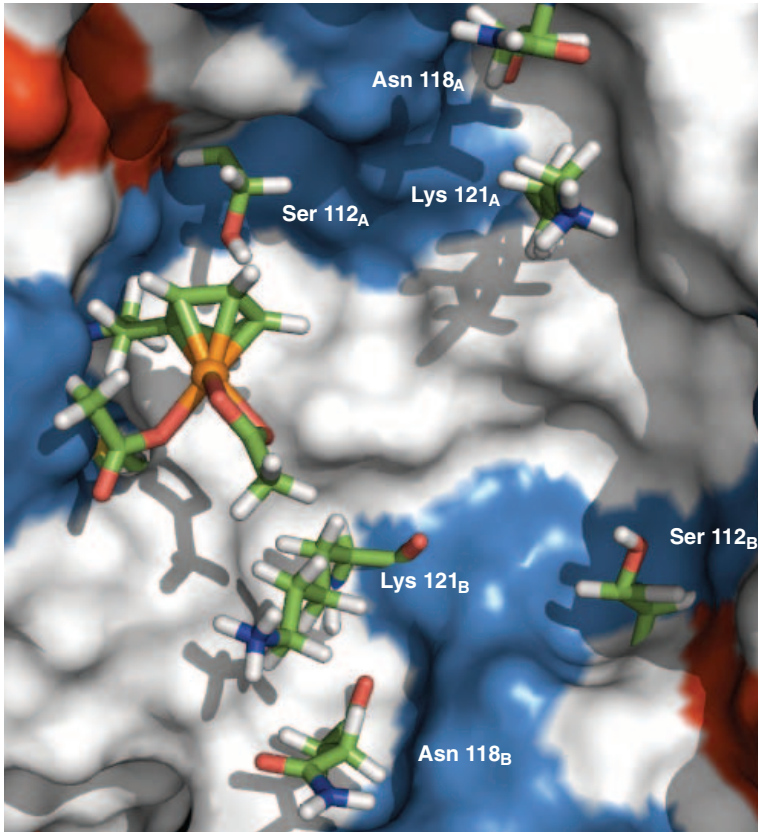
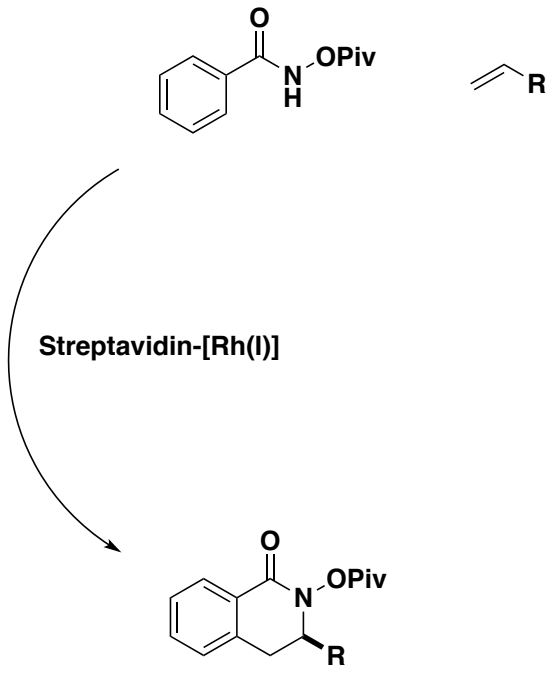
# Artificial Metalloenzymes: A Catalysis Upgrade?



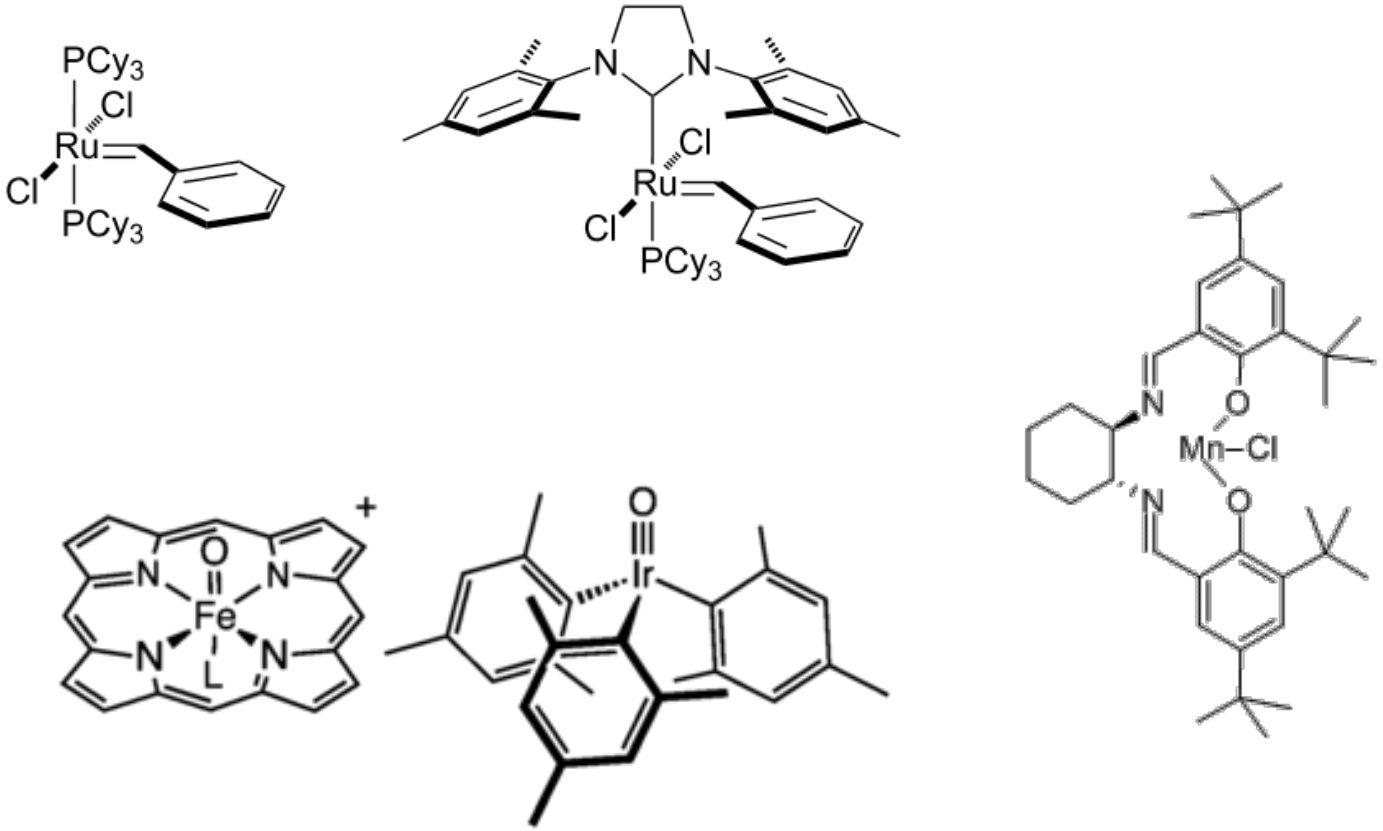




***Soumitra Athavale***

***SED Group Meeting***

***17 May 2016***

# Transition metal complexes and their sphere of influence




*Ligands around the metal modulate electronic properties and provide the architecture for steric influence.*

# Transition metal complexes and their sphere of influence

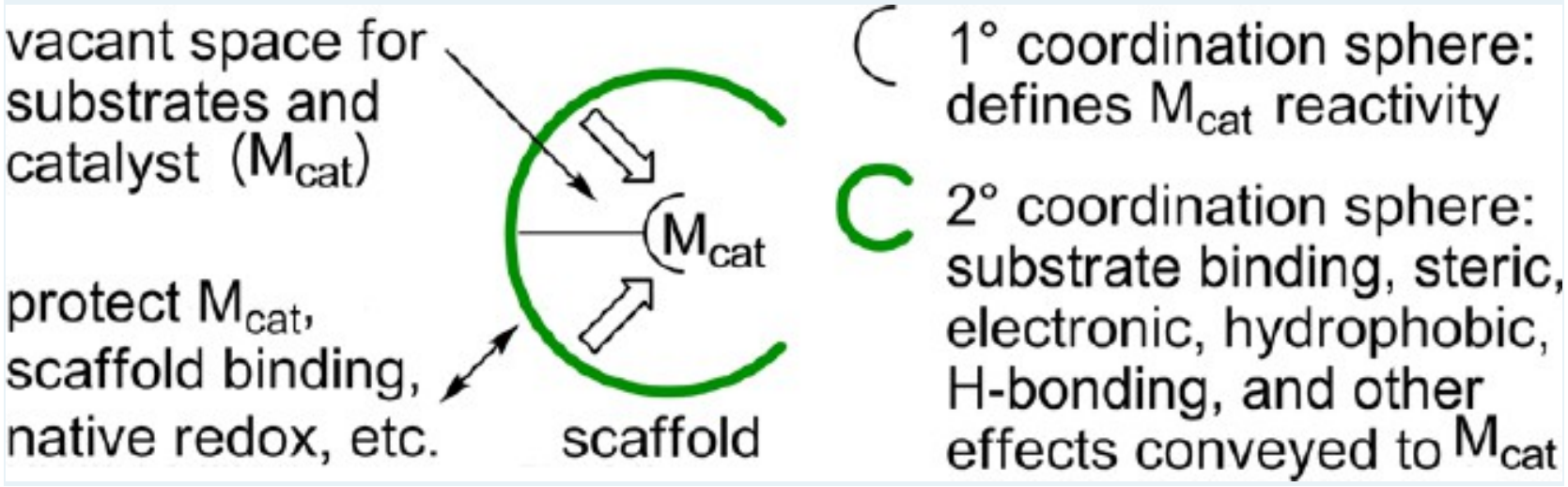
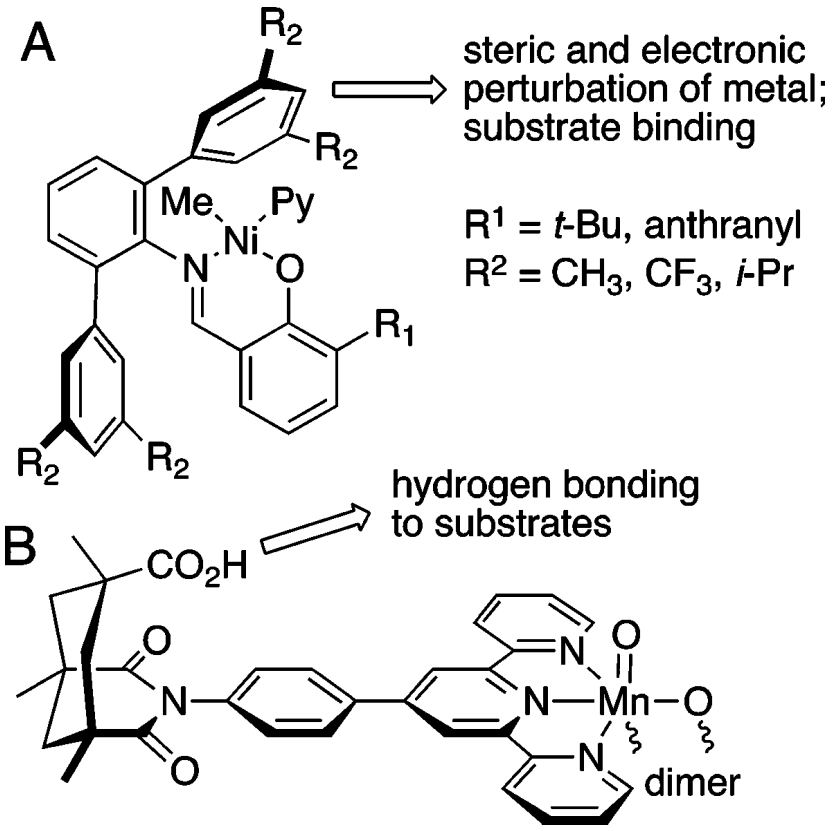



Figure from *ACS Catal.,* 2013, 3, 2954.

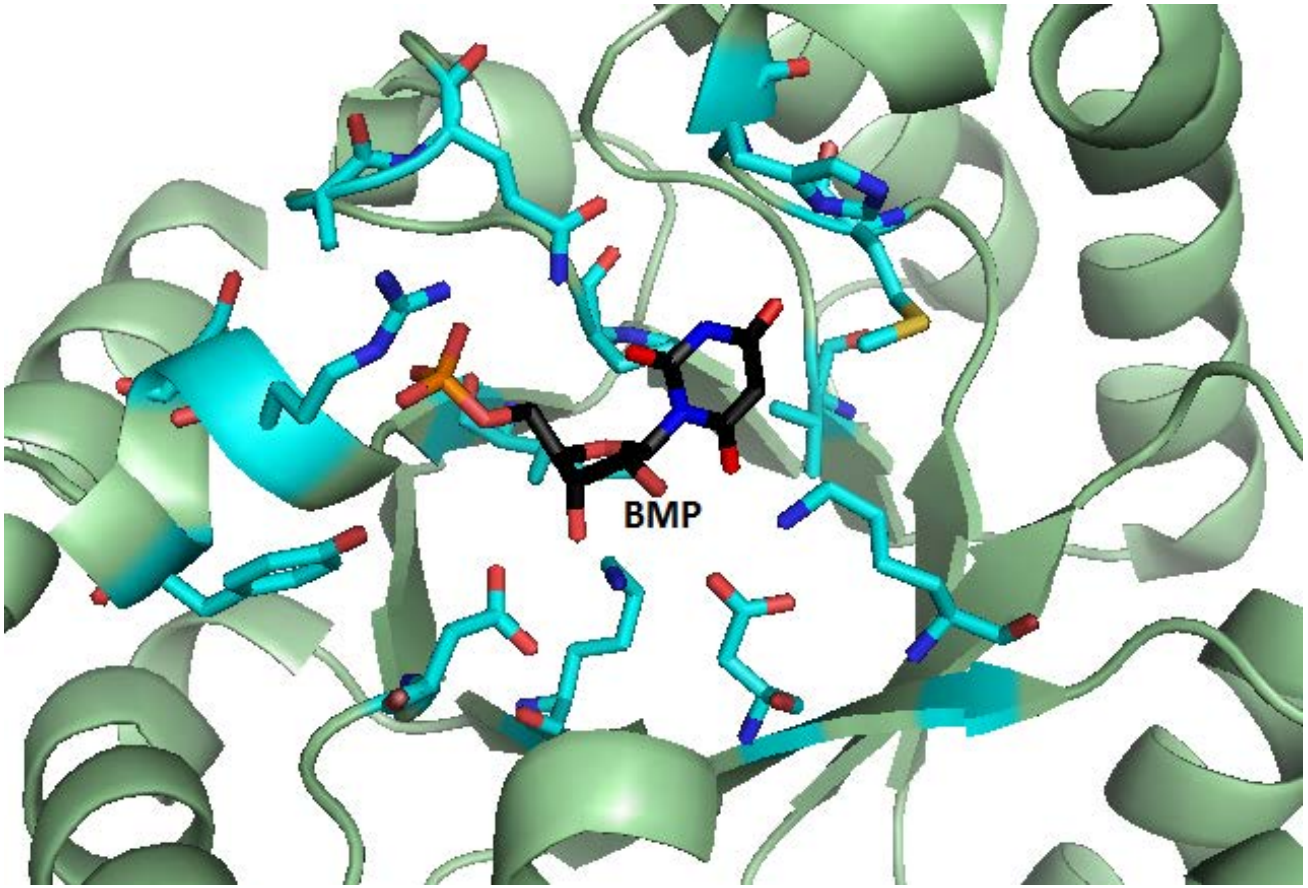
# The 2nd Coordination Sphere




In conventional homogenous catalysts, the design of this 2nd coordination sphere is generally challenging. Since the architecture is also connected to the primary binding ligands, only a limited control is possible.

