
Unlike peroxygenase, NH_2OH aminating enzyme requires reducing environment

Gao, S.; Das, A., *J. Am. Chem. Soc.* 2023, *145*, 20196.

This led to discovery of new aminating reagents: NO_2^-, NO, HNO all drive enzymatic amination



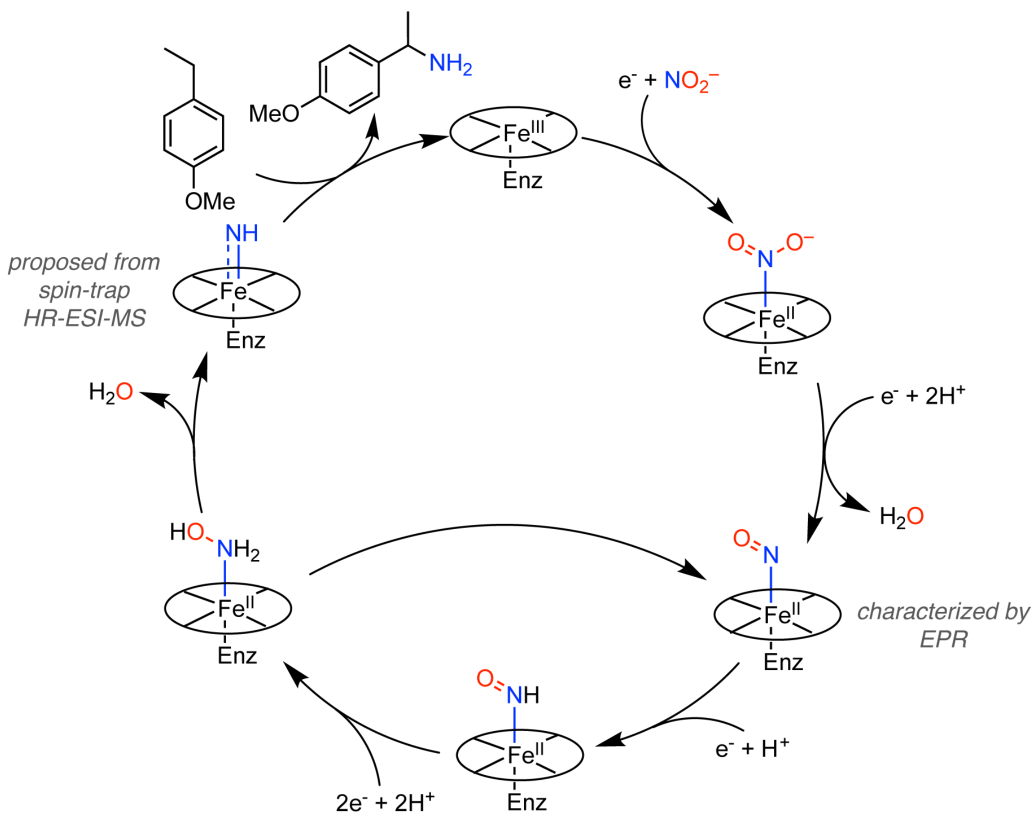
Near-perfect enantioselectivity maintained across all N-sources



