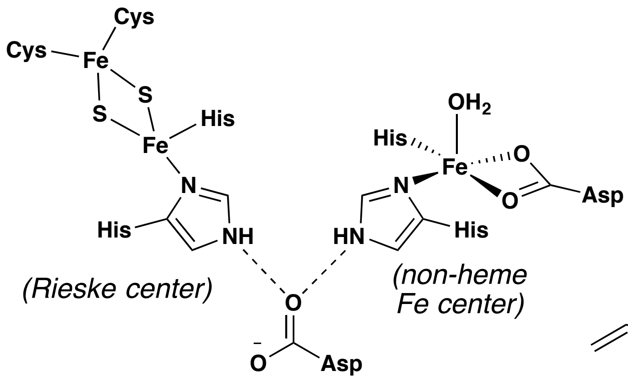
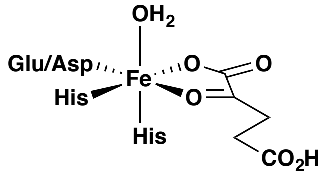
Natural and Artificial Metalloenzymes

Cofactor diversity of natural metalloenzymes

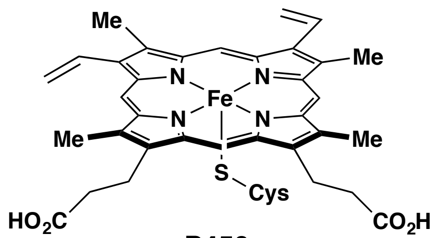
Oxygenases



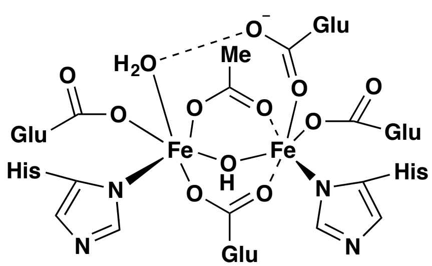
Rieske oxygenases



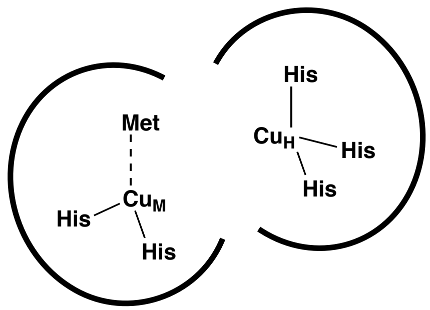
Non-heme dioxygenases



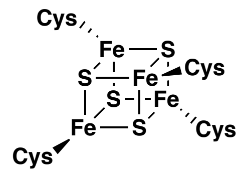
P450s



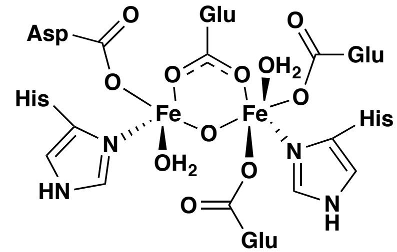
Methane monooxygenases



Cu-dependent monooxygenases

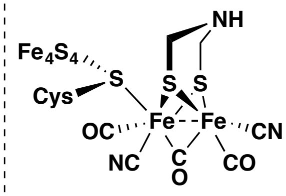


[4Fe4S] cluster

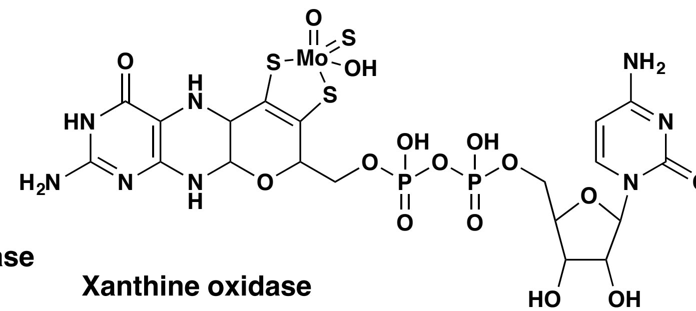


Ribonucleotide reductase

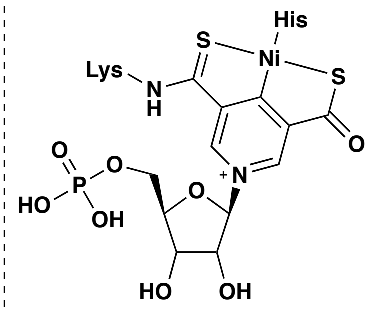
More exotic cofactors



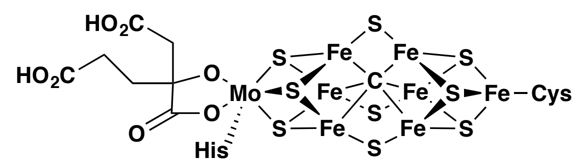
FeFe hydrogenase



Xanthine oxidase



Ni pincer lactate racemase



FeMoco nitrogenase

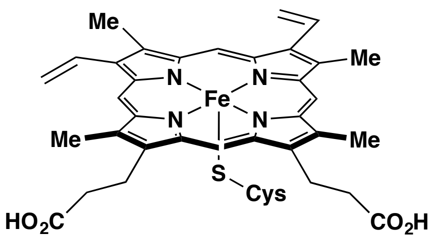
Definition:

Monooxygenase – only one oxygen atom from O_2 is incorporated into the substrate, the other being reduced to H_2O

Dioxygenase – both oxygen atoms are incorporated into the substrate(s)

Natural and Artificial Metalloenzymes

The P450s

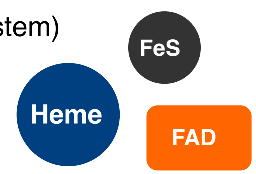


- Presence of heme (protoporphyrin IX) cofactor
- Axial Cys ligation
- Characteristic Soret peak at 450 nm for ferrous-CO complex

Different domain organizations of P450

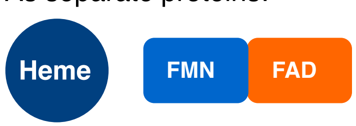
Trends Biotechnol. **2012**, *30*, 26; *Biochim. Biophys. Acta* **2007**, *1770*, 330; *Trends Biochem. Sci.* **2013**, *38*, 140

Class I (three-protein system)



Class II (FAD- and FMN-containing reductase)

As separate proteins:



Reductase

Fused (e.g. P450-BM3):

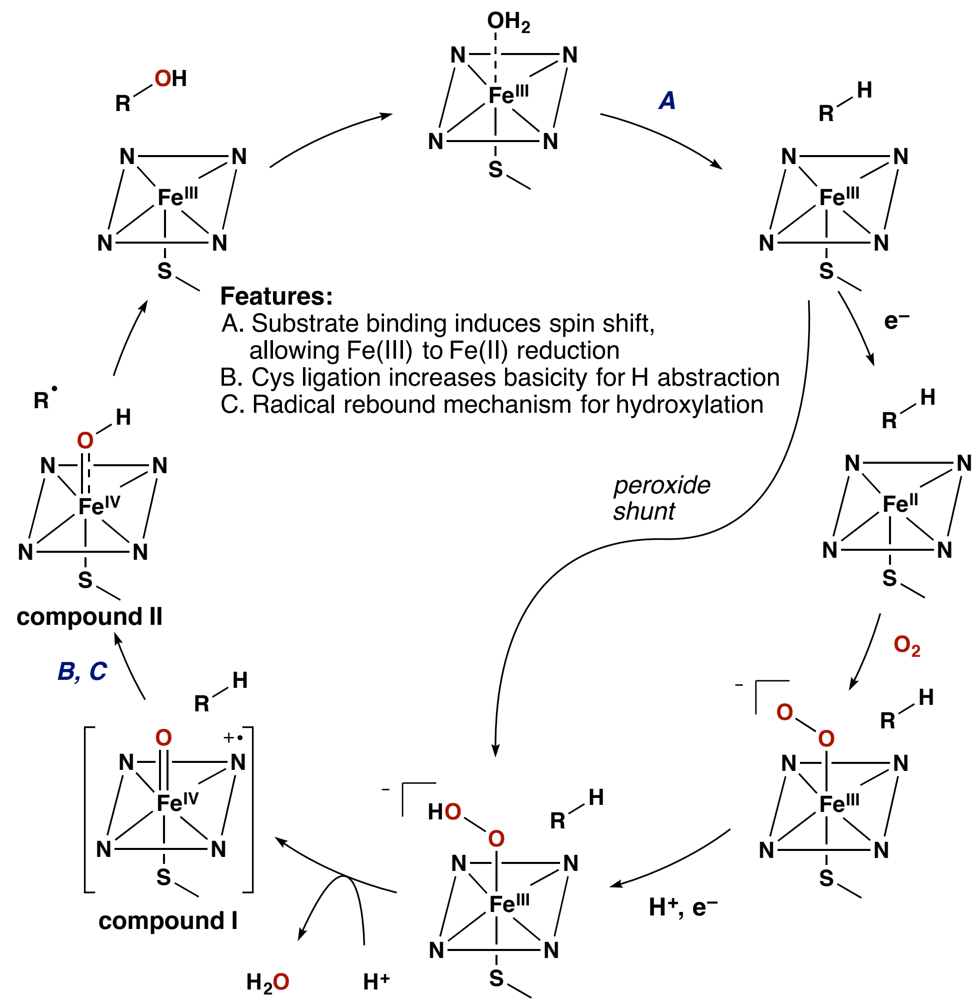
Terminology

FAD domain: flavin adenine dinucleotide binding domain
FMN domain: flavin mononucleotide binding domain