Figure from *ACS Catal.,* 2013, 3, 2954.

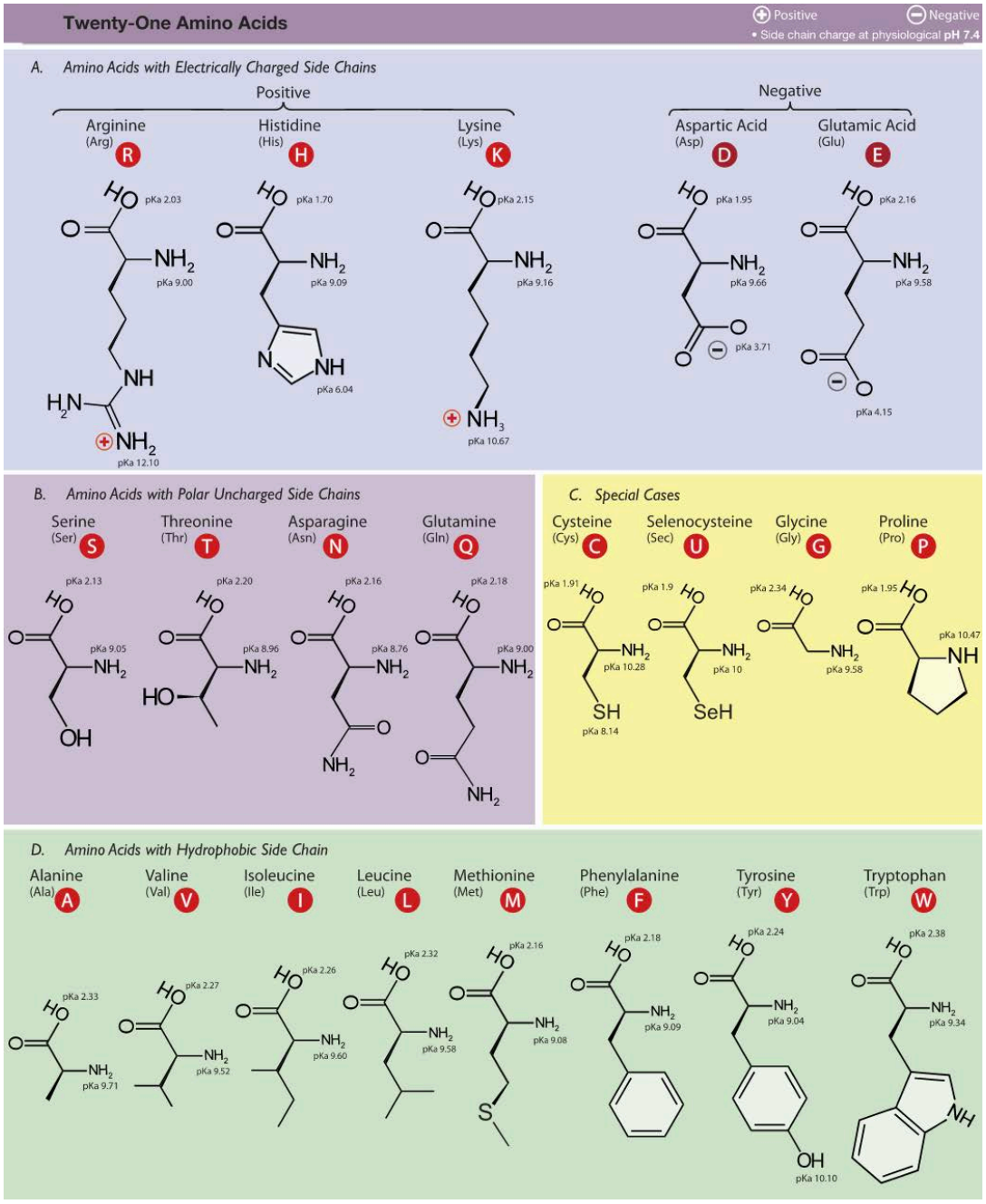
# 2nd coordination sphere in Enzymes.




Apart from the reactive residues, influence from amino acid side chains in the surrounding microenvironment aid in catalysis and selectivity. In a sense, subtle contributions from the entire protein structure provide enzymes with their exquisite selectivity.

https://en.wikipedia.org/wiki/Orotidine_5%27-phosphate_decarboxylase#/media

# The Amino Acid Arsenal

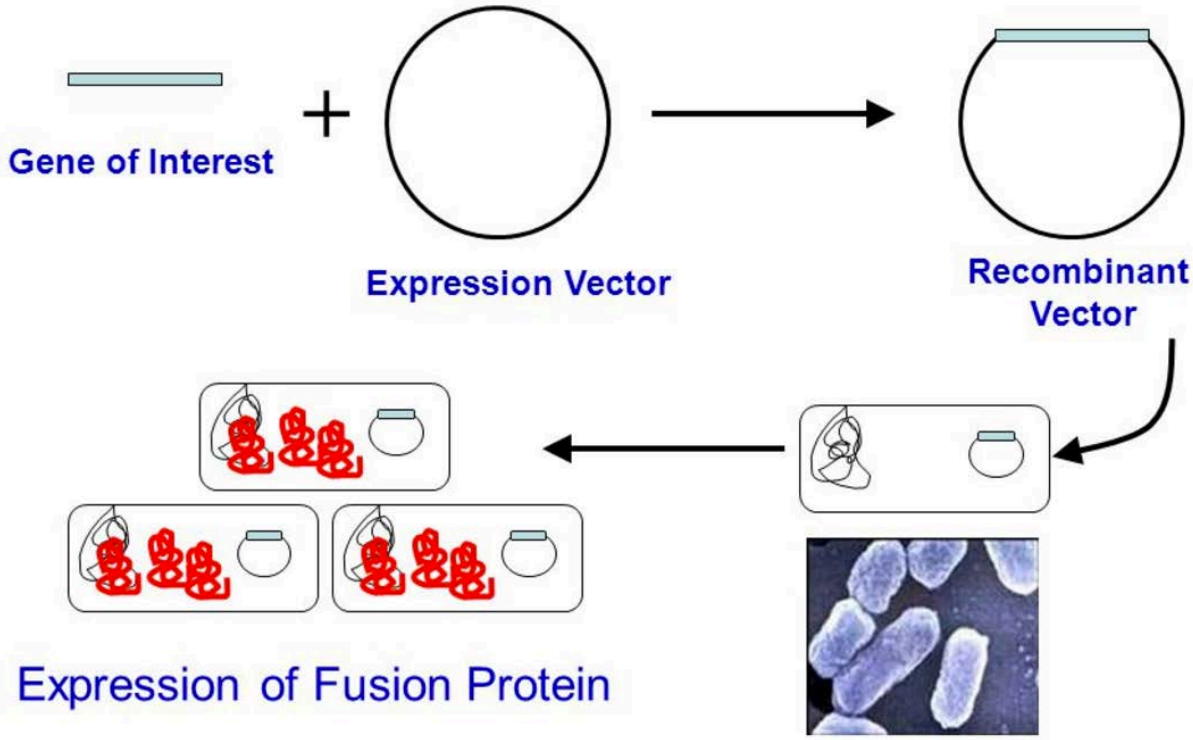



The 21 natural amino acids provide a palette of coordinating and scaffolding ligands for engineering the protein microenvironment.

Decades of seminal advances in molecular biology allow site specific modifications of enzymes.

http://ccsb2.dfci.harvard.edu/workshop/amino-acid/

# Protein Expression and Engineering

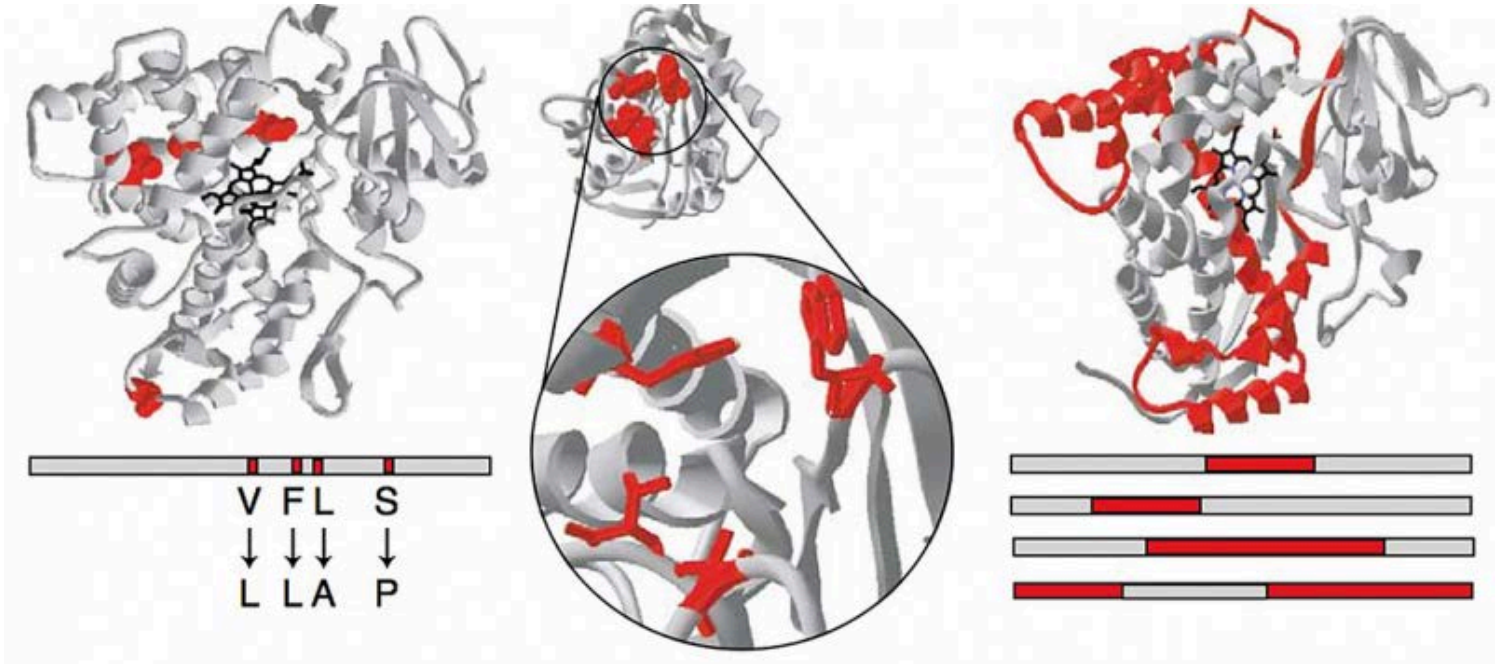



- Proteins are *always* produced by their synthesis in different expression systems. Most commonly, bacteria are used.

- The protein sequence is controlled by the *input* gene (DNA) sequence. By manipulating the DNA sequence, various modifications in the target protein can be achieved.

- Small molecules are modified synthetically; proteins are modified *genetically*.

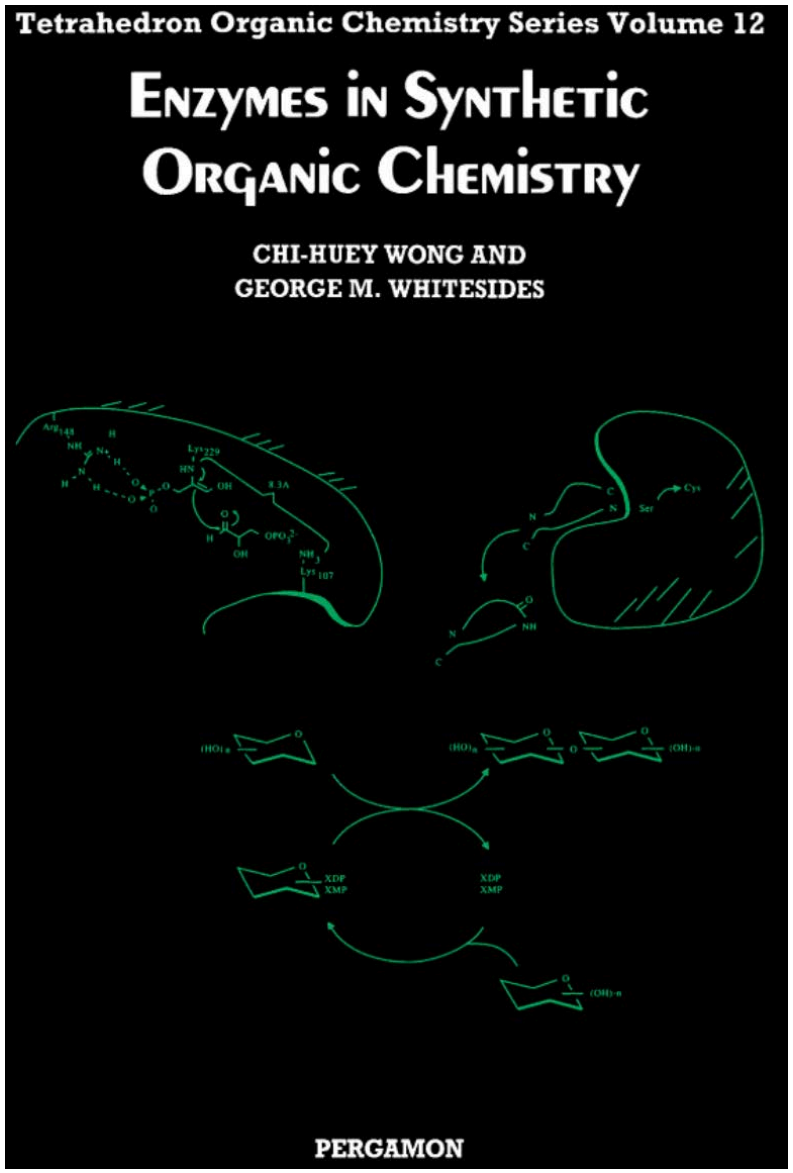
# Protein Expression and Engineering




- Protein modifications can includes amino acid substitutions, mutations and deletions. Non-natural amino acids can be incorporated.
- An extended peptide sequence can also be inserted or deleted.
- However, the effect of such modifications on protein function is generally challenging to predict.

http://biomedicalcomputationreview.org/content/computing-better-enzymes-optimizing-directed-evolution

# Enzymes in Industry and Organic synthesis




(2013)

| Enzyme | Application |
|---|---|
| Glucose isomerase | Isomerization of glucose to fructose (high fructose corn syrup; 8 million tons) |
| Aminoacylase | Optical resolution of DL-amino acids |
| B-galactosidase | Hydrolysis of lactose to galactose and glucose (treatment of milk and whey) |
| Lipase | Interesterification of fats |
| Nitrile hydratase | Production of acrylamide from acrylonitrile; 15,000 tons |

- A number of industrial transformations use enzymes.
- Environmently friendly and extremely mild reaction conditions additionally favour their use in industry.