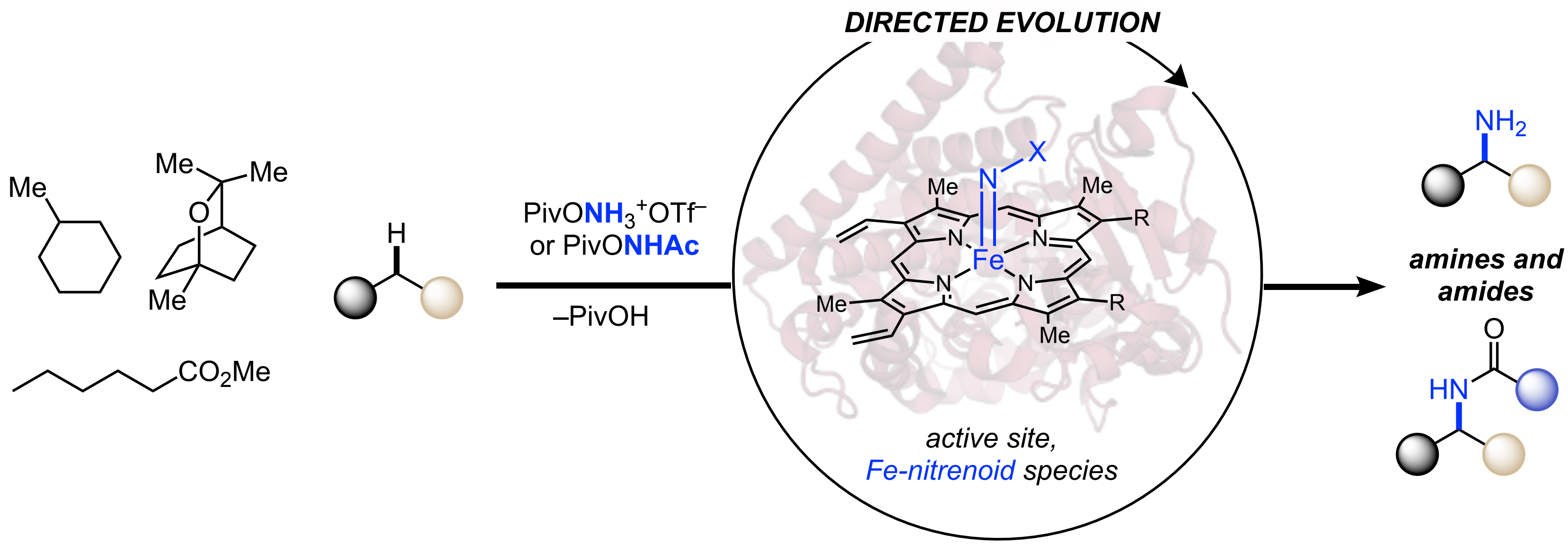
Evolved protoglobin nitrene transferase

Das, A.; Gao, S. et al. *J. Am. Chem. Soc.* 2024, *in press*

Proposed mechanism inspired by nitrite reductase



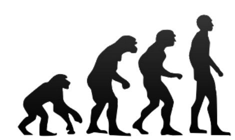
Anuvab Das; Gao, S. et al. *J. Am. Chem. Soc.* 2024, *in press*



Derek Lowe (Opinion for *Science*):

“Here’s one of those reactions that looks like alchemy to most of us in the synthetic organic community…

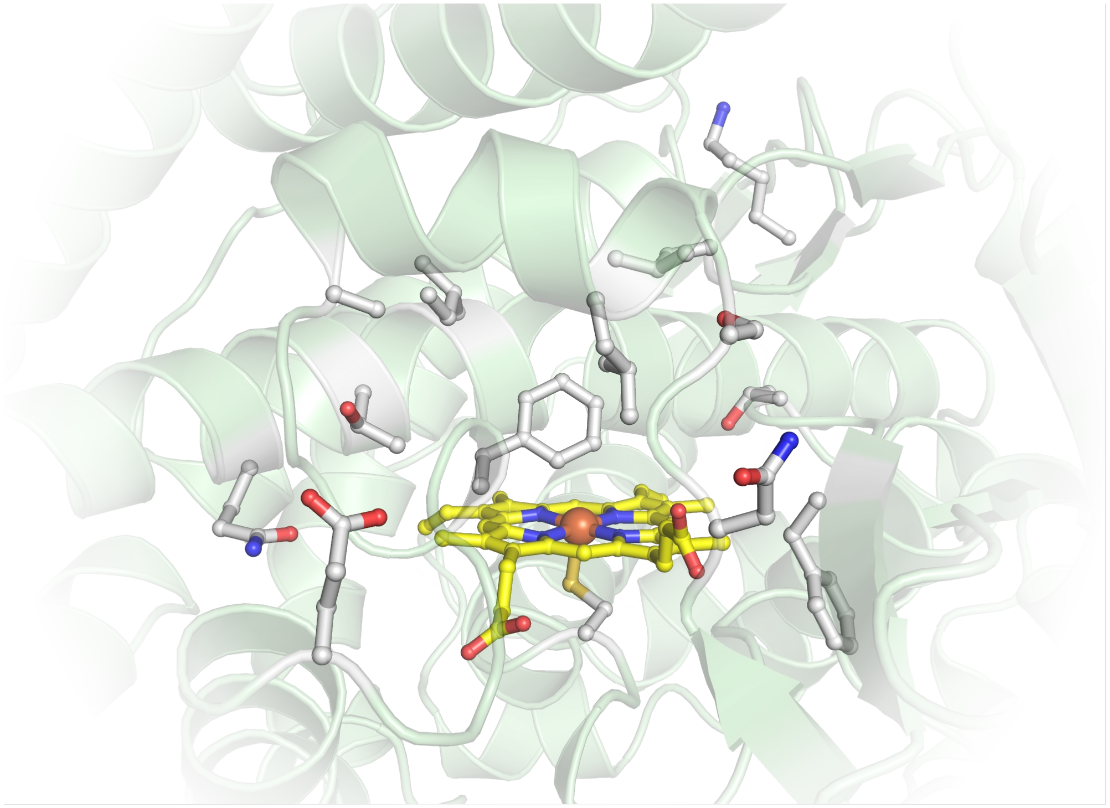
…these reactions really do look like magic, and they do chemistry that just can't be done with the reagents we have now. “