New electron transfer chain mechanisms have recently been discovered in P450s

Catalytic cycle of P450 hydroxylation

Chem. Rev. **2004**, *104*, 3947

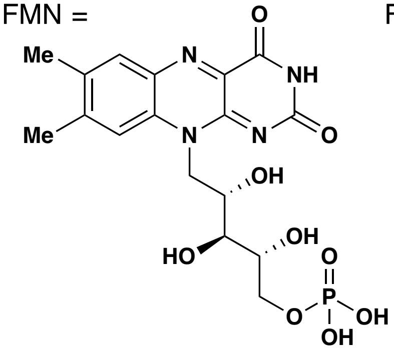


Compound I basicity: *Science* **2004**, *304*, 1653
Compound I characterization: *Science* **2010**, *330*, 933
Radical rebound overview: *Eur. J. Inorg. Chem.* **2004**, 207

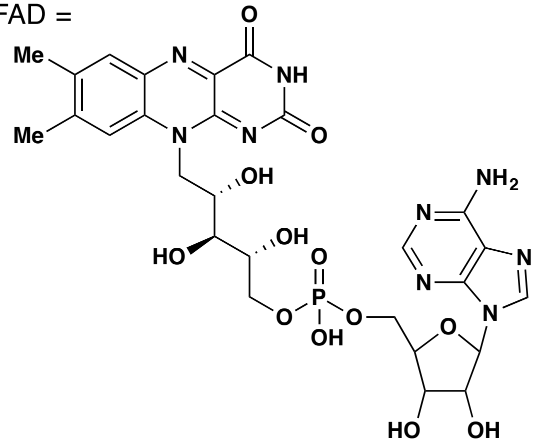
Natural and Artificial Metalloenzymes

Electron transport chain

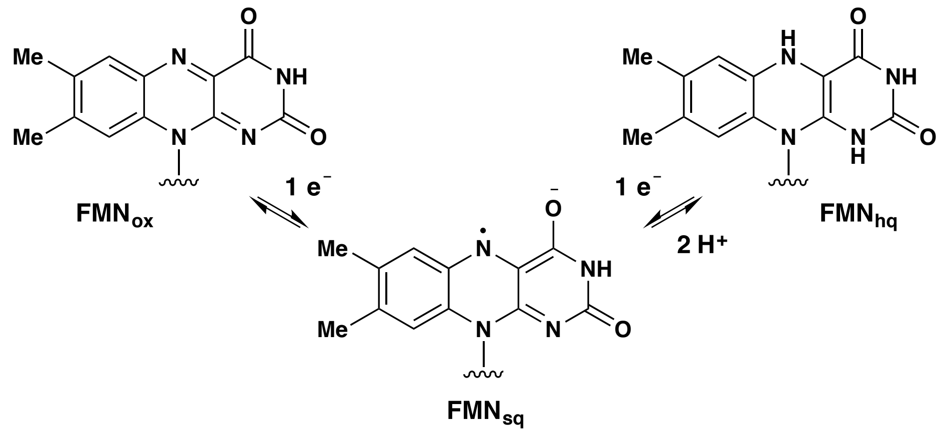
FMN =



FAD =

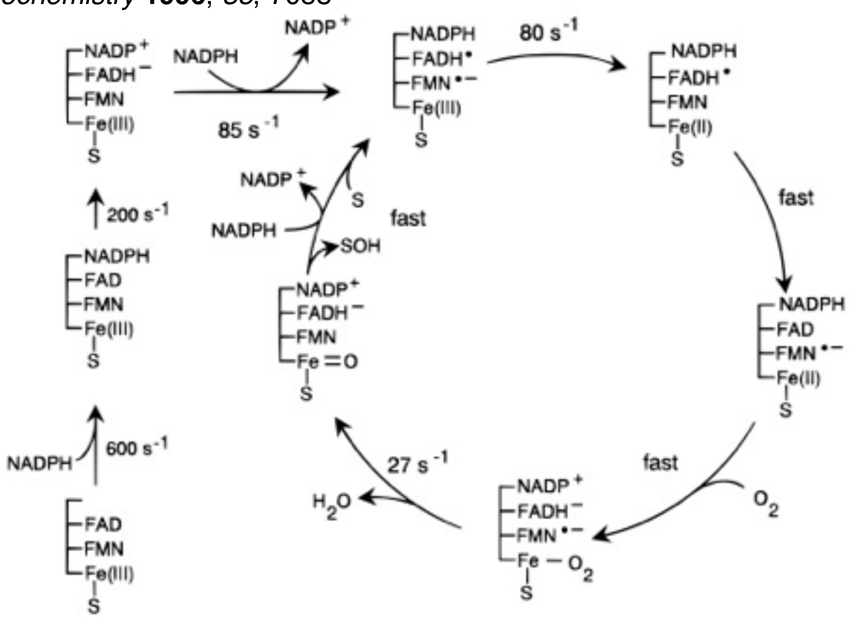


Reduced flavin species

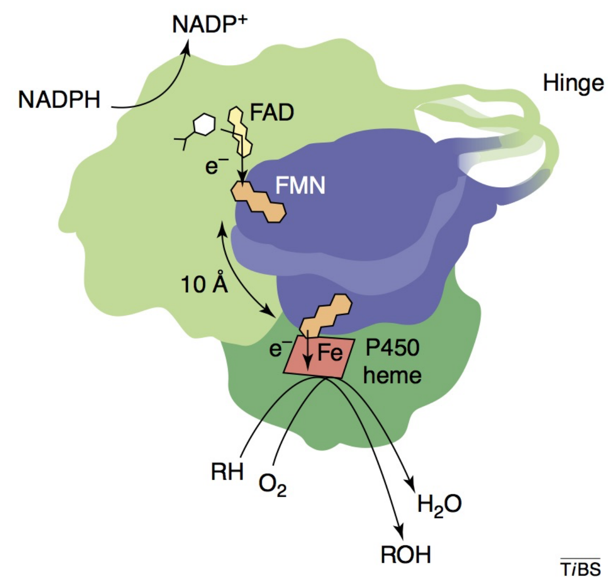


Electron transfer cycle in P450BM3

Biochemistry **1996**, *35*, 7058



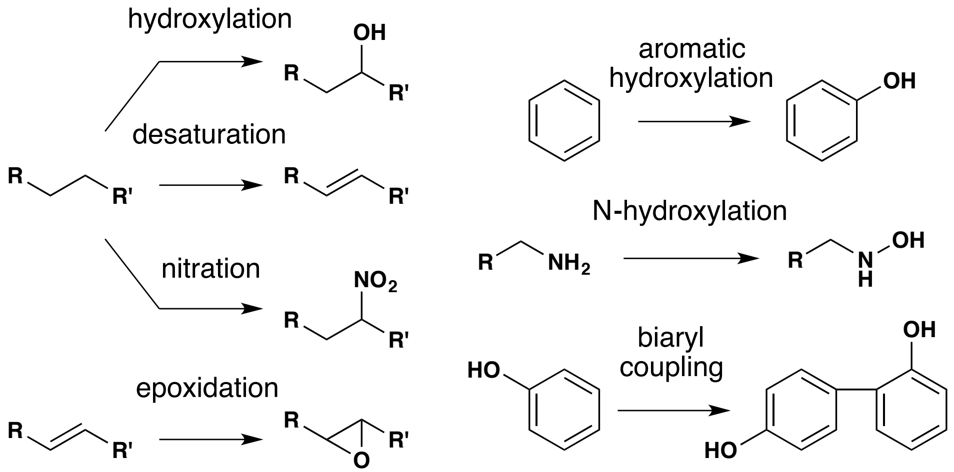
Protein dynamics of electron transfer



Trends Biochem. Sci. **2002**, *27*, 250

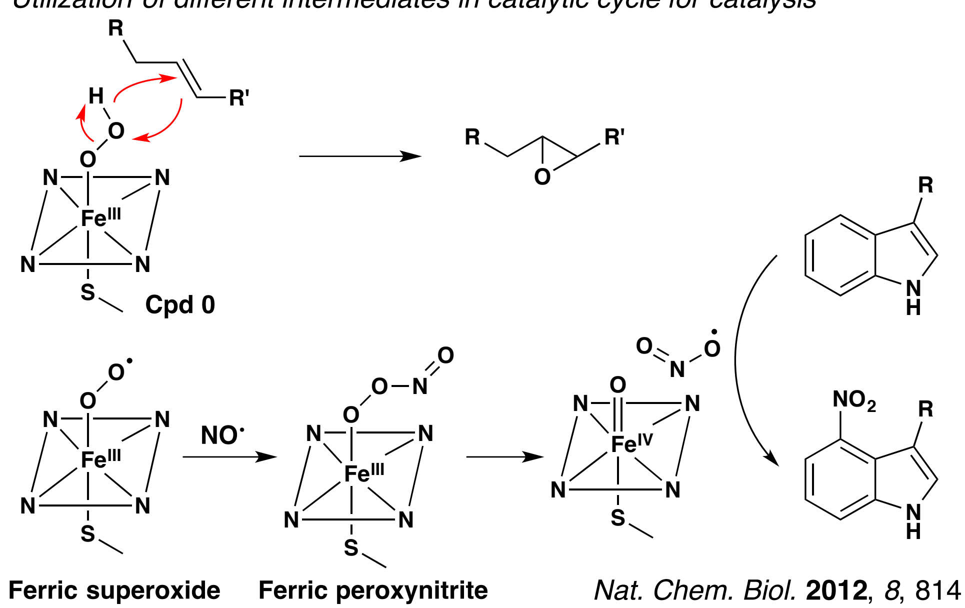