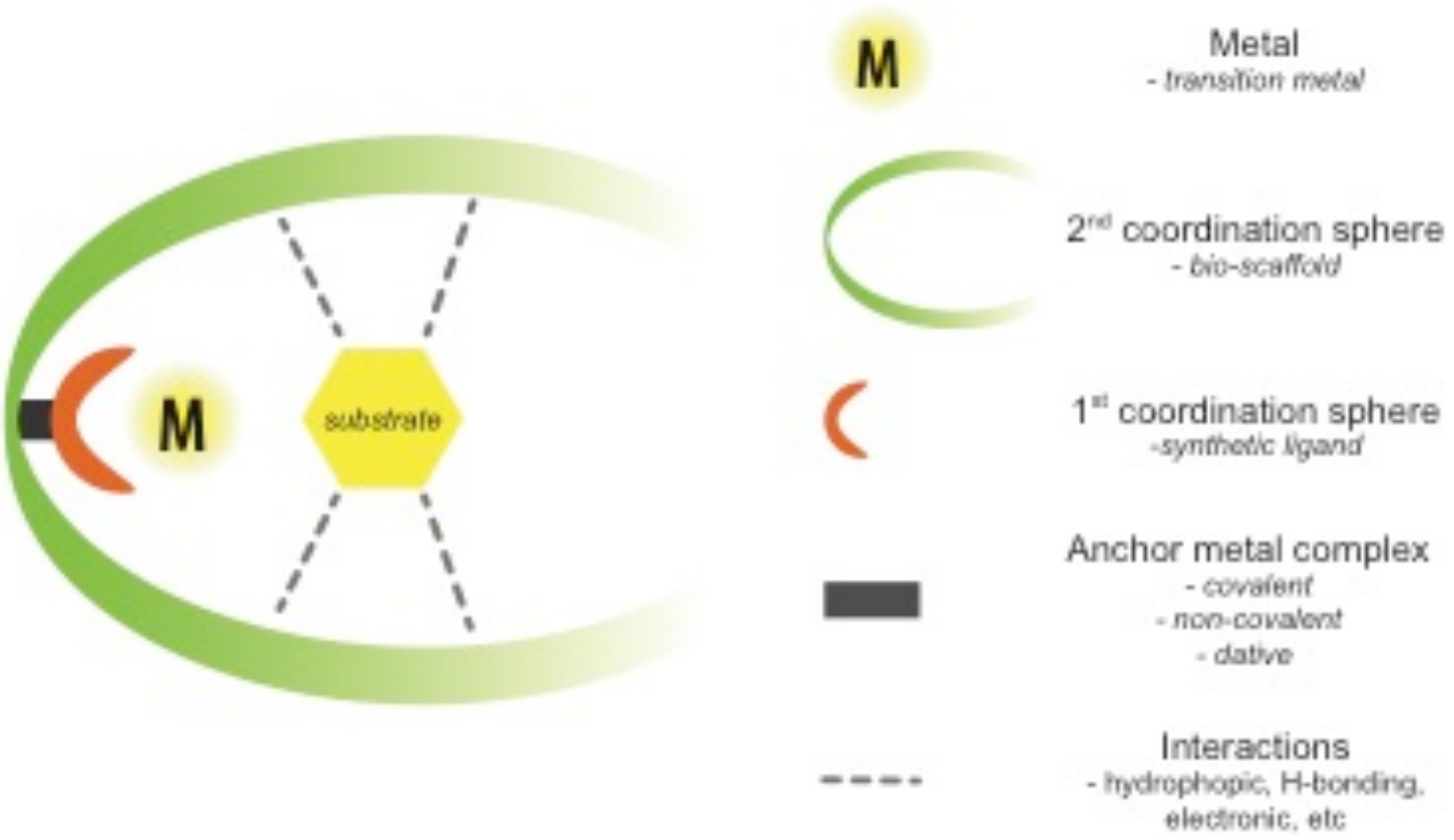
# Comparing Homogenous Catalysis to Enzyme Catalysis

| | homogeneous catalysis | enzymatic catalysis |
|---|---|---|
| substrate scope | large | limited |
| enantiomers | both enantiomers accessible | single enantiomer |
| functional group tolerance | small | large |
| reaction repertoire | large | small |
| turnover numbers | small | large |
| solvent compatibility | large | small (aqueous) |
| optimization | chemical | genetic |
| second coordination sphere | poorly-defined | well-defined |

*A desirable goal is to combine the large reaction repertoire of organometallic catalysis with the specificity and mild reaction conditions offered by enzyme catalysis.*

Ward, T. R. et al., *JACS.,* 2004, 126, 14411.

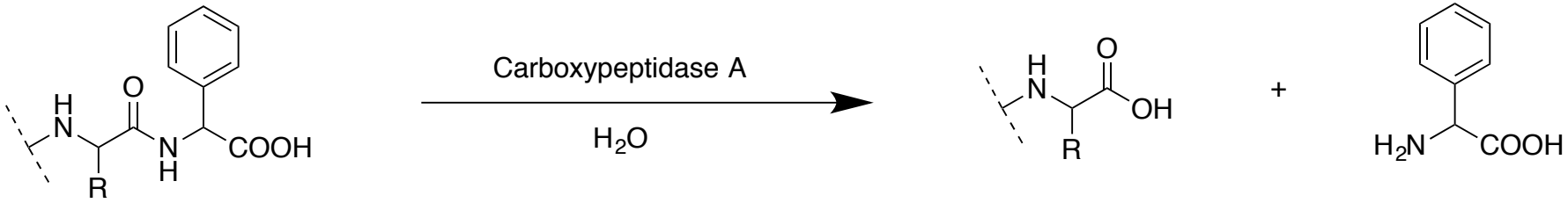
# Metal Complexes with Protein Cloaks?

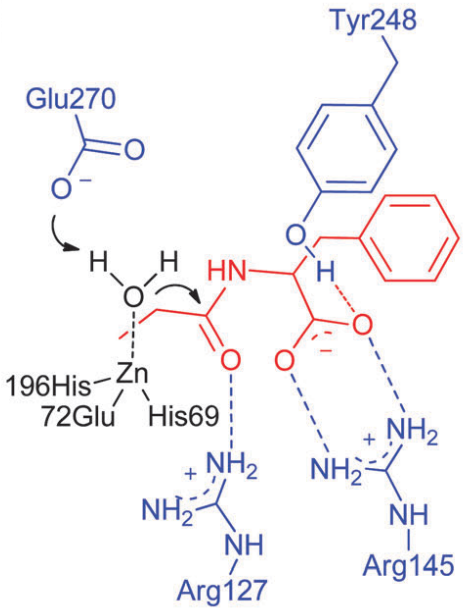



*Envisioning an artificial metalloenzyme with various parameters amenable to modification.*

G. Roelfes, Curr. Opin. Chem. Biol., 2014, 19, 135-143.

# The First Modified Metalloenzyme- Carboxypeptidase A



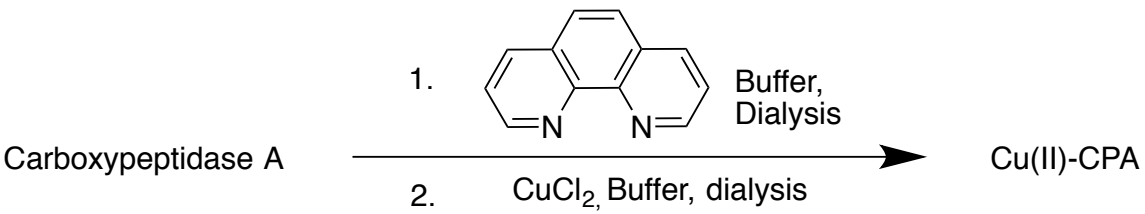



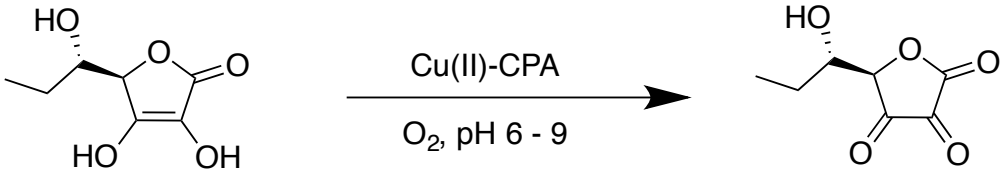

- Carboxypeptidase A is a zinc-containing peptidase.
- Specifically hydrolyzes the C-terminus of a polypeptide.
- Participation of side chain groups in the active site and the precisely positioned zinc ion provides a general acid-base catalyzed mechanism.



*Figure:* Chem. Soc. Rev. 2013, 42, 8360.

Kaiser (1976), replaced the zinc ion by Cu(II). The wild type peptidase activity was completely destroyed but surprisingly, an oxidase activity was gained!




Ascorbic acid

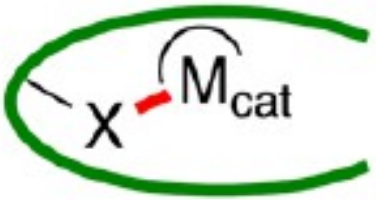
dehydroascorbate

"Our observations demonstrate that the replacement of one active-site metal ion by another in a given protein matrix can result in the conversion of a powerful hydrolytic catalyst into an oxidase."



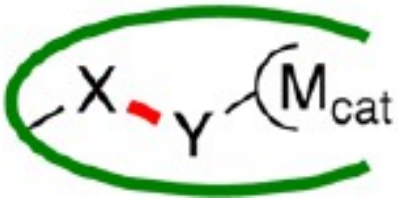
Yamamura, K. and Kaiser, ET. *J.C.S. Chem. Comm.,* 1976, 107.

# Key Strategies for Metal Incorporation



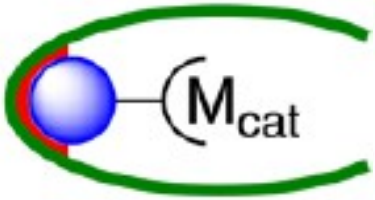

A:

Direct coordination of metal ion or catalyst




B:

Covalent attachment of metal complex via a linker.




C:

Non-Covalent attachment of a metal complex.

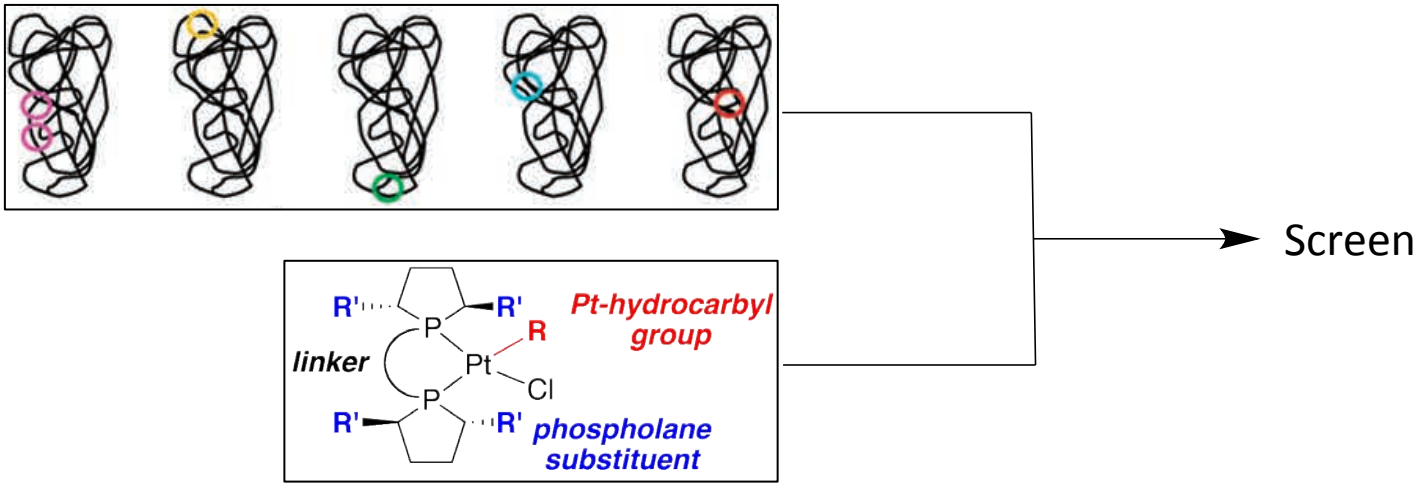
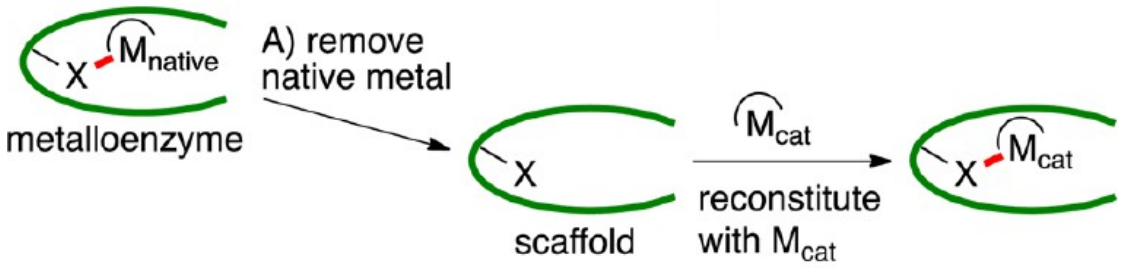



Figure from *ACS Catal.,* 2013, 3, 2954.

# General Strategy A. Replacement of Native ion.

- Kaiser's results demonstrated a simple approach to engineer metalloenzymes by replacing the original metal ion by a new one.




- This approach was explored in some detail recently by various groups by using the Carbonic Anhydrase enzyme as the protein scaffold.



Figure adapted from *ACS Catal.,* 2013, 3, 2954.