A heme protein is a

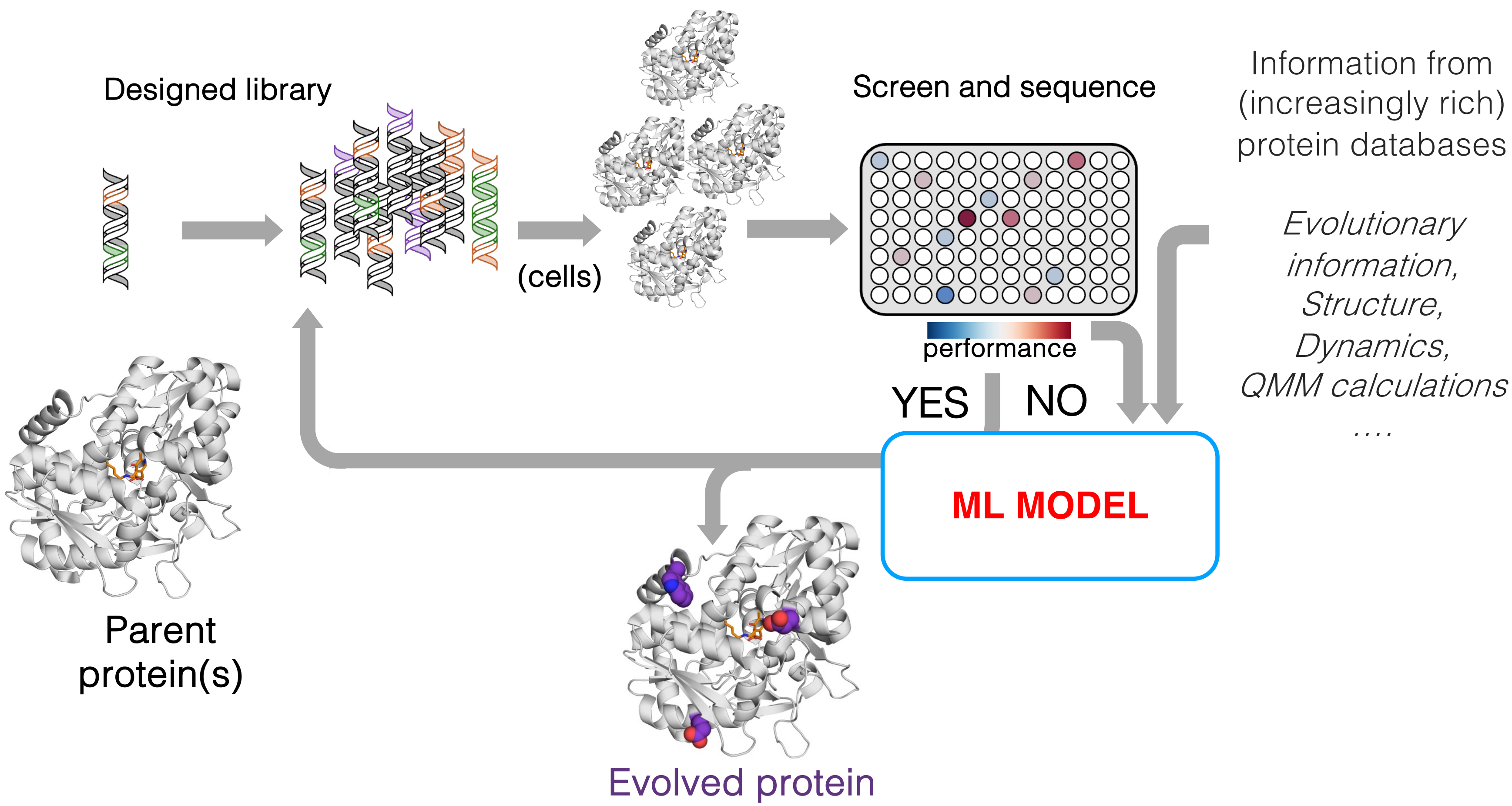
self-assembling, DNA-encoded, chiral metal complex

whose structure and electronic properties can be tuned by evolution.