Natural and Artificial Metalloenzymes

Catalytic diversity of P450s



Nat. Prod. Rep. **2012**, *29*, 1251
Nat. Prod. Rep. **2017**, *34*, 1141

Utilization of different intermediates in catalytic cycle for catalysis

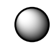


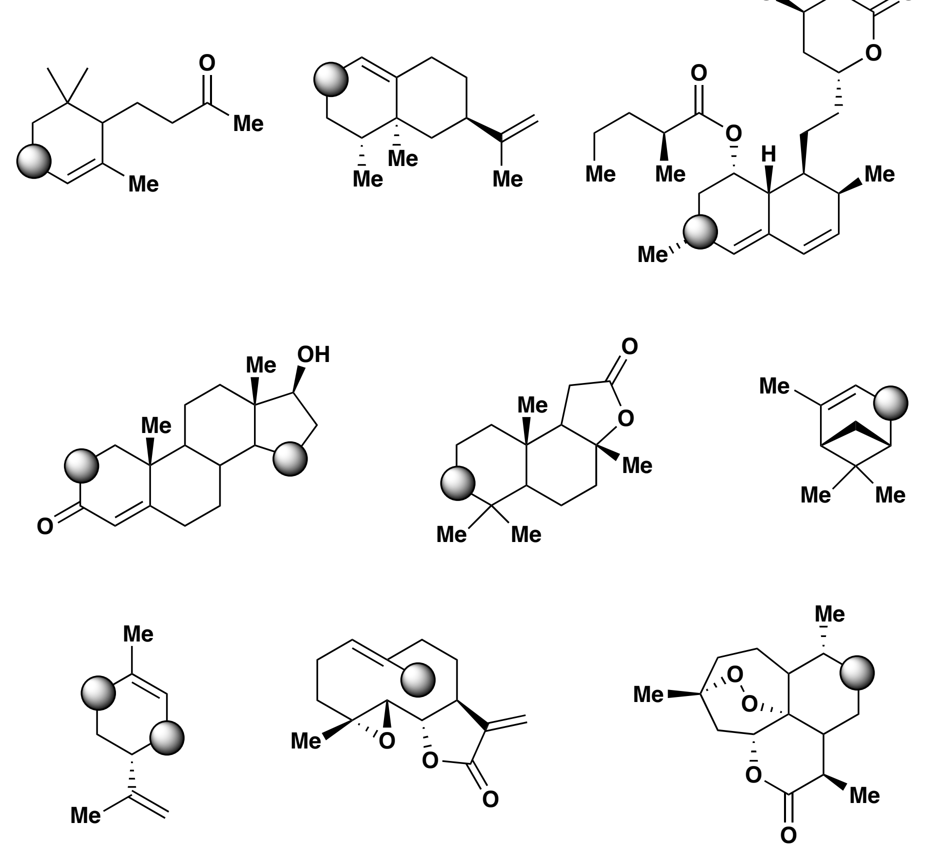
Nat. Chem. Biol. **2012**, *8*, 814

P450-BM3 (CYP102A1)

- Has been extensively studied due to the "fused" nature of the protein
- Native activity: long-chain fatty acid hydroxylase

Examples of site-selective hydroxylation by P450-BM3 variants

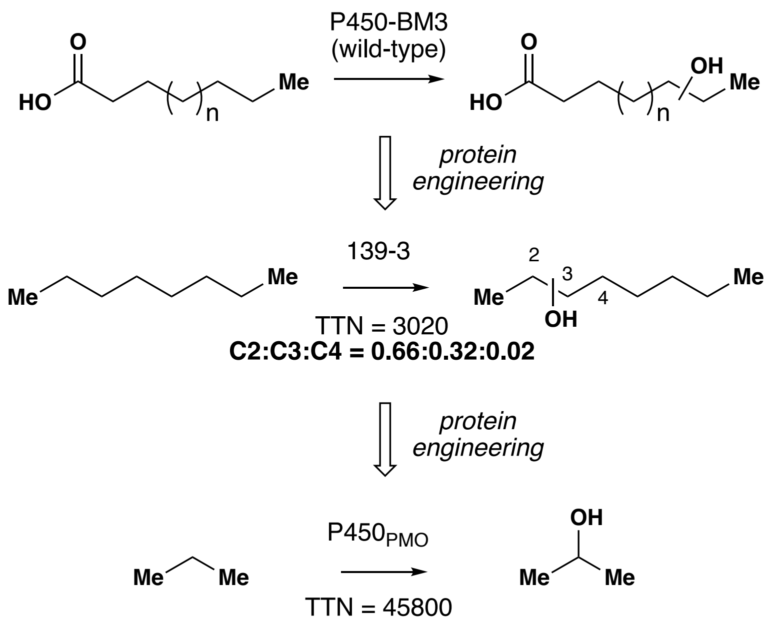
 = site of oxidation



Chem. Soc. Rev. **2012**, *41*, 1218

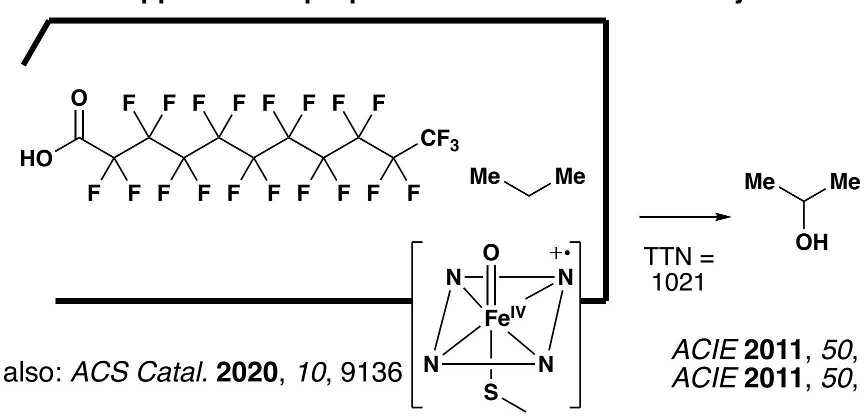
Natural and Artificial Metalloenzymes

Altering the site-selectivity of BM3 oxidation by enzyme engineering



Nature Biotechnol. **2002**, *20*, 1135
JACS **2003**, *125*, 13442
ACIE **2007**, *46*, 8414