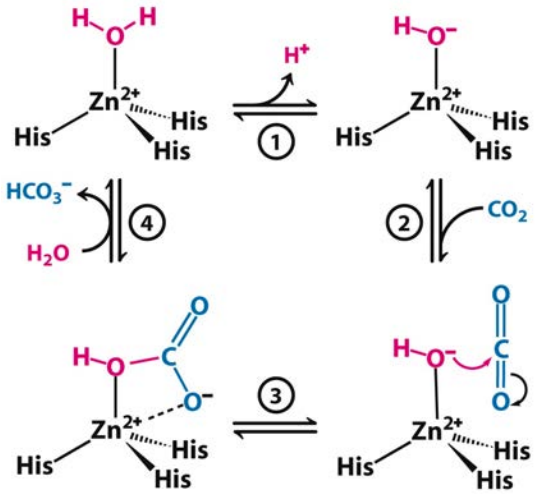
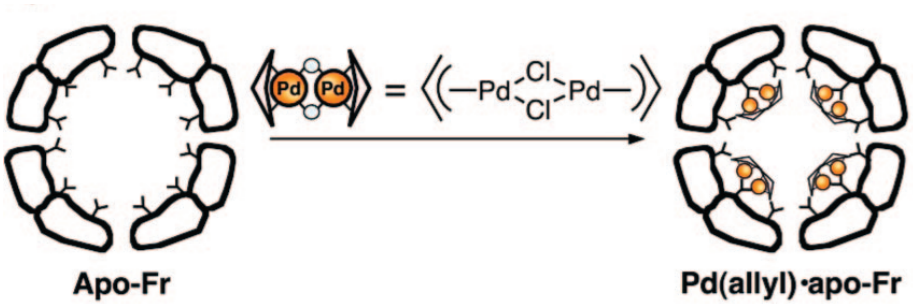
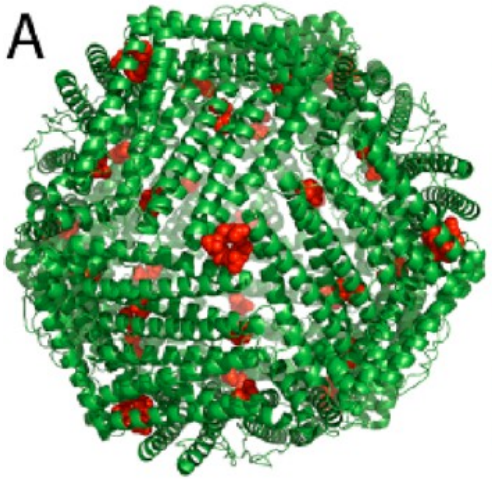


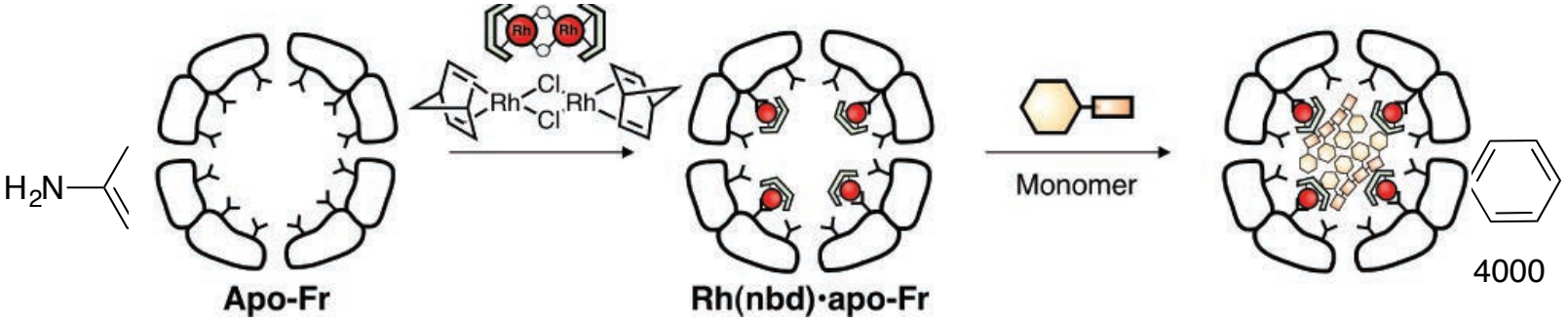

Figure 9.25
*Biochemistry*, Seventh Edition


- The wild type Zn(II)-enzyme catalyzes decomposition of carbonic acid.
- The metal ion has been substituted by Mn(II) and Rh(I) to invoke alternate reactivities.

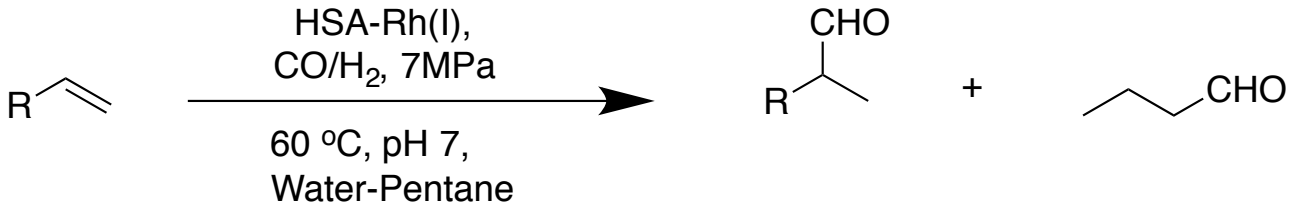



- Ferritin is a ubiqitous Iron storage protein made up of 24 subunits.
- Watanabe (2008, 2009) incorporated Pd complexes (4 Pd atoms per subunit). Pd is coordinated by Cys, His side chains. Resultant assembly showed catalytic properties for a Suzuki reaction and alkene polymerization.



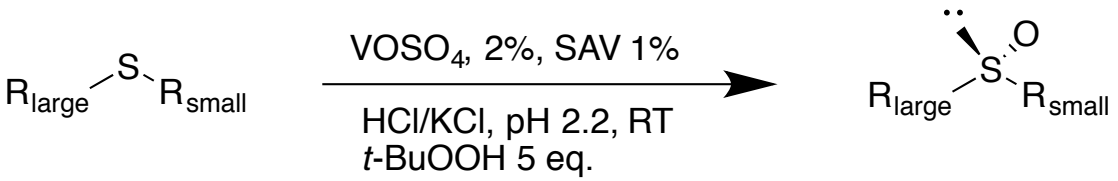

Watanabe et. al., *JACS*, 2008, 130, 10512.
Watanabe et. al., *JACS*, 2009, 131, 6958.




| R | conv. (%) | B/L |
|---|---|---|
| Ph | 99 | 9 |
| OPh | 70 | - |
| *n*-hex | 99 | - |

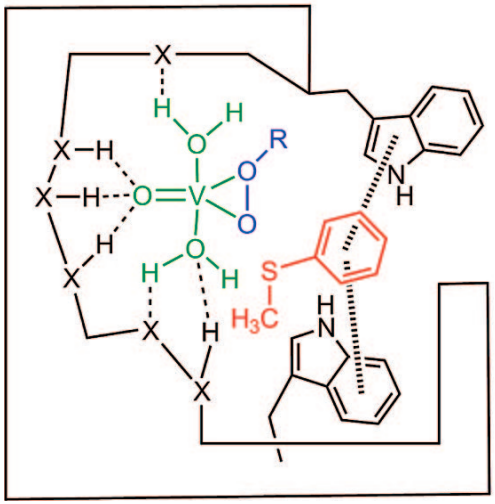
- Serum albumin does not contain any metal ions but binds non selectively to hydrophobic compounds. Highly abundant and available commercially.
- Marchetti and coworkers (2002) showed exceptional efficiency for HSA-Rh complex in styrene hydroformylation with TON > 500,000.
- *Although not demonstrated, immobilized albumin may be amenable to easy recovery and recyclability.*

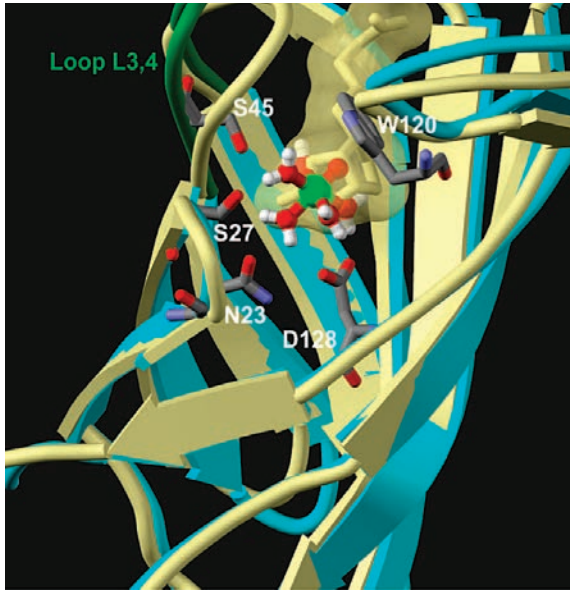
Marchetti et. al., *Adv. Synth. Cat.,* 2002, , 344, 556.




| $R^1$ | $R^2$ | conv (%) | ee (%) |
|---|---|---|---|
| p-Tol | Me | 96 | 87 |
| Ph | Et | 96 | 90 |
| Bn | Me | quant | 73 |
| Cy | Me | 61 | 86 |

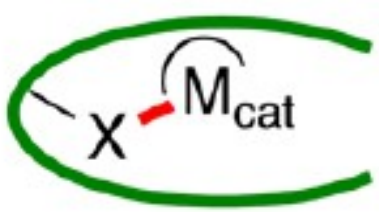
- Ward (2008) demonstrated that streptavidin, which has no native catalytic activity can be transformed into an enantioselective biocatalyst for sulfoxidation.
- Binding studies indicated that outer-sphere interactions were responsible for inducing selectivity.




Ward, T. et al., *JACS.,* 2008, , 130, 8085.

# Key Strategies for Metal Incorporation




A:

Direct coordination of metal ion or catalyst

- Metal is directly coordinated to a nucleophilic sidechain in the protein interior.

- Pros:

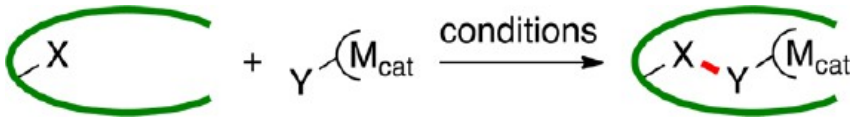
-Technically straightforward protocol.
-Minimal perturbation of the enzyme microenvironment

- Cons:

-Multiple binding sites possible
-Electronic properties of the metal centre may be affected in an adverse way.

- Since the metal is directly modified by the enzyme architecture, original purpose of delinking reactivity (metal) and scaffolding (protein) is lost.

# Strategy B: Covalent Anchoring

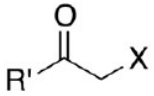
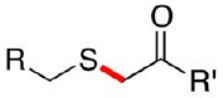
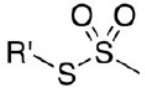
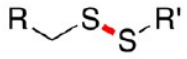
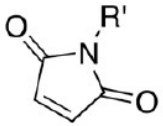
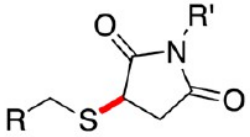
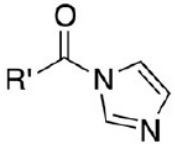
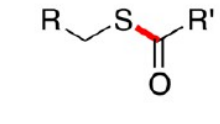
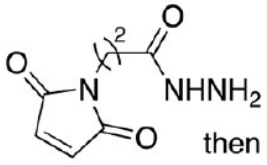
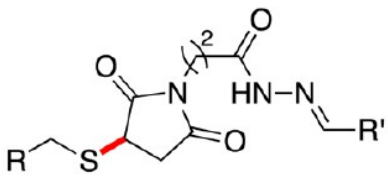
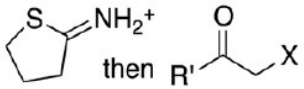
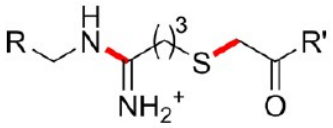
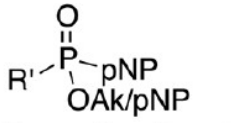
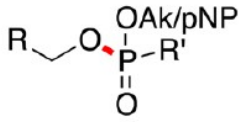
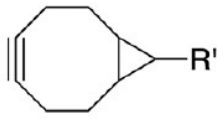
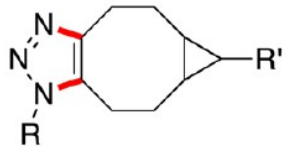


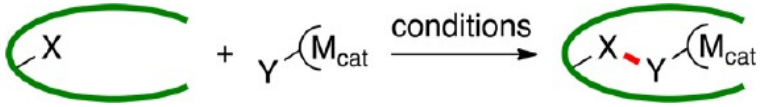

- Methods have been developed to covalently link synthetic, catalytically active transition metal ligands/cofactors to proteins.
- At a minimum, such bioconjugation requires a scaffold protein containing a uniquely reactive residue (typically a nucleophile) and the corresponding reaction partner (typically an electrophile).
- Cysteine alkylation is the most utilized method.



Figure adapted from *ACS Catal.,* 2013, 3, 2954.

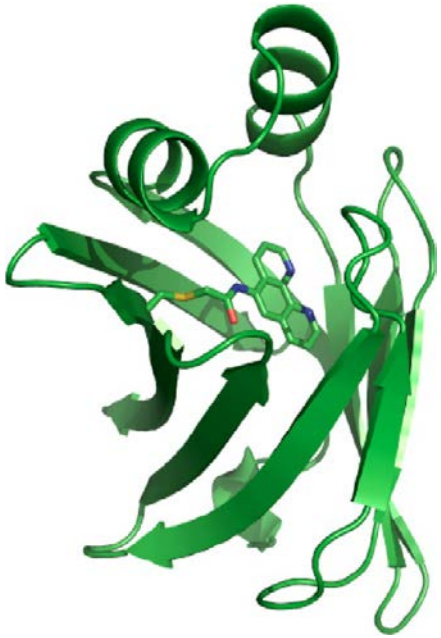
# Covalent anchoring

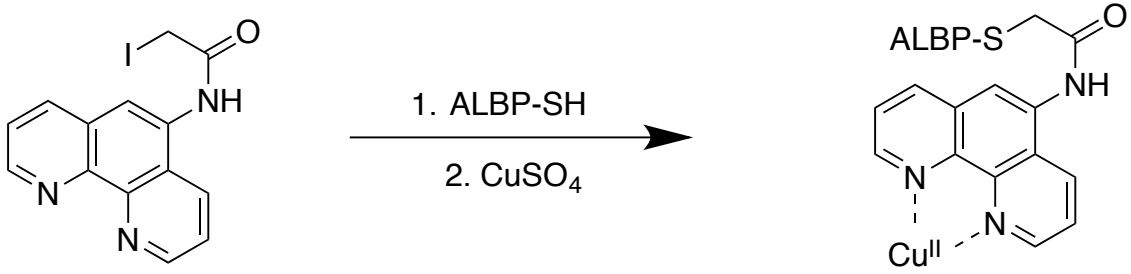
| X (residue) | Y | X-Y |
|---|---|---|
| R–SH (cysteine) | R'–Br | R–S–R' |
| R–SH (cysteine) |  X = Br, I |  |
| R–SH (cysteine) |  |  |
| R–SH (cysteine) |  |  |
| R–SH (cysteine) |  |  |
| R–SH (cysteine) |  then  |  |
| R–NH$_2$ (lysine) |  |  |
| R–OH (serine hydrolase) |  pNP = *p*-nitrophenol |  |
| R–N$_3$ (*p*-azidophenylalanine) |  |  |






Lewis, J. C., *ACS Catal.,* 2013, 3, 2954.

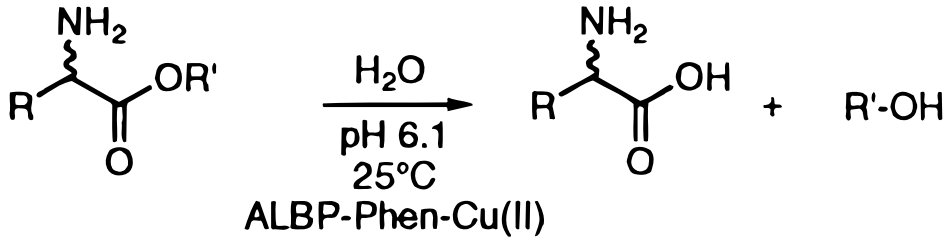





- An early example was provided by Distefano and coworkers who utilized the single Cys containing Adipocyte Lipid Binding Protein (ALBP) for Phenanthroline conjugation and subsequent Cu(II) catalyzed hydrolysis.