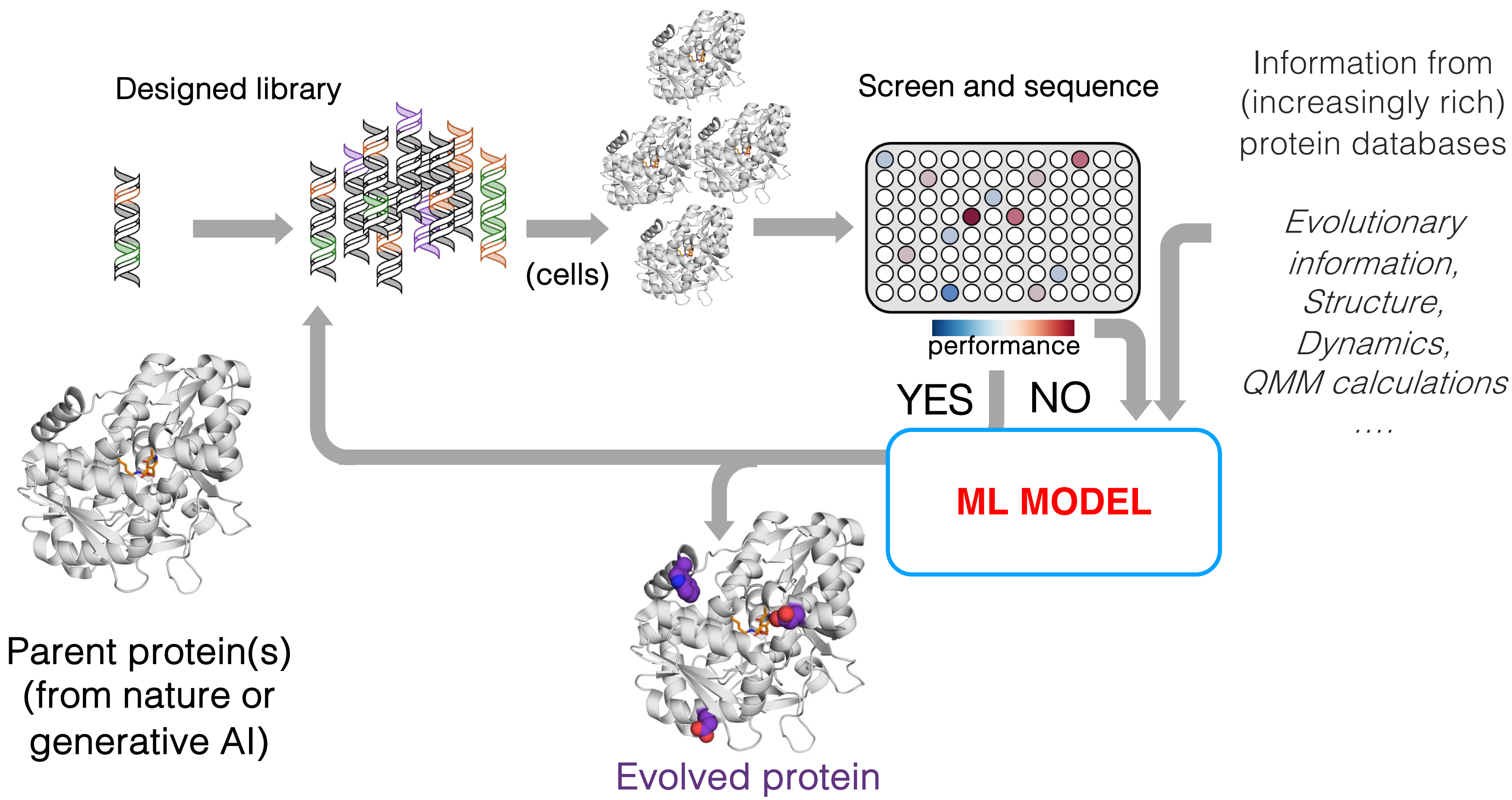


Future: reducing the time and expense of directed evolution ("push the button")