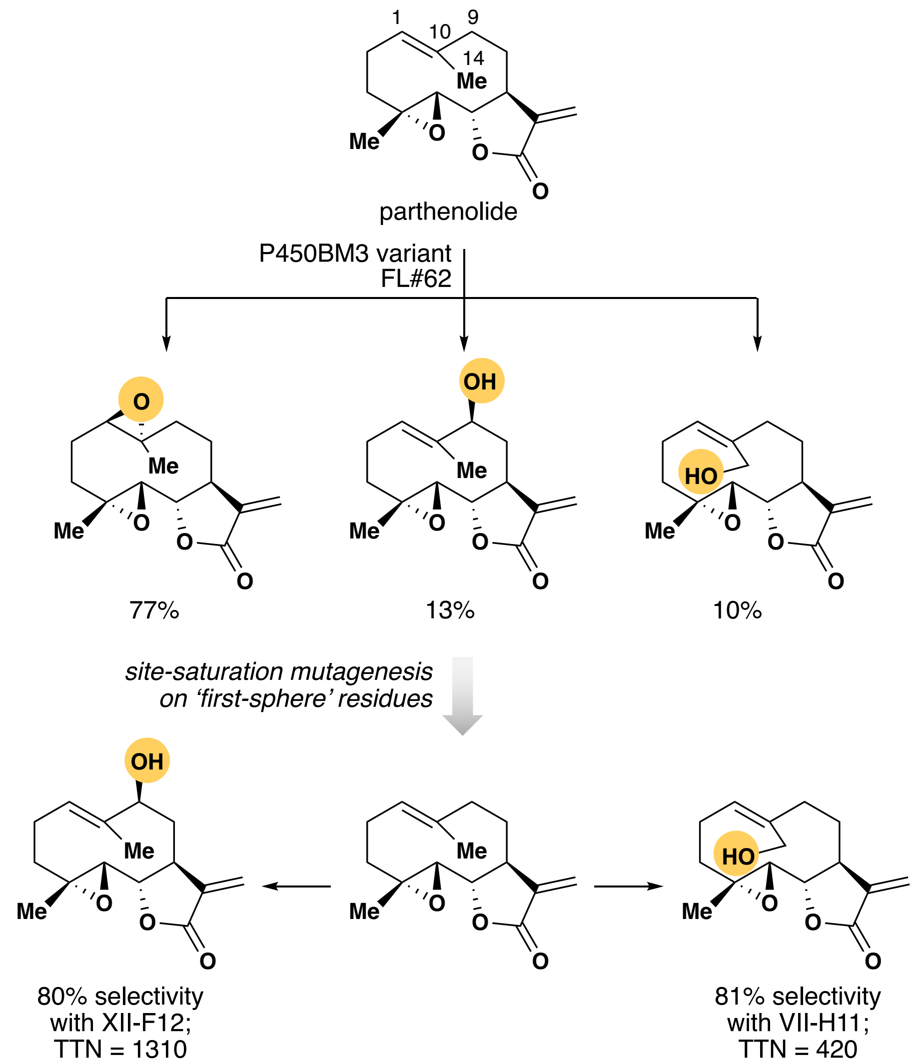
Alternative approach for propane oxidation – use of decoy



See also: *ACS Catal.* **2020**, *10*, 9136

ACIE **2011**, *50*, 2720
ACIE **2011**, *50*, 5315

Application in terpene oxidation

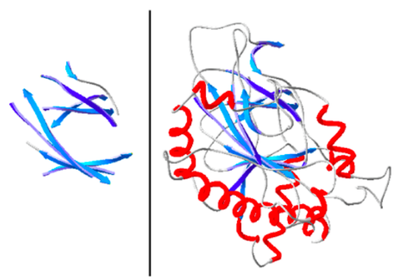


Bioorg. Med. Chem. **2018**, *26*, 1365
Bioorg. Med. Chem. **2016**, *24*, 3876
ACS Chem. Biol. **2014**, *9*, 164

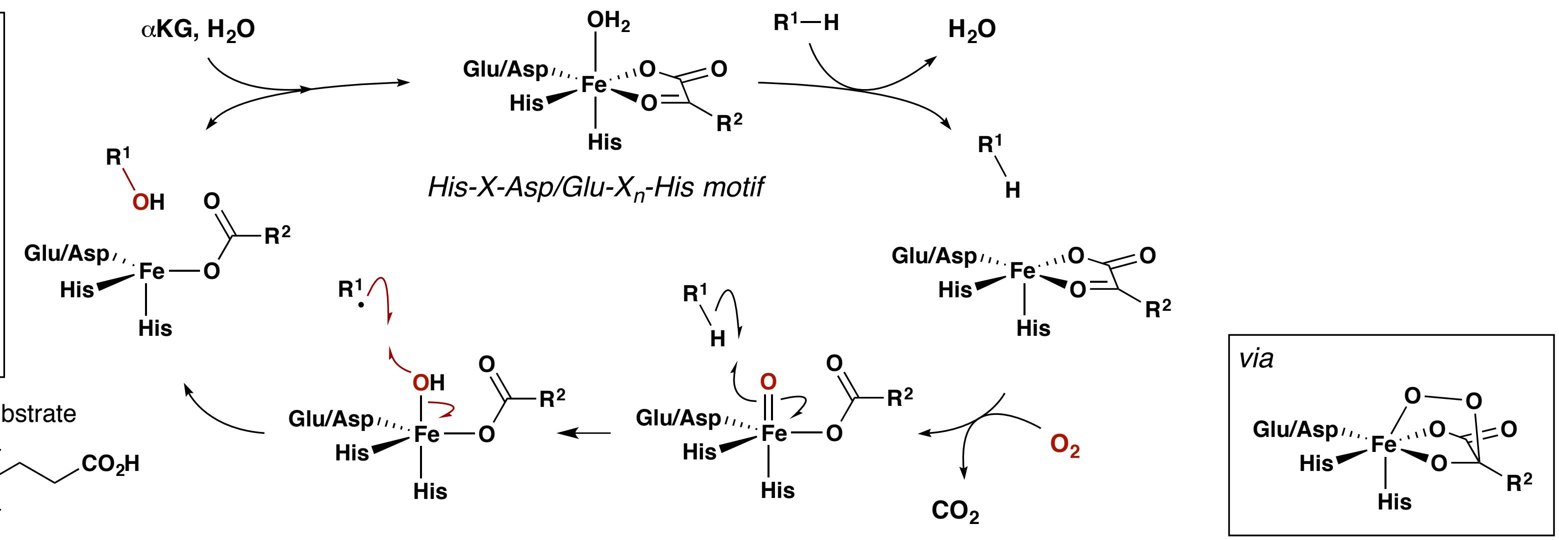
Natural and Artificial Metalloenzymes

Fe-αketoglutarate (Fe-αKG) dioxygenase

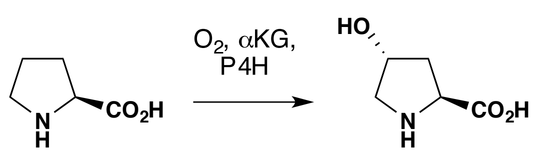
Crit. Rev. Biochem. Mol. Biol. **2004**, *39*, 21



Conserved jellyroll fold among Fe-αKGs

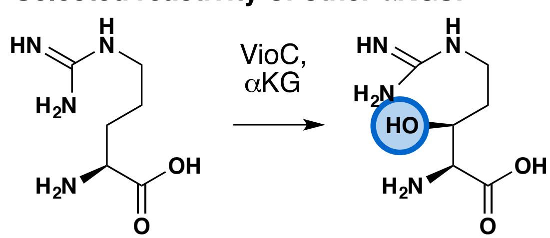


First discovery of Fe-αKG: Prolyl 4-hydroxylase

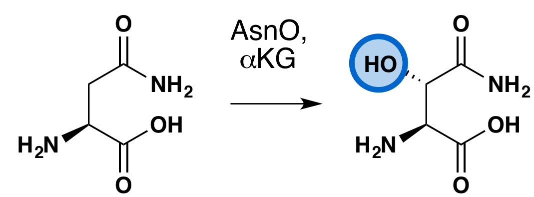


Biochem. Biophys. Res. Commun. **1966**, *24*, 179

Selected reactivity of other αKGs:



L-arginine

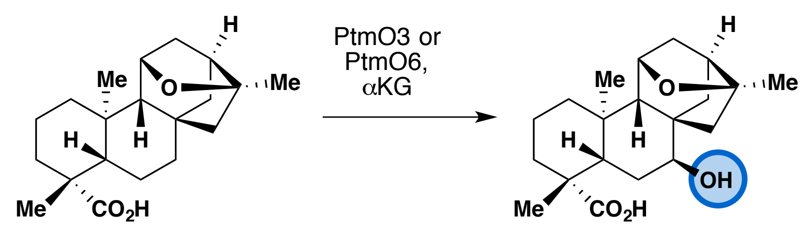


L-asparagine

FEBS J. **2009**, *276*, 3669

ACS Chem Biol. **2007**, *2*, 187

Plant Mol. Biol. **1997**, *34*, 935

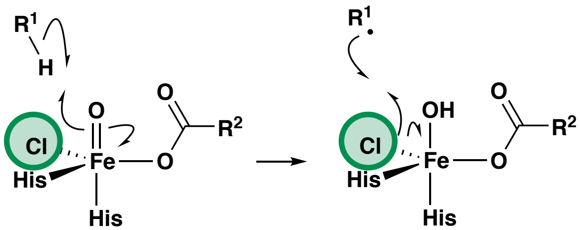


J. Am. Chem. Soc. **2019**, *141*, 4043

Natural and Artificial Metalloenzymes

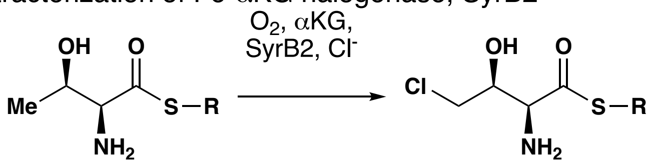
Fe-αKG halogenases

In Fe-αKG halogenases, the carboxylate ligand is replaced by a halide:

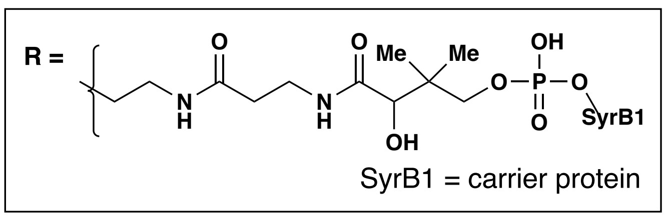


Chem. Rev. **2006**, *106*, 3364

First characterization of Fe-αKG halogenase, SyrB2



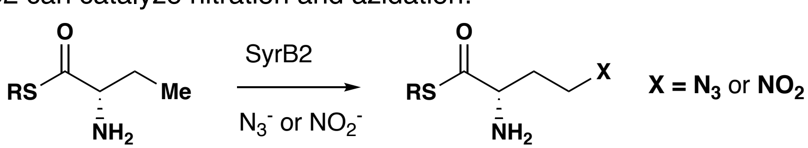
Threonine-SyrB1



Note: Norvaline-SyrB1 gives primarily hydroxylation *PNAS* **2005**, *102*, 10111
PNAS **2009**, 106, 17723

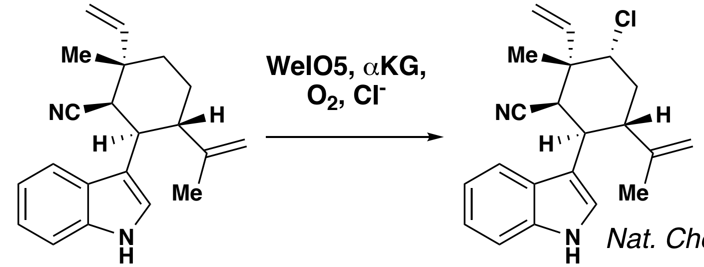
For mechanistic studies with EPR: *JACS* **2015**, *137*, 6912
For QM/MM: *ACS Catal.* **2019**, *9*, 4930

Extensive mechanistic study of this enzyme has been performed by Bollinger-Krebs group (PSU). Under stoichiometric conditions, they also observed that SyrB2 can catalyze nitration and azidation:



Nat. Chem Biol. **2014**, *10*, 209

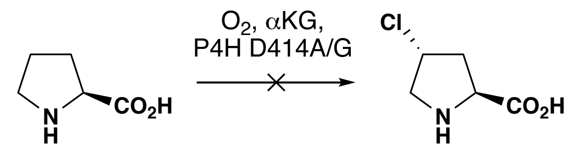
A standalone Fe-αKG halogenase was recently characterized:



Nat. Chem Biol. **2014**, *10*, 921
ACIE **2016**, *55*, 5780

A related enzyme, AmbO5 (79% sequence identity), was characterized and shown to have less-stringent substrate specificity than WelO5. WelO5-AmbO5 fusion showed similar promiscuity but with altered regioselectivities.

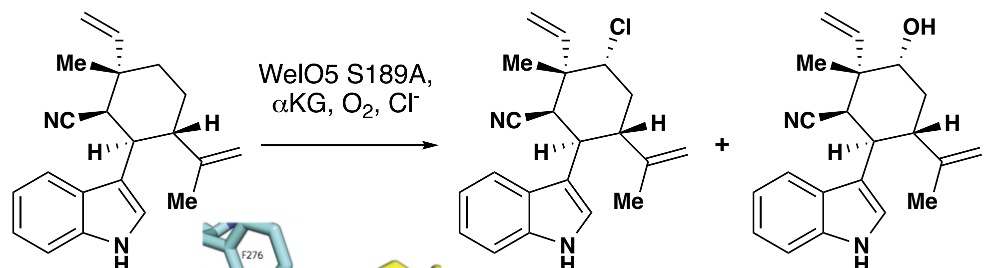
Converting Fe-αKG hydroxylase to a halogenase is not trivial

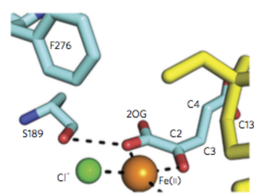


PLoS ONE **2009**, *4*, e7635

swapping out Glu to non-coordinating residue gave non-functional enzyme

Rational engineering was recently performed on WelO5 based on solved crystal structure:

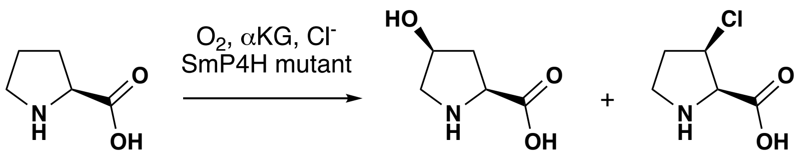




Nat. Chem Biol. **2016**, *12*, 636

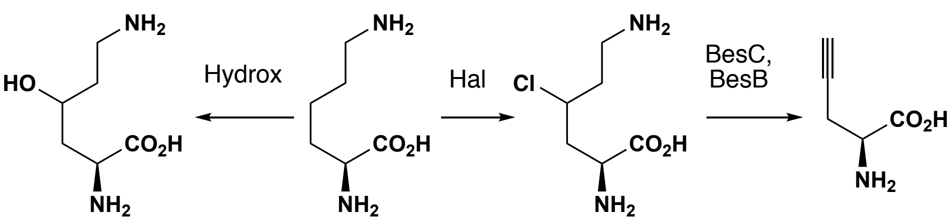
Natural and Artificial Metalloenzymes

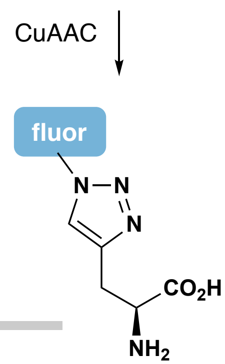
Improving halogenase activity on hydroxylase by engineering



mutant	hydroxylation:halogenation	k_{cat}/K_m (min^{-1}/M^{-1})
SmP4H-0	24:1	0.23
SmP4H-7	12:1	22