- ALBP is a small (130aa) protein with a large 600$A^3$ hydrophobic cavity. It was hoped that the resulting scaffolding environment around the Cu(II) centre would provide basis for selective transformations.

Distefano, M.D. et al., *JACS.,* 1997, , 119, 11643.




**6a** R = $CH_3$, R' = $CH_3$
**6b** R = $CH_3$, R' = $CH_3CH_2$
**6c** R = $CH_3$, R' = $(CH_3)_2CH$
**8a** R = $HOC_6H_5CH_2$, R' = $CH_3$
**8b** R = $HOC_6H_4CH_2$, R' = $CH_3CH_2$
**10** R = $HOCH_2$, R' = $CH_3$

**7** R = $CH_3$

**9** R = $HOC_6H_4CH_2$

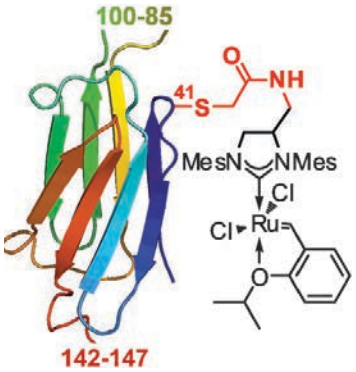
**11** R = $HOCH_2$

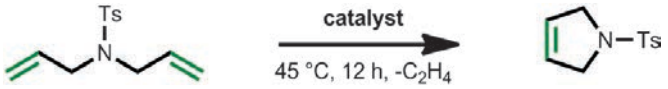
- Amino acid esters were used as substrates. Modest enantioselectivities (30 – 92% ee) were observed. The catalyzed rate was >100 times faster than background hydrolysis and selective for the L-isomer.

- A low TON of ~7.5 was seen that was attributed to the highly buried position of the phenanthroline ligand and possible product inhibition.

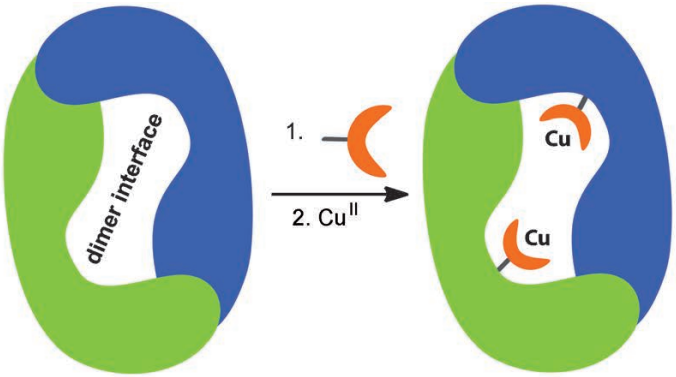
Distefano, M.D. et al., *JACS.,* 1997, , 119, 11643.

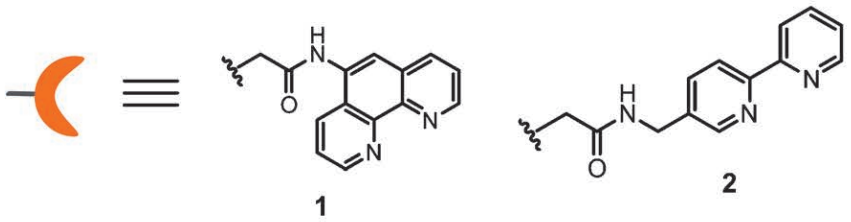
# Other examples



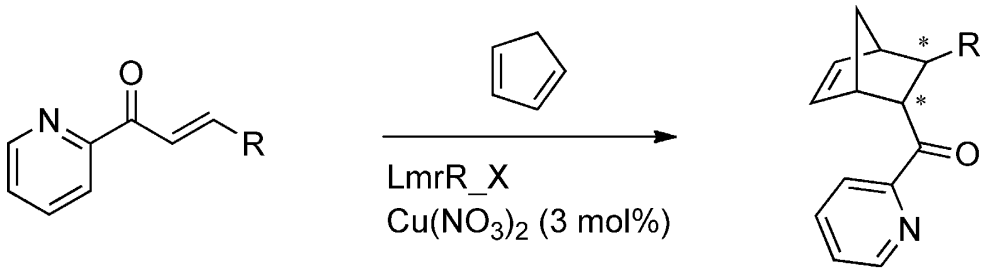




*HSP from bacteria*
*Hilvert, 2011*
*TON ~ 33*




- Roelfes and coworkers utilized the Lactococcal multidrug resistance regulator (LmrR), a dimeric protein to prepare a Cu(II) modified metalloenzyme for a representative Diels-Alder reaction. The dimer interface provides the chiral microenvironment for catalysis.

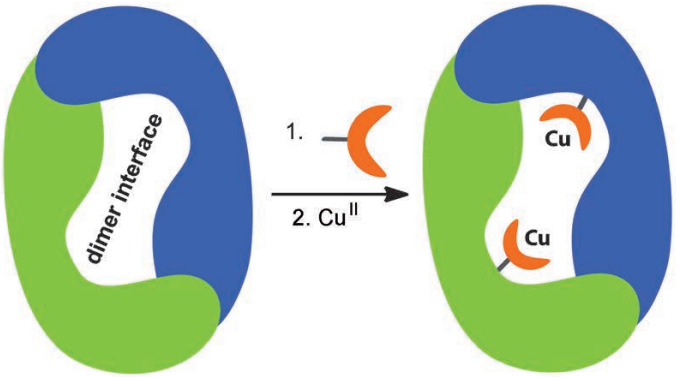


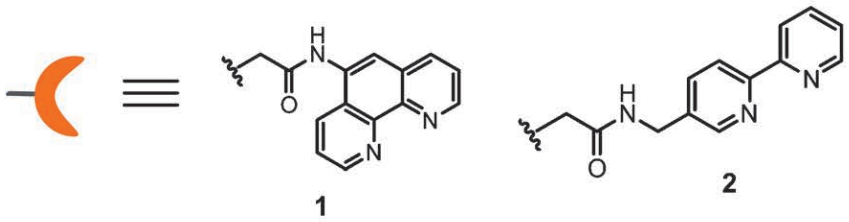

**3 a-c** **4 a-c**

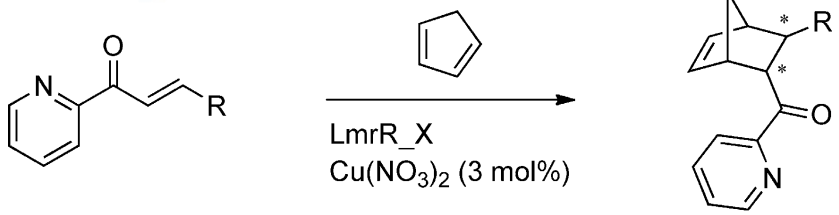
**a**: = Ph
**b**: = *m*-MeOPh
**c**: = Me

Roelfes, G. et al., *ACIE.*, 2012, 51, 7472.

# Diels-Alder reaction




**3 a-c**

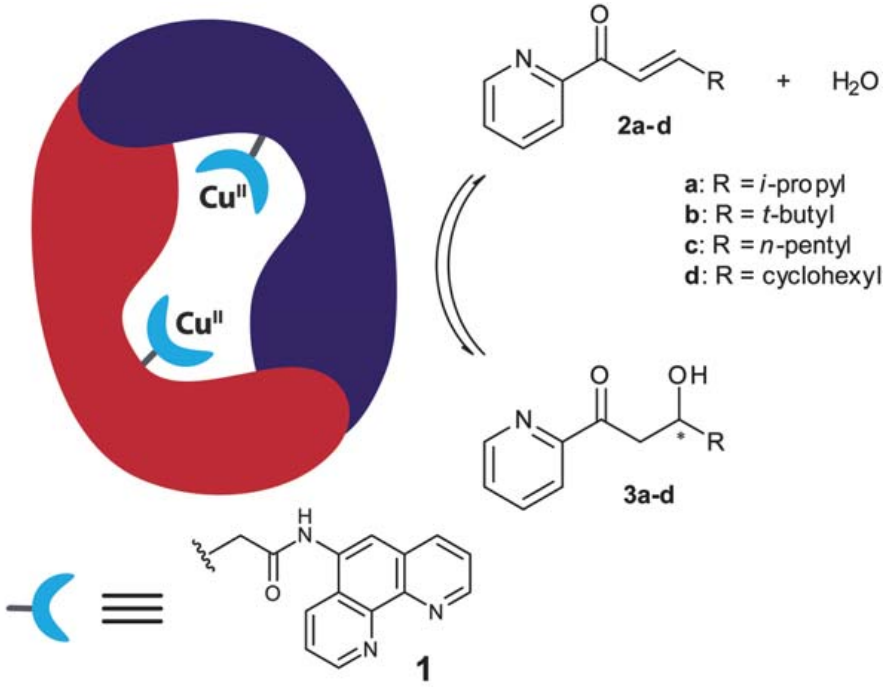
**4 a-c**

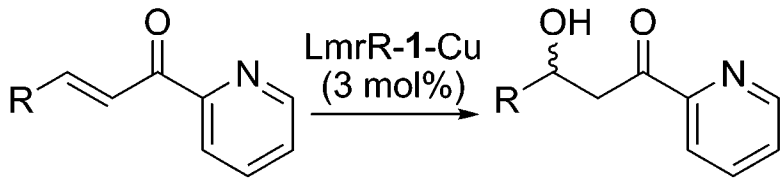
**a**: = Ph
**b**: = *m*-MeOPh
**c**: = Me

| Entry | Catalyst | Product | Conversion [%] | *endo*:*exo* | *ee* (*endo*) [%] |
|---|---|---|---|---|---|
| 3[b] | LmrR_M89C_**1**_$Cu^{II}$ | **4a** | 98 ± 1 | 90:10 | 95 ± 1 (+) |
| 4 | LmrR_M89C_**1**_$Cu^{II}$ | **4b** | 56 ± 9 | 96:4 | 93 ± 1 (+) |
| 5 | LmrR_M89C_**1**_$Cu^{II}$ | **4c** | 97 ± 1 | n.a.[c] | < 5 |
| 6 | LmrR_M89C_**2**_$Cu^{II}$ | **4a** | 55 ± 8 | 63:37[d] | 66 ± 2 (−) |

Roelfes, G. et al., *ACIE.*, 2012, 51, 7472.

# Hydration of azachalcone



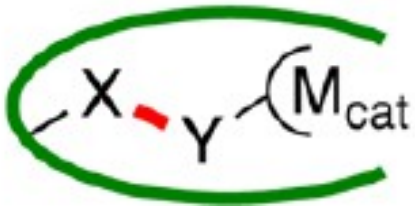




| R | LmrR variant | conv (%) | ee (%) |
|---|---|---|---|
| *i*-Pr | M89C | 67 | 77 |
| *t*-Bu | M89C | 80 | 84 |
| *n*-pent | M89C | 57 | 67 |
| *i*-Pr | M89C,D100A | 28 | <5 |

- The Roelfes group (2013) demonstrated that the same system could afford a hydration reaction with high conversions (>90%) and moderate enantioselectivities (upto 86: 14 e.r.)
- Mutagenesis studies indicated that amino acids in the inner as well as outer sphere participated in reactivity and selectivity.

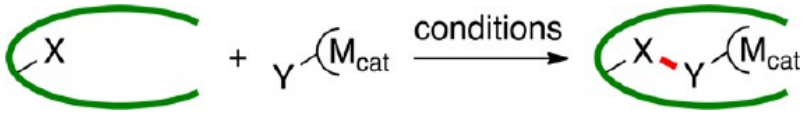
Roelfes, G. et al., *Chem Sci.,* 2013, 4, 3578.

# Key Strategies for Metal Incorporation



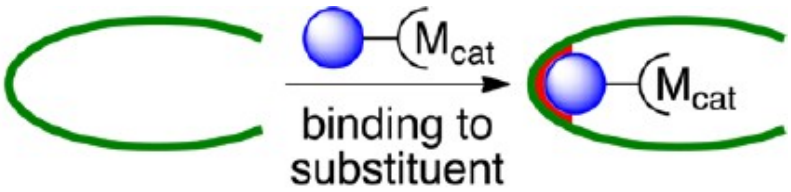

B:

Covalent attachment of metal complex via a linker.




- In general, covalent anchoring has shown limited success in producing useful reactivities.
- Examples from the Roelfes group are rare cases where appreciable selectivity and reactivity was seen.
- The factors resulting in low selectivities have been rationalized in terms of poor control over cofactor/scaffold (secondary coordination sphere) interactions, resulting from large active site volumes, linker length, and linker flexibility.

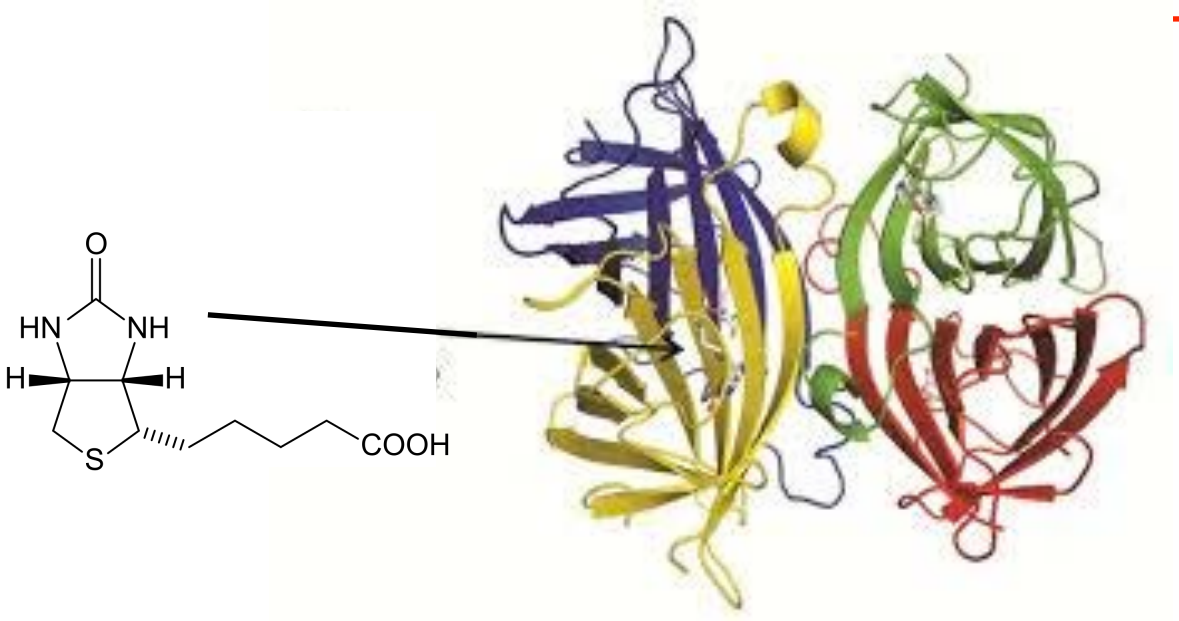
# Strategy C: Non-covalent anchoring



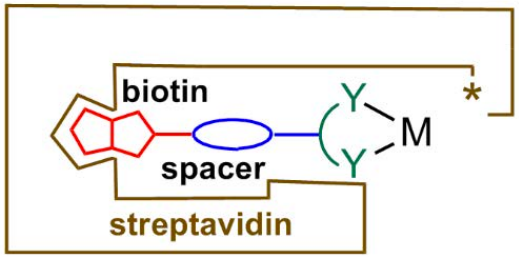

- The inherent binding capabilities of an enzyme to a ligand or cofactor can be utilized for non-covalently anchoring a metal centre.
- This eliminates the need for scaffold modification. The idea is to modify a known binding partner of the enzyme with a metal centre so as to aid its localization to the active site.
- Such an approach naturally brings a restriction on the enzyme-cofactor combinations that can be used but presumably leads to minimal structural perturbation at the attachment site.