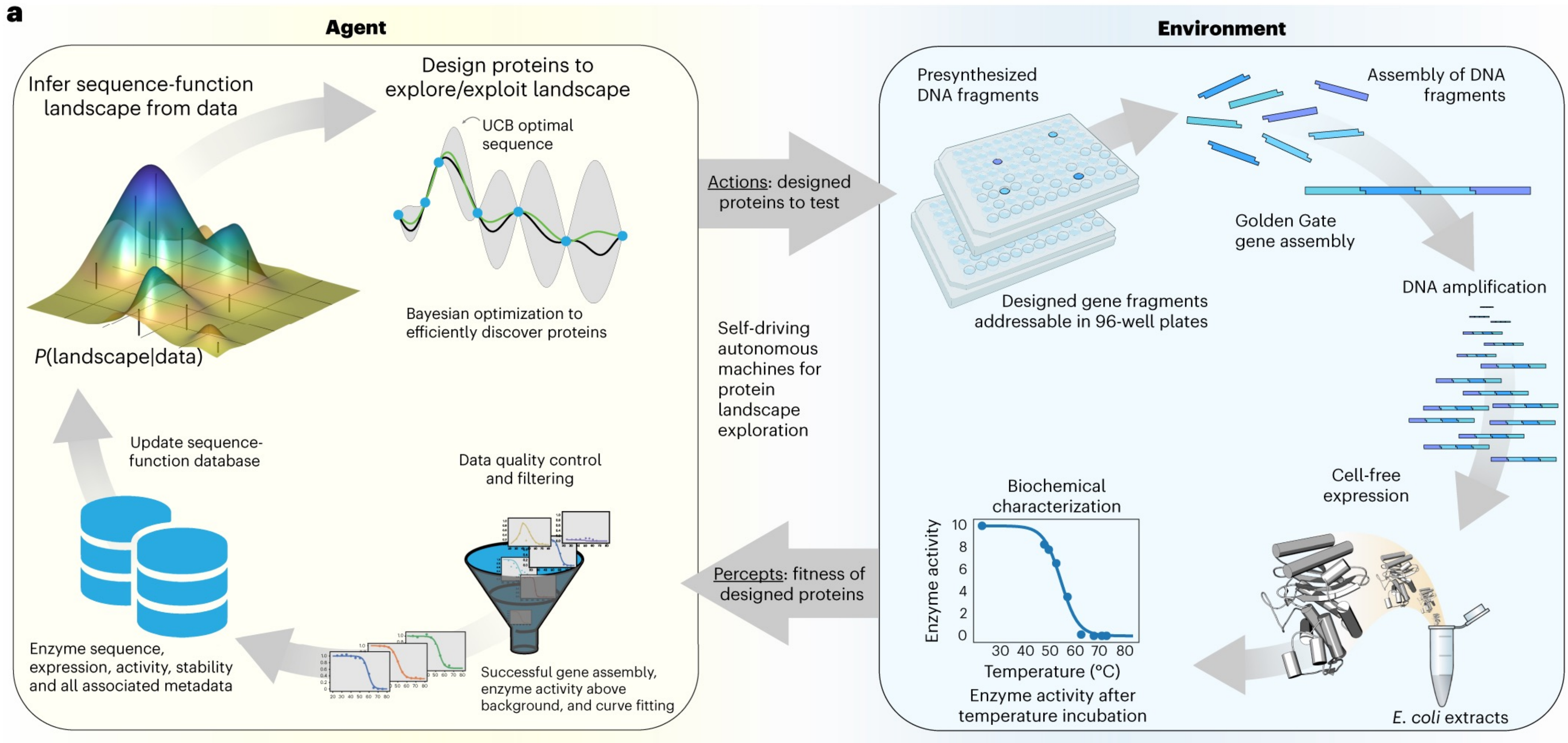
Improving directed evolution with machine learning



All these steps can be automated….



A fully autonomous system for protein engineering/evolution (P. Romero, U. Wisconsin)



Rapp et al., Self-driving laboratories to autonomously navigate the protein fitness landscape. *Nat Chem Eng* **1**, 97–107 (2024).