ChemBioChem **2021**, *13*, 3914



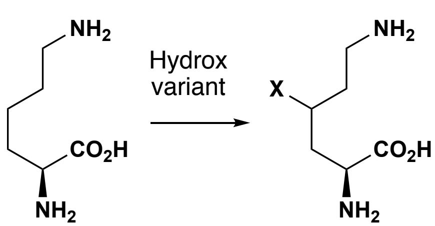


Nat. Chem. Biol. **2022**, *18*, 171

strategy to engineer halogenase activity in Hydrox

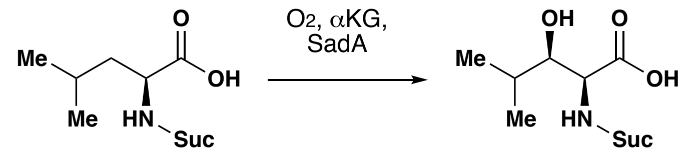
shuffle library from Hydrox D144G and Hal → HTS → sequence-function insights → rational engineering of Hydrox

engineering result



variant	% Cl:OH selectivity
WT	0
D144G-N151I-V225N	84

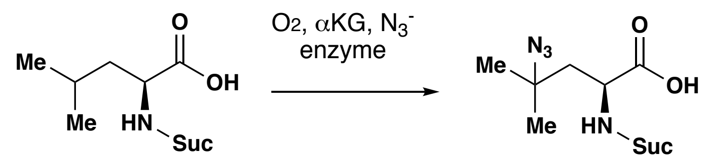
Optimization of C–H azidation on SadA



single turnover experiment hydroxylation predominates

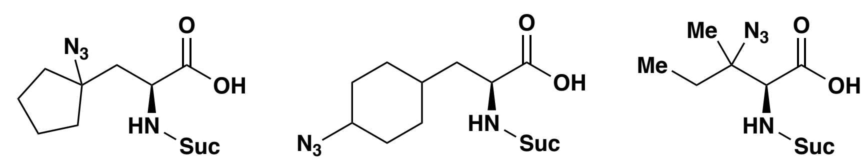
R = OH or Cl

Biochemistry **2017**, *56*, 441



4-IC = SadX V38I R48C I71V I138V F152L R172H Q233L F261L

representative product scope

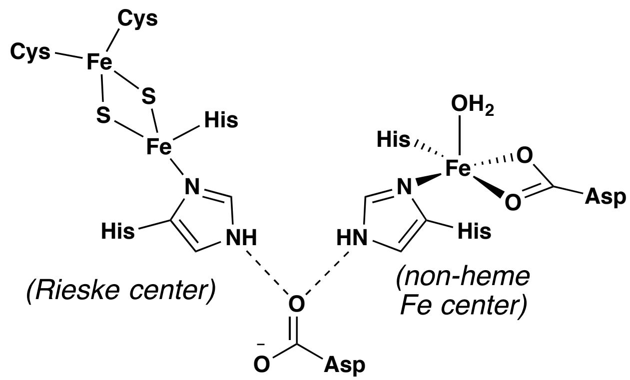


75% yield (4-IC) | 81% yield (1-VH) | 48% yield (1-VH)

ACIE **2023**, e202301370

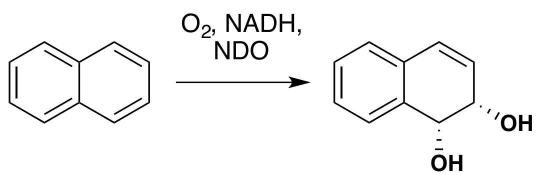
Natural and Artificial Metalloenzymes

Rieske Dioxygenases



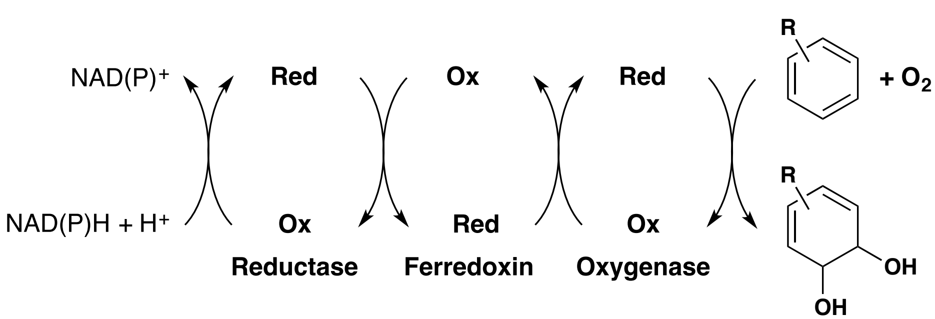
ACS Catal. **2013**, *3*, 2362

- First identified in degradation of aromatic compounds by *P. putida*.
- Identified to be three-component system naphthalene and toluene dioxygenase

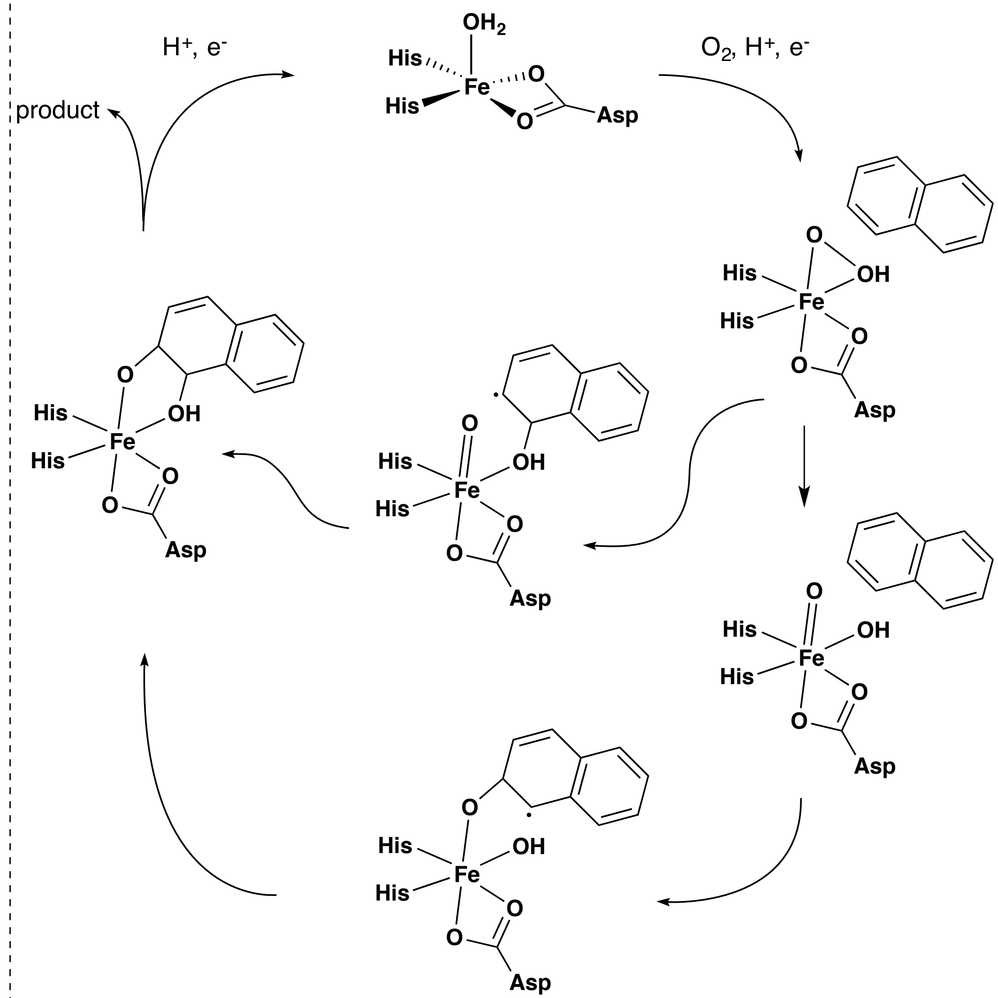


J. Bacteriol. **1982**, *149*, 948
J. Bacteriol. **1983**, *155*, 505

- Components: flavin-dependent reductase, ferredoxin, and terminal oxygenase



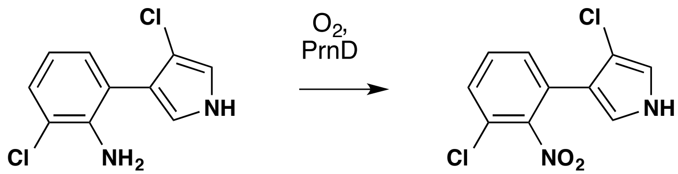
Postulated mechanism for arene dihydroxylation



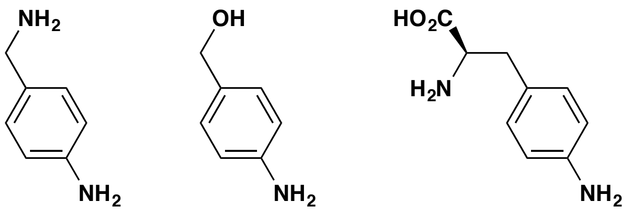
Challenges in studying Rieske oxygenases:
- Multi-component system
- Oxygen-sensitive nature of [2Fe-2S] cluster
- Lack of chromophore for spectroscopic studies (cf. P450)

Natural and Artificial Metalloenzymes

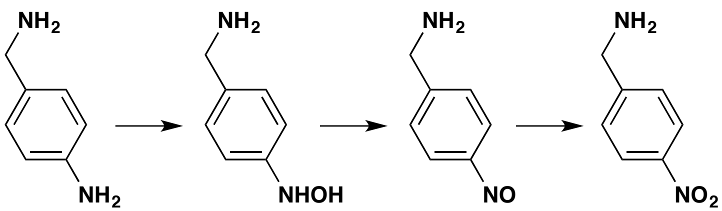
Pyrrolnitrin biosynthesis



Other reported substrates (*J. Biol. Chem.* **2005**, *280*, 36719)