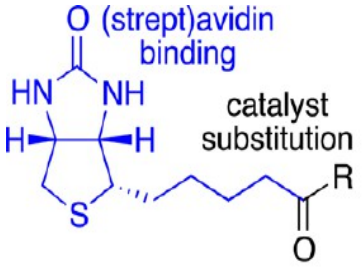
Figure adapted from *ACS Catal.,* 2013, 3, 2954.

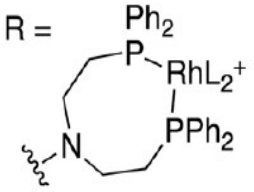



- Avidin (or streptavidin) is a protein derived from birds and amphibians which shows an extremely high affinity for biotin (vitamin B7).
- With a $K_d \sim 10^{-15}$ M, this is among the strongest non-covalent interaction known. For all practical purposes, the binding can be considered irreversible.
- The distant carboxylate can be derivatized without affecting these binding properties.

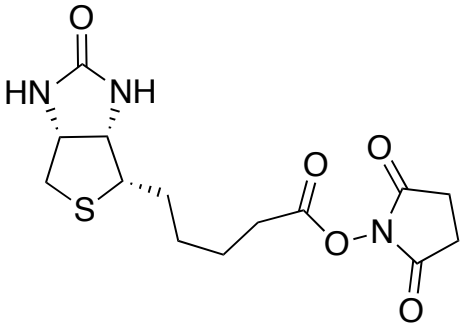


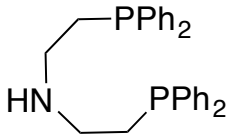

artificial metalloenzyme



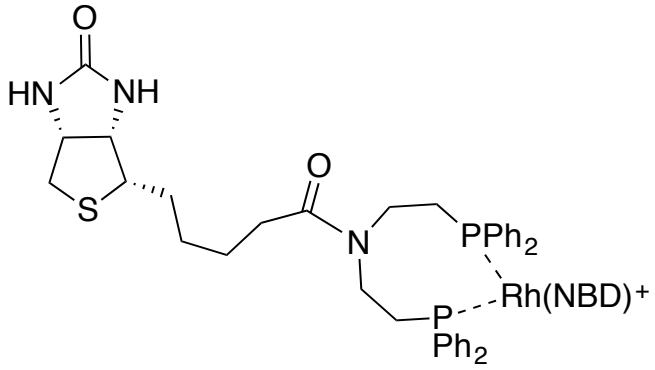

**24**, $L_2$=NBD

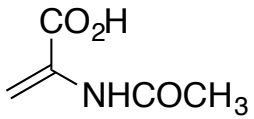



1. 


DMF, $Et_3N$
84%

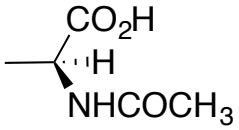
2. NBDRh(I)Tf, THF




Avidin-$RhL_2^+Tf^-$ (0.2 mol%)

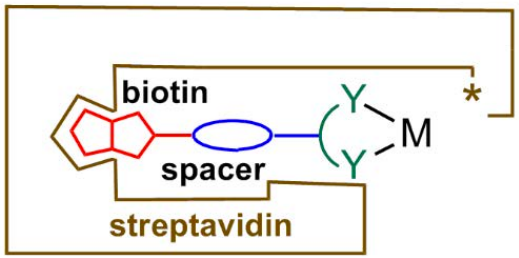
$H_2$ (1.5 atm),
Phosphate Buffer, pH 7
0 °C, 48 h



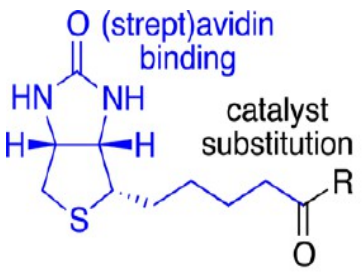

quantitative
70:30 e.r.

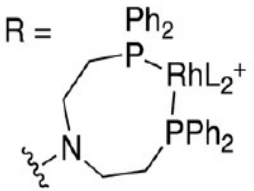


Whitesides, G. M. et al., *JACS.,* 1978, 100, 306.

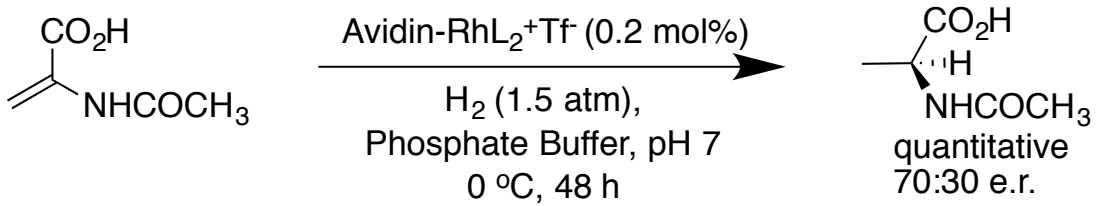



artificial metalloenzyme




**24**, $L_2$=NBD

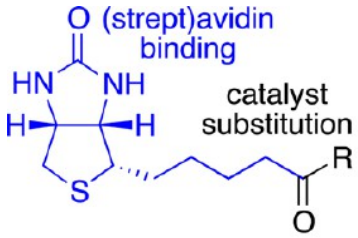


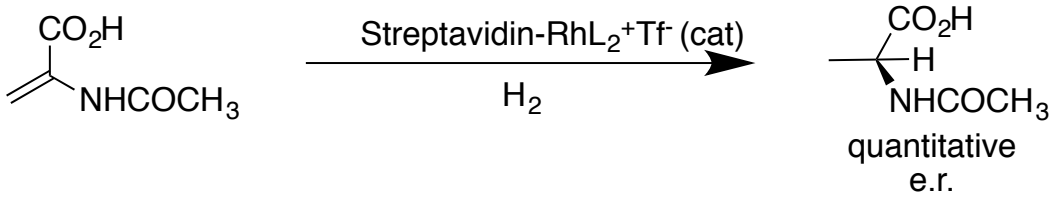

- Whitesides (1978) provided the first proof of principle example utilizing the avidin-biotin combination for design of an artificial metalloenzyme.
- A cationic Rh(I) complex, aided by the chiral environment in the enzyme pocket, provided moderate enantioselectivity for hydrogenation.
- No other substrates were tested and the exact geometrical restrains imposed by the protein scaffold were not analyzed.



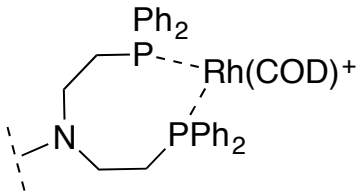
Whitesides, G. M. et al., *JACS.,* 1978, 100, 306.

# Improvements in Hydrogenation

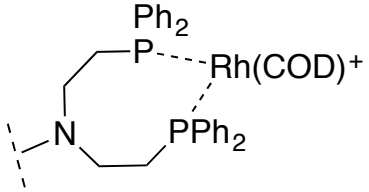


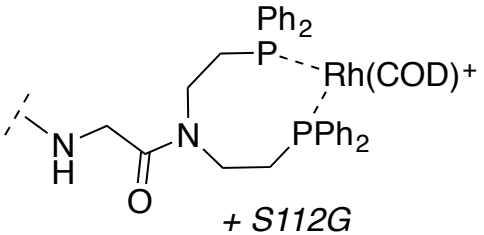

Catalysts and corresponding selectivities:




96:4 e.r.
R-isomer
*Ward (2003)*

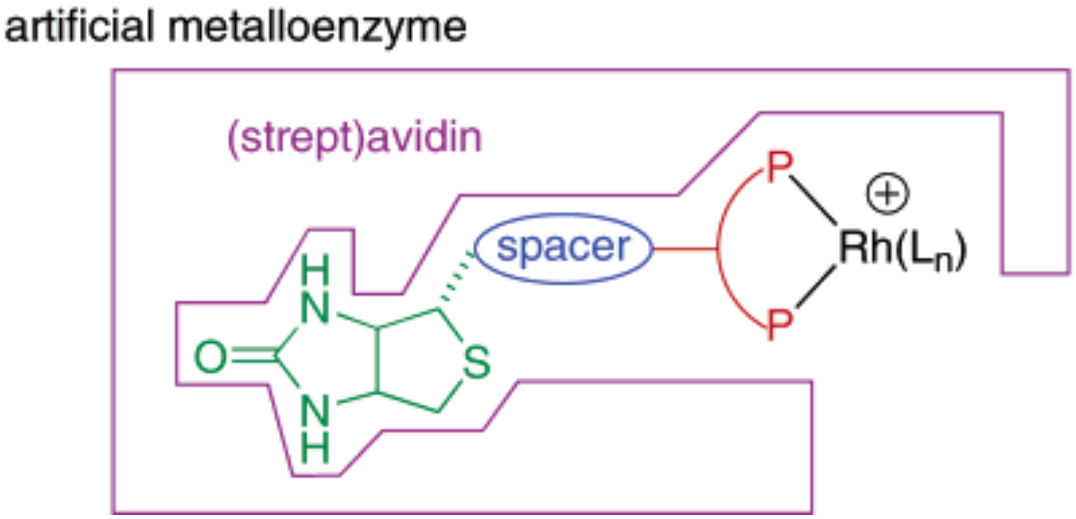



*+ S112G*
98:2 e.r.
R-isomer
*Ward (2004)*




*+ S112G*
90:10 e.r.
S-isomer
*Ward (2007)*

- Ward and coworkers made significant improvements in selectivity by modifying the ligand, linker length and amino acids proximal to the metal centre.

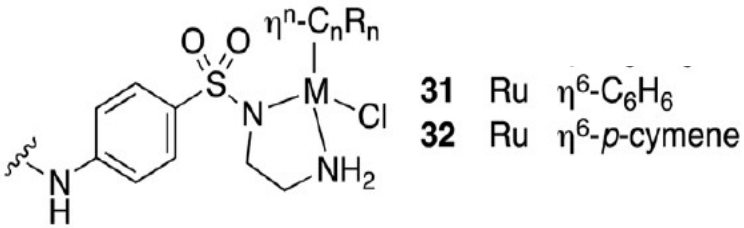


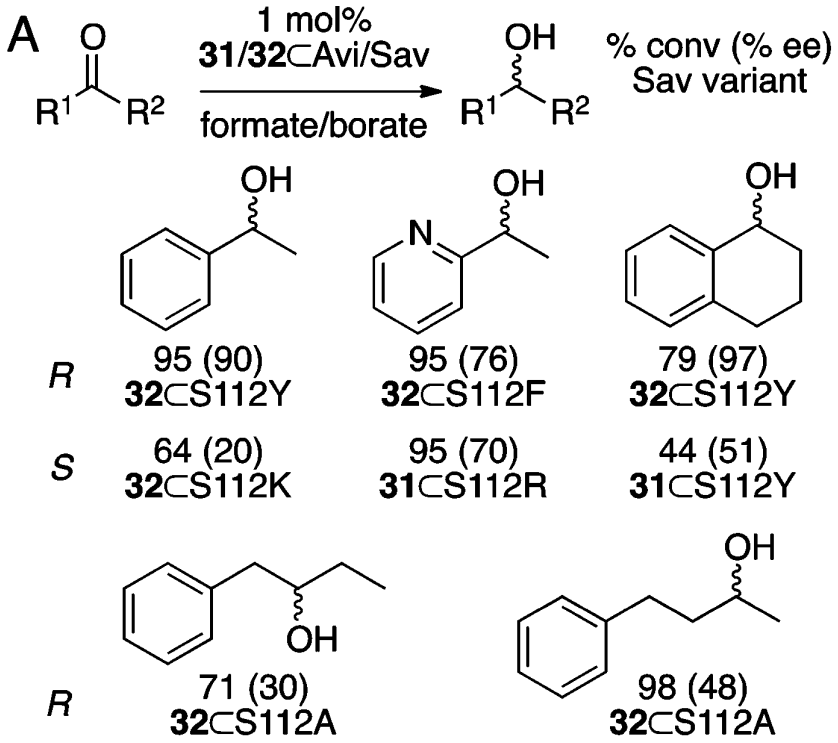

- Three parameters can be varied for systematic screening of candidate enzymes:

1. Ligand on the metal
2. Spacer between metal/biotin
3. Amino acid mutations in the metal vicinity.

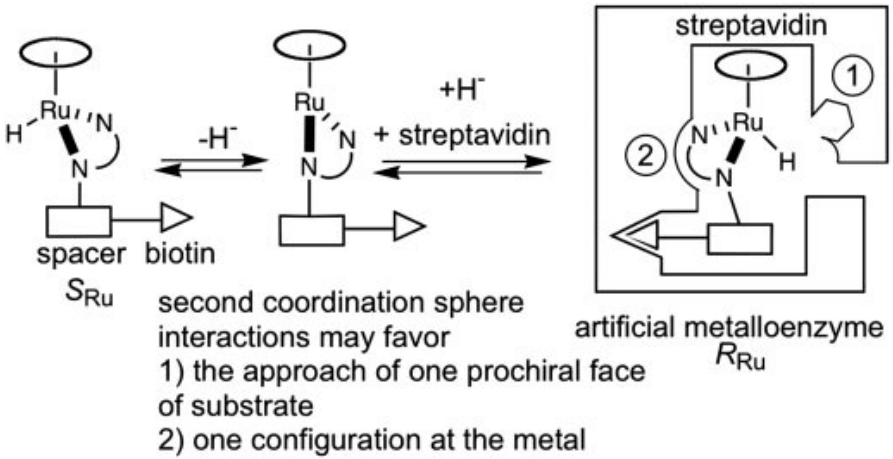
- The Ward group systematically explored the resulting 'chemogenetic' space to come up with successful hits.

Ward, T. R. et al., *JACS.,* 2004, 126, 14411.

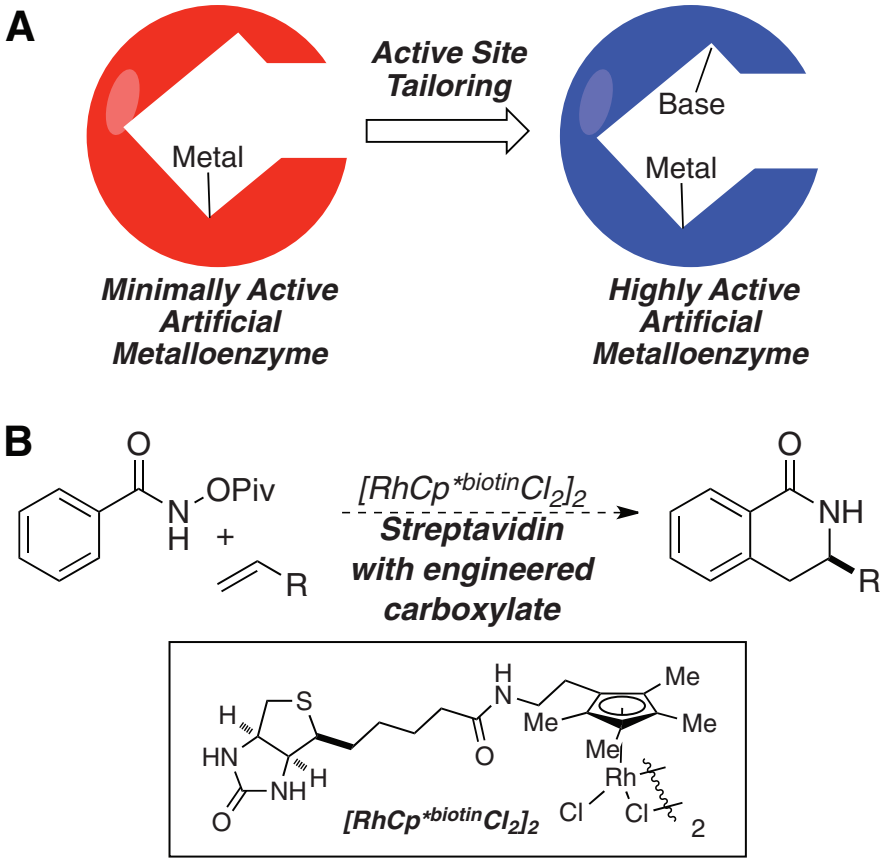



- The Ward group (2005) then extended their reaction discovery methodology to transfer hydrogenation using Ru(II) catalysts.
- A number of alkyl-aryl and alkyl-alkyl ketones were reduced in good yields and good to excellent enantioselectivities.

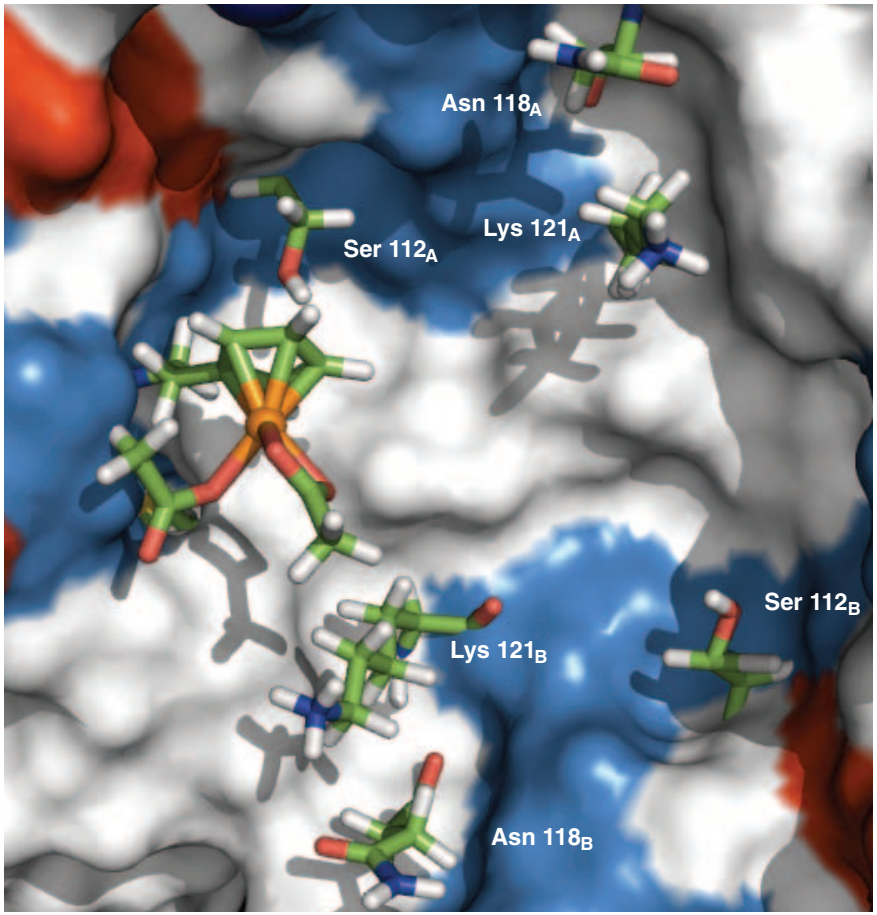


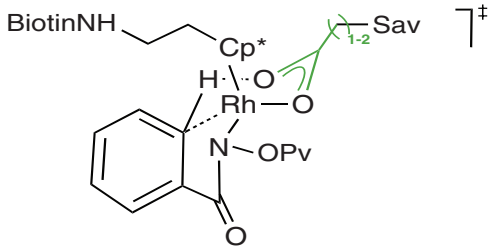

Ward, T. R. et al., *PNAS.,* 2005, 102, 4683.




- The Ward group (2012) demonstrated how rational tuning of the protein microenvironment can enable design of a catalytic asymmetric C-H activation reaction which had no methodological counterpart in mainstream organic synthesis.

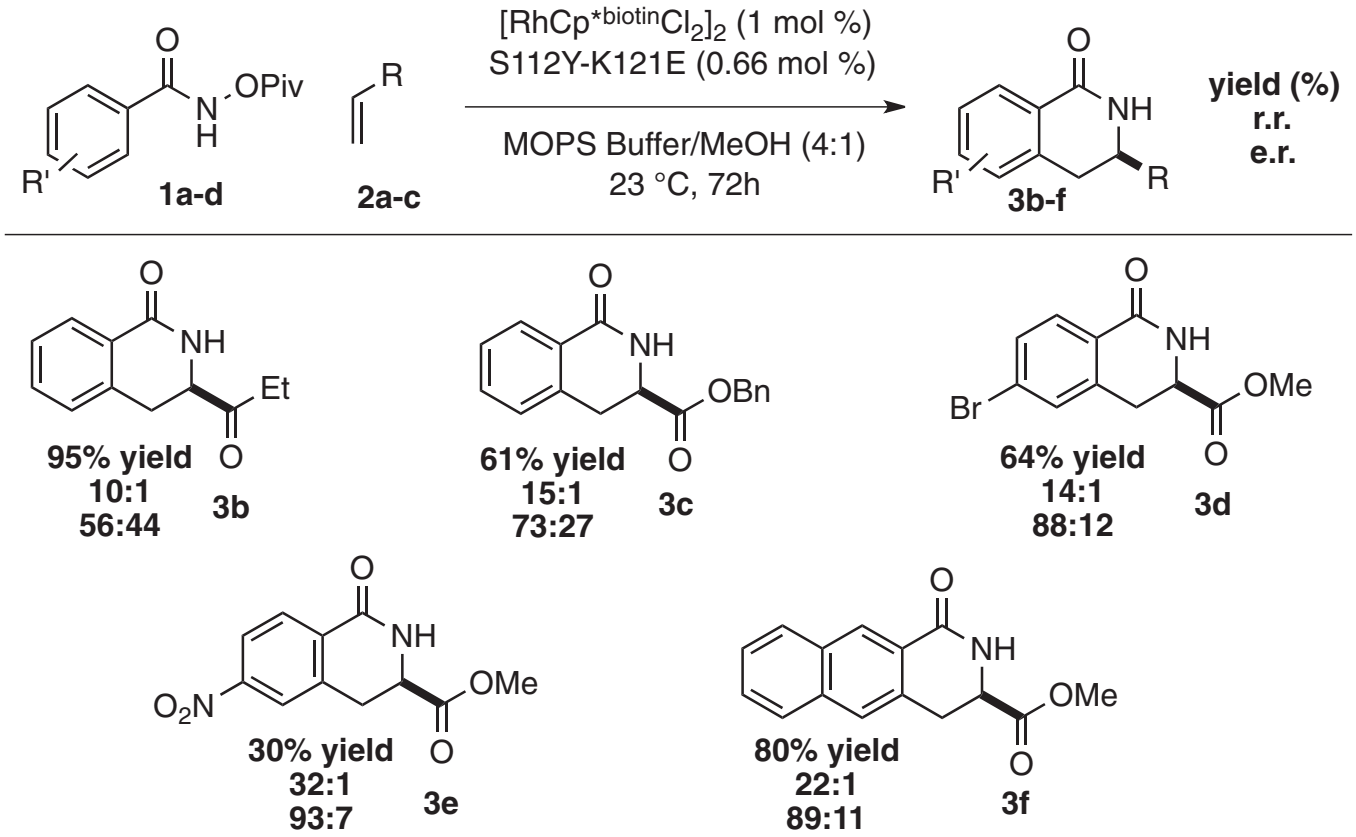
Ward, T. R. et al., *Science.,* 2012, 338, 500.




- Lys121 was mutated to glutamic acid to provide a proximal carboxylate.
- Further fine tuning by S112Y mutation provided a superior metalloenzyme.

Ward, T. R. et al., *Science.,* 2012, 338, 500.

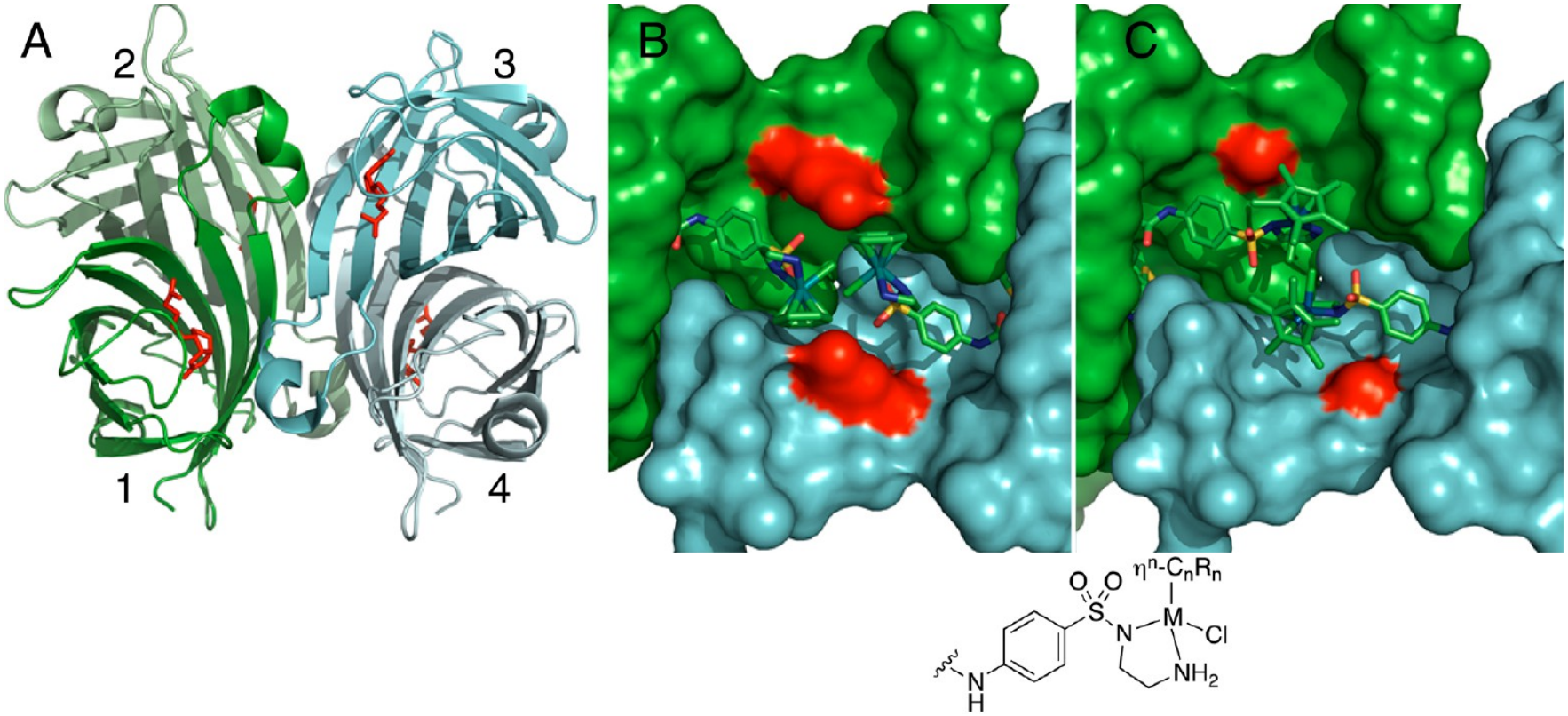



- The proximal carboxylate and the specific microenvironment assist in a 100 fold higher rate than the background benzannulation.

- Convincing demonstration of the potential of a tunable protein scaffold in affecting reactivity.

Ward, T. R. et al., *Science.,* 2012, 338, 500.

# Insights into Sav Binding

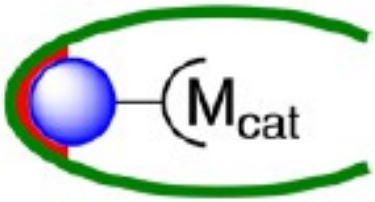



- The Sav quaternary structure is a dimer of dimers in which the biotin binding pockets from alternating pairs face each other. Biotin binds deep within these pockets.
- The metal complexes are solvent exposed and lie in a shallow cleft at the dimer interface.
- The metal complexes project from the scaffold via only a couple of rotatable bonds.



Ward, T. R. et al., *ACIE.,* 2011, 50, 3026.

# Key Strategies for Metal Incorporation




C:

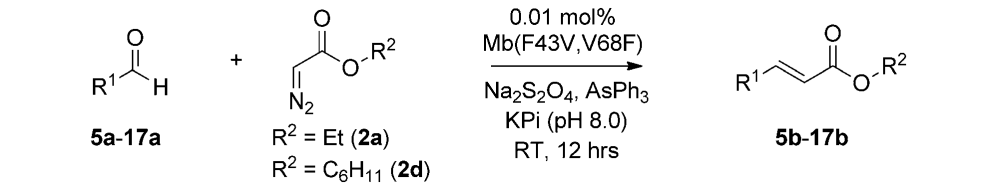
Non-Covalent attachment of a metal complex.

- The Ward group has systematically explored the Sav-Biotin platform as a new paradigm in metalloenzyme scaffold development.
- Biotin incorporation in the organometallic structure is generally not synthetically challenging.
- The Sav binding has been well characterized and structural data can help in rational engineering of the active site microenvironment.