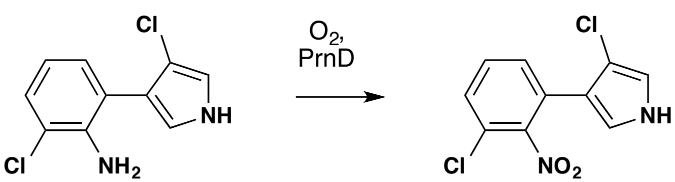


Mechanistic studies indicated presence of various intermediates



ACIE **2006**, *45*, 622

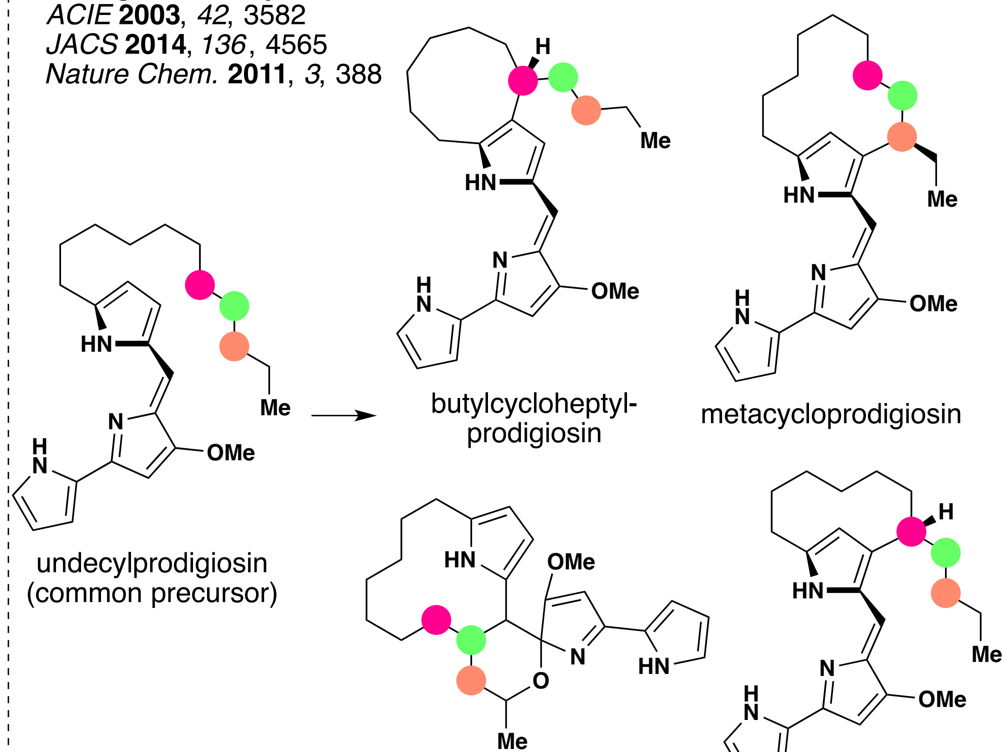
Engineering based on molecular modeling was shown to improve the catalytic efficiency of the enzyme (*J. Bacteriol.* **2006**, *188*, 6179)



k_{cat}/K_m (WT) = 0.036 $min^{-1}/\mu M^{-1}$

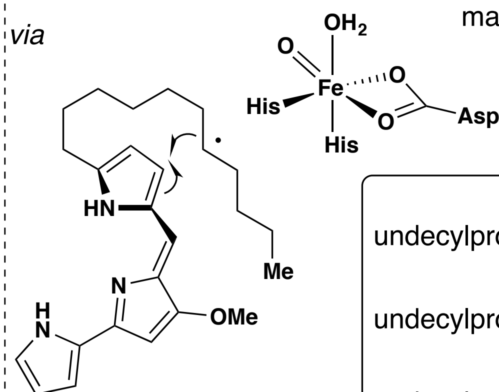
k_{cat}/K_m (F312A L277V) = 0.243 $min^{-1}/\mu M^{-1}$

Prodigiosin biosynthesis
ACIE **2003**, *42*, 3582
JACS **2014**, *136*, 4565
Nature Chem. **2011**, *3*, 388



undecylprodigiosin (common precursor) → butylcycloheptylprodigiosin, metacycloprodigiosin, marineosin, streptorubin B

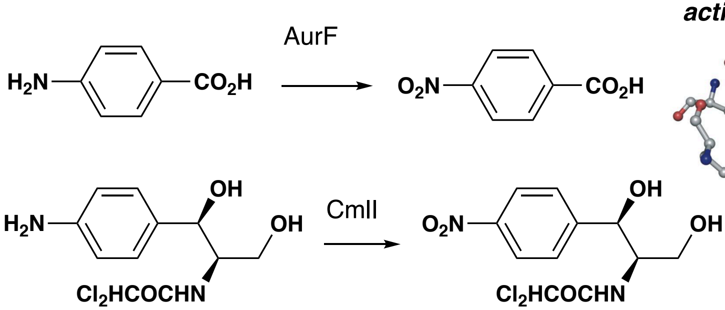
via



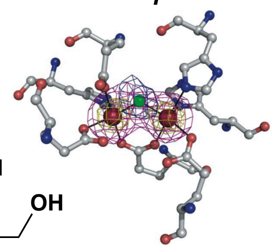
undecylprodigiosin —RedG→ streptorubin B
undecylprodigiosin —McpG→ metacycloprodigiosin
undecylprodigiosin —MarG→ premarineosin

Natural and Artificial Metalloenzymes

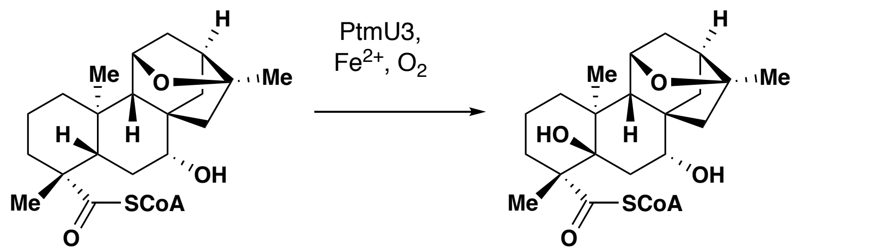
Nonheme diiron monooxygenase in oxidation chemistry



active-site picture:

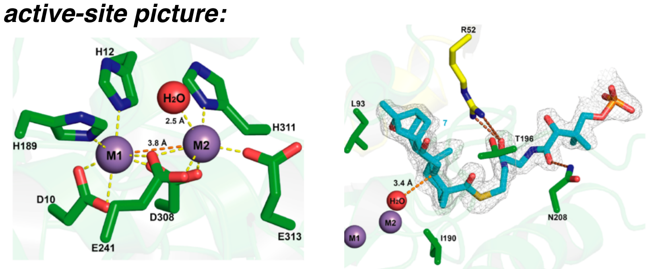


JACS **2004**, *126*, 3694; *PNAS* **2008**, *105*, 6858; *JACS* **2015**, *137*, 1608



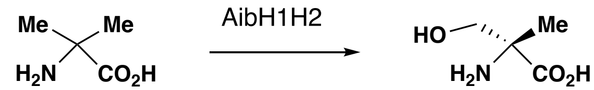
JACS **2019**, *141*,12406

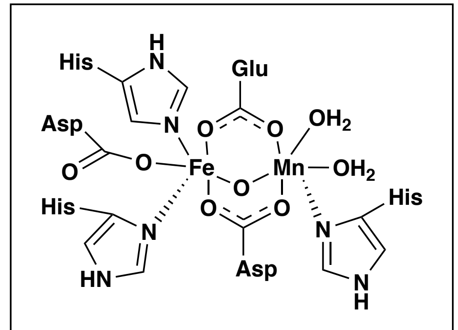
active-site picture:



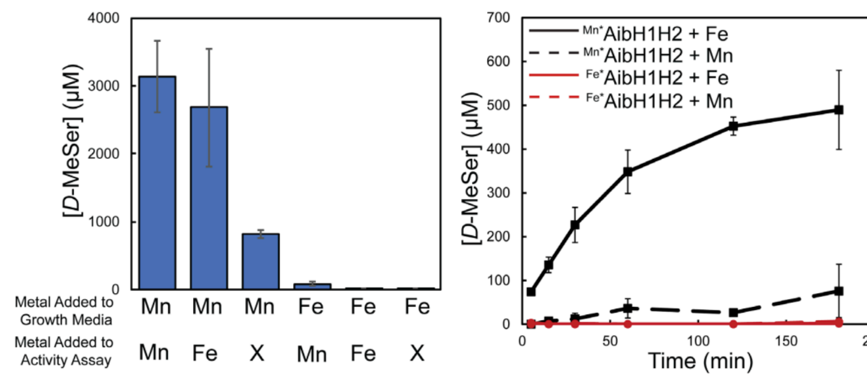
For mechanistic proposal: *Inorg. Chem.* **2021**, *60*, 17783

Beyond Fe….