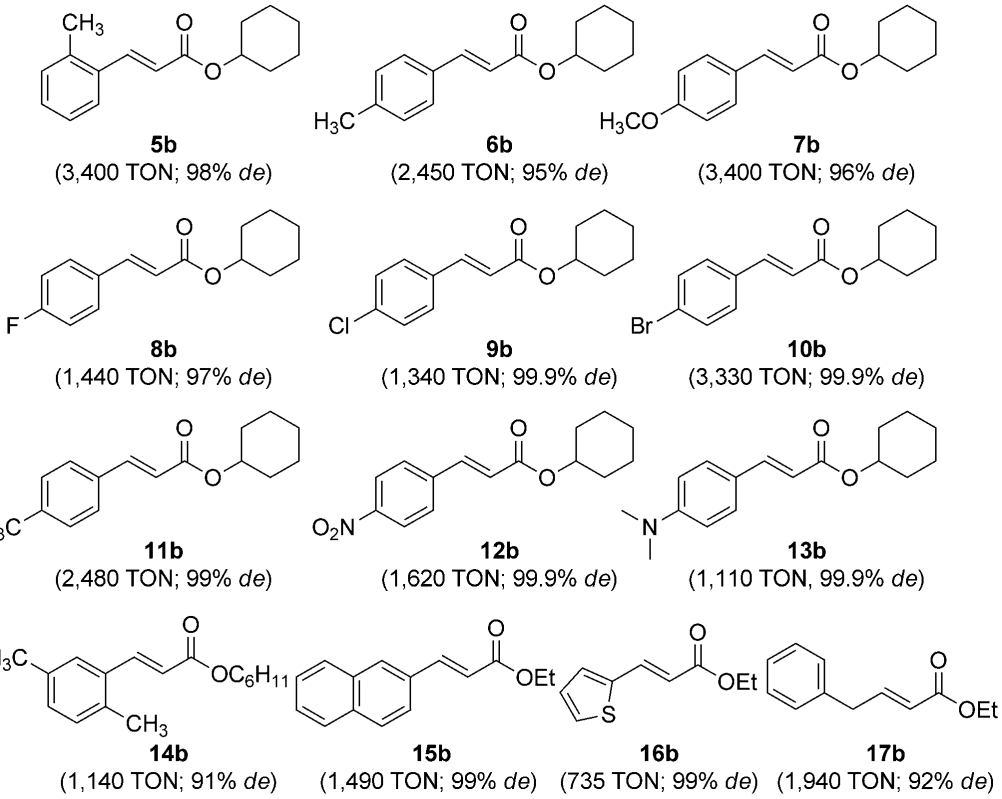
# Other Related Examples

## Myoglobin-Catalyzed Olefination of Aldehydes

*Vikas Tyagi and Rudi Fasan** *Angew. Chem.* **2016**, *128*, 2558 –2562




**5a-17a** $R^2$ = Et (**2a**), $R^2$ = $C_6H_{11}$ (**2d**) **5b-17b**



**5b** (3,400 TON; 98% *de*)

**6b** (2,450 TON; 95% *de*)

**7b** (3,400 TON; 96% *de*)

**8b** (1,440 TON; 97% *de*)

**9b** (1,340 TON; 99.9% *de*)

**10b** (3,330 TON; 99.9% *de*)

**11b** (2,480 TON; 99% *de*)

**12b** (1,620 TON; 99.9% *de*)

**13b** (1,110 TON, 99.9% *de*)

**14b** (1,140 TON; 91% *de*)

**15b** (1,490 TON; 99% *de*)

**16b** (735 TON; 99% *de*)

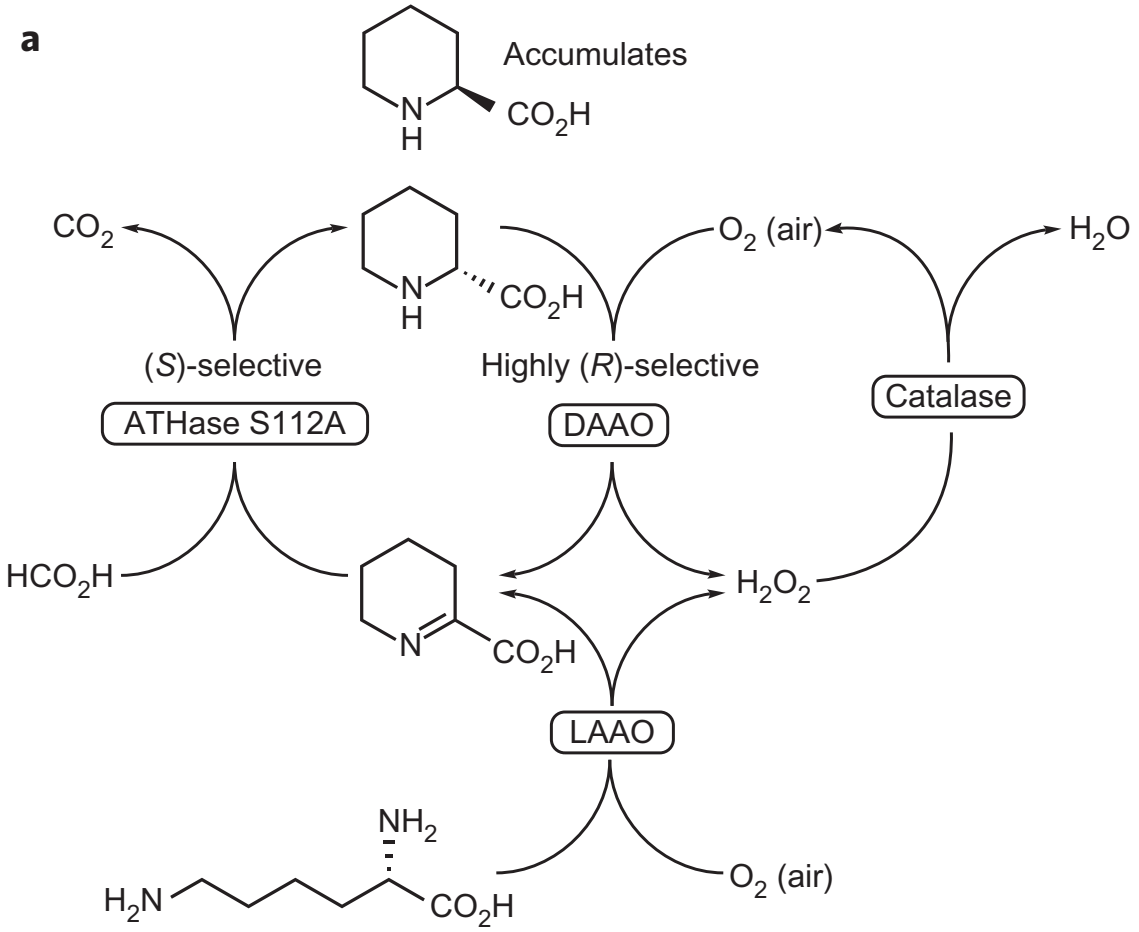
**17b** (1,940 TON; 92% *de*)

nature chemistry
ARTICLES
PUBLISHED ONLINE: 25 NOVEMBER 2012 | DOI: 10.1038/NCHEM.1498



# Synthetic cascades are enabled by combining biocatalysts with artificial metalloenzymes

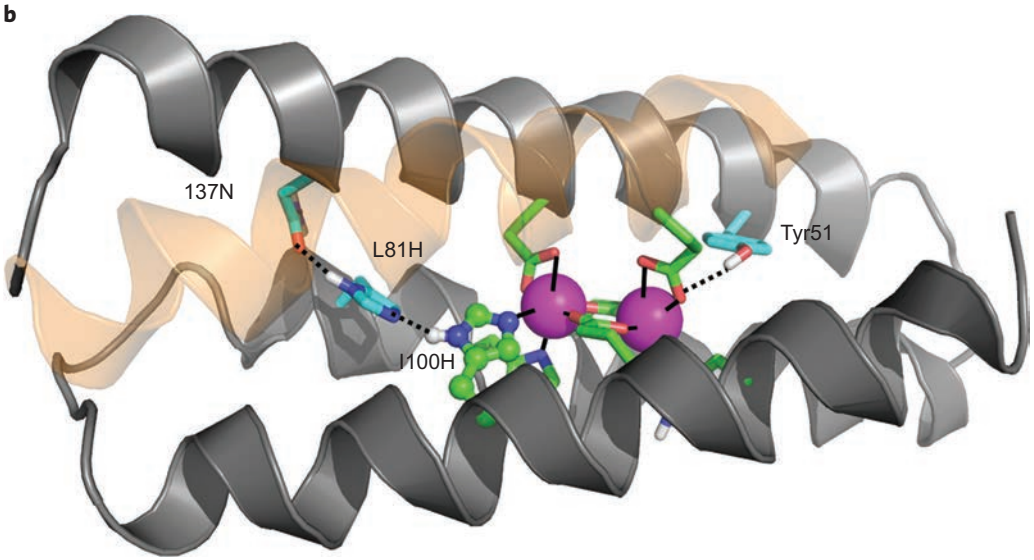
V. Köhler[1], Y. M. Wilson[1], M. Dürrenberger[1], D. Ghislieri[2], E. Churakova[3], T. Quinto[1], L. Knörr[1], D. Häussinger[1], F. Hollmann[3]*, N. J. Turner[2]* and T. R. Ward[1]*

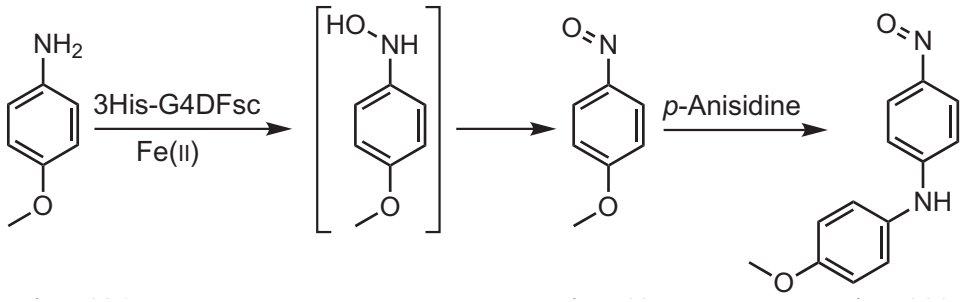


ARTICLES
PUBLISHED ONLINE: 23 SEPTEMBER 2012 | DOI: 10.1038/NCHEM.1454
nature chemistry

# Alteration of the oxygen-dependent reactivity of *de novo* Due Ferri proteins

Amanda J. Reig[1,2]*, Marcos M. Pires[1†], Rae Ana Snyder[3], Yibing Wu[1,4†], Hyunil Jo[1†], Daniel W. Kulp[1†], Susan E. Butch[2], Jennifer R. Calhoun[1], Thomas Szyperski[4,5], Edward I. Solomon[3] and William F. DeGrado[1†]*

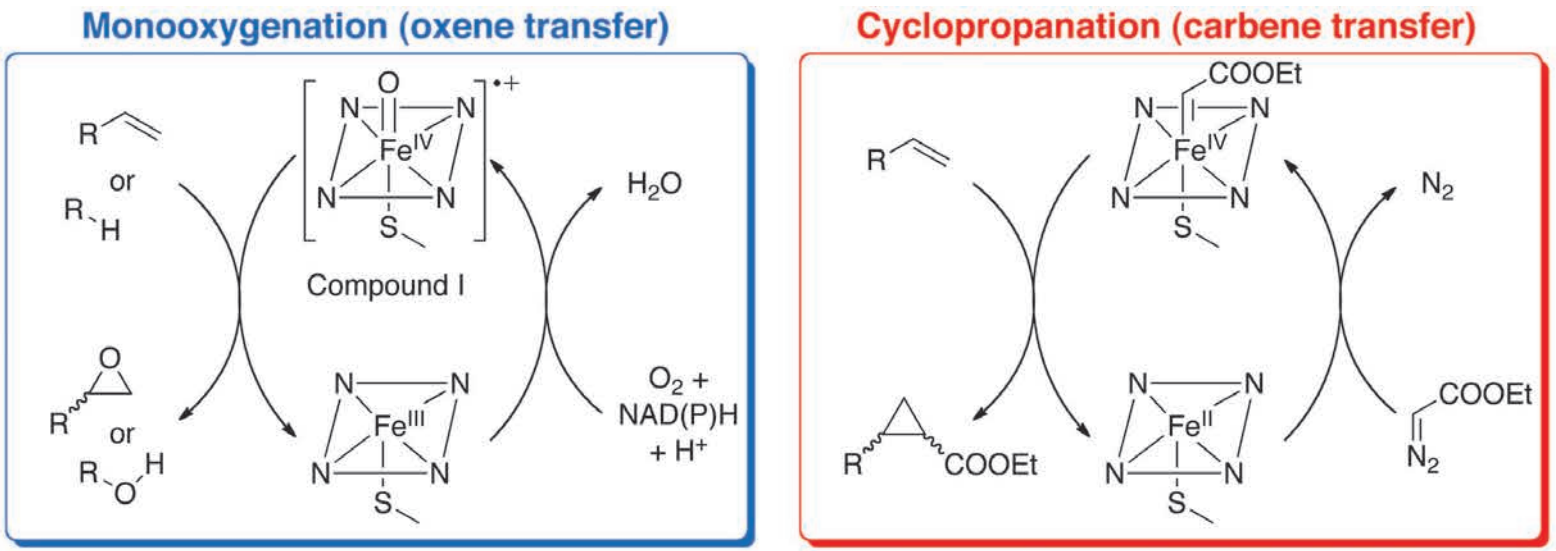


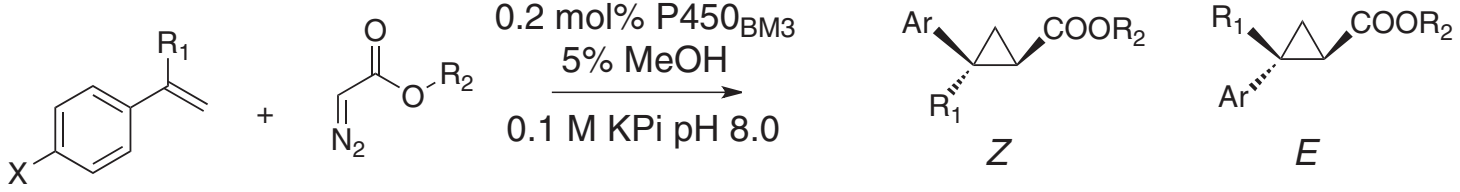



# Olefin Cyclopropanation via Carbene Transfer Catalyzed by Engineered Cytochrome P450 Enzymes

Pedro S. Coelho,[1]* Eric M. Brustad,[2]* Arvind Kannan,[1] Frances H. Arnold[1]†

www.sciencemag.org SCIENCE VOL 339 18 JANUARY 2013



# Summary and Outlook

- Protein scaffolds provide a method to introduce a well defined 2$^{nd}$ coordination sphere around an organometallic complex.
- The resulting artificial metalloenzyme has the promise to combine metal reactivity with enzyme selectivity under mild reaction conditions.
- Three major anchoring strategies have been used to introduce the organometallic motif in the enzyme active site.
- Protein engineering methods can enable precise tailoring of the active site microenvironment.
- The overall development pipeline is highly interdisciplinary and borrows from expertise in synthesis, molecular biology, protein structure and computational modeling.

# Summary and Outlook

- The Sav-biotin system has been identified as a platform amenable to metalloenzyme design. Thousands of protein systems exist in nature which have the potential to be even better suited for these applications.

- Advances in protein engineering technologies and directed evolution methods will aid the field in the future.

- Computational approaches to identify/predict lead structures is expected to play a crucial role going forward.

- A collaborative effort among synthetic chemists and molecular biologists is necessary to effectively utilize the specialized expertise needed from both fields.

- Impressive lead results have been obtained in this decade and methods to systematically screen for successful catalysts have been articulated. With more researchers entering the field, the full potential of the artificial metalloenzyme approach seems to have a promising future.

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