Fe/Mn cofactor

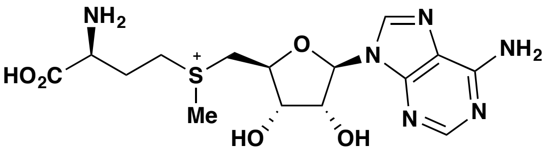


bioRxiv DOI:10.1101/2023.03.10.532131

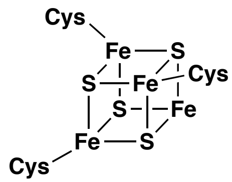
Natural and Artificial Metalloenzymes

Radical SAM enzymes

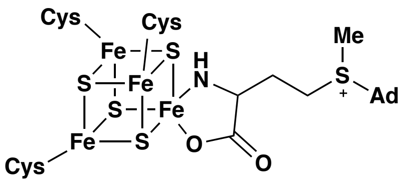
- Cofactor components:

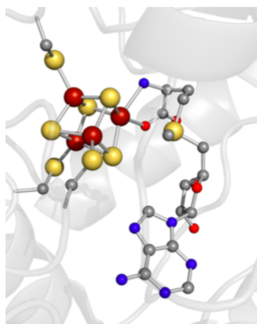


S-Adenosylmethionine



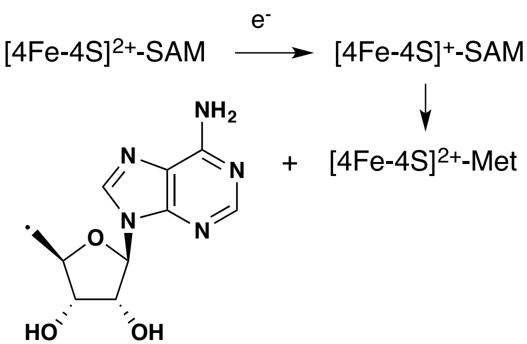
[4Fe-4S] cluster





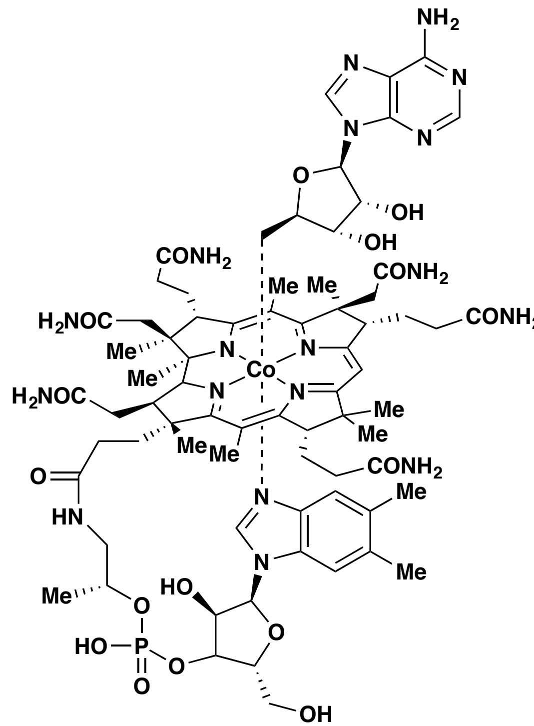
Chem. Rev. **2014**, *114*, 4229

- General mechanism for radical generation



5'-deoxyadenosyl radical

- The same radical intermediate can be generated from adenosylcobalamin (AdoCbl)



Energetic considerations for radical SAM enzyme reduction

- Reduction potential of free SAM ~ –1800 mV
- Reduction potential of [4Fe-4S] ~ –500 to –600 mV
- Radical generation is energetically unfavorable when considered in isolation!

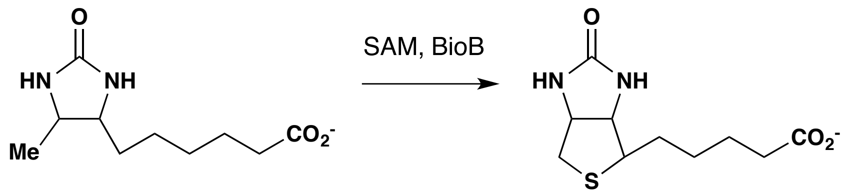
Selectivity considerations in C–S bond cleavage

- Spectroscopic studies suggest direct orbital overlap between Fe-S cluster and sulfonium S; orbital overlap determines which C–S bond is cleaved

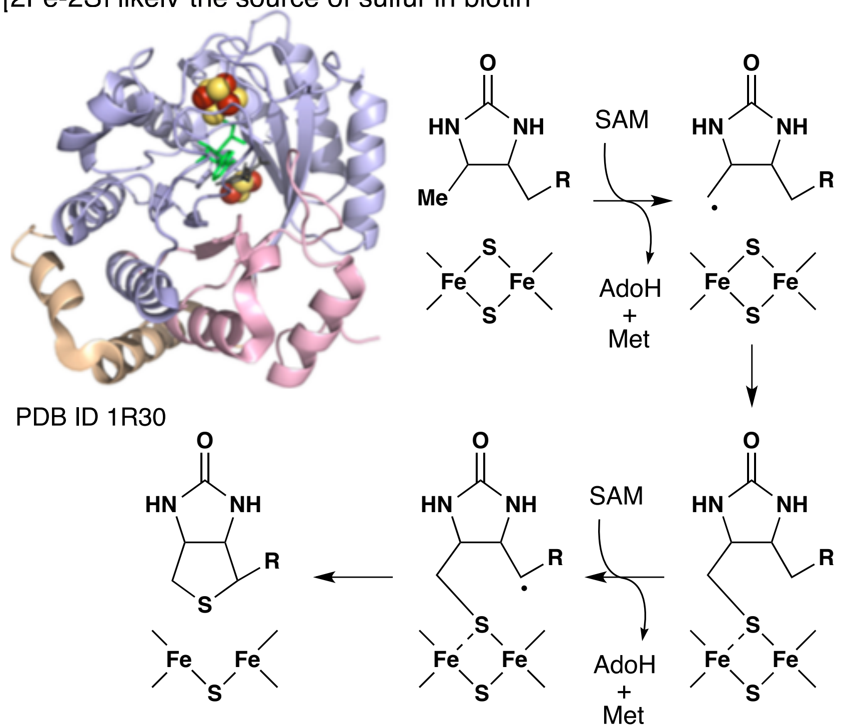
Natural and Artificial Metalloenzymes

Examples of radical SAM in action

Sulfur insertion – biosynthesis of biotin



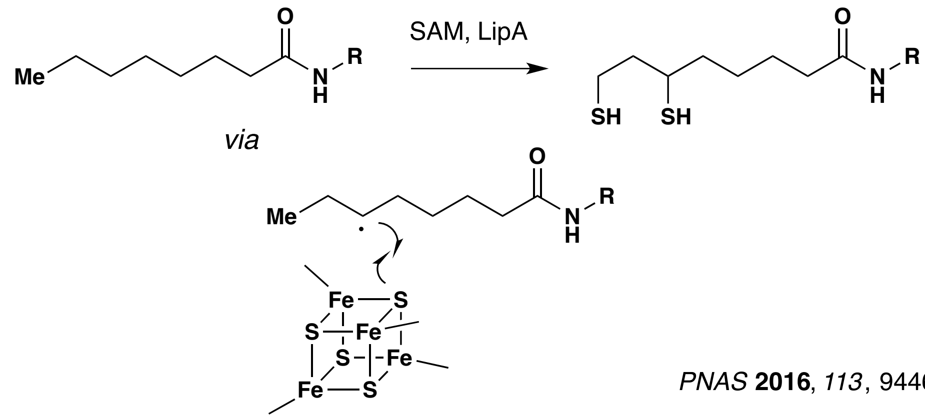
- Successful reconstitution showed presence of one [4Fe-4S] and one [2Fe-2S] cluster per enzyme monomer
- [4Fe-4S] was retained during turnover, and [2Fe-2S] degraded
- [2Fe-2S] likely the source of sulfur in biotin



PDB ID 1R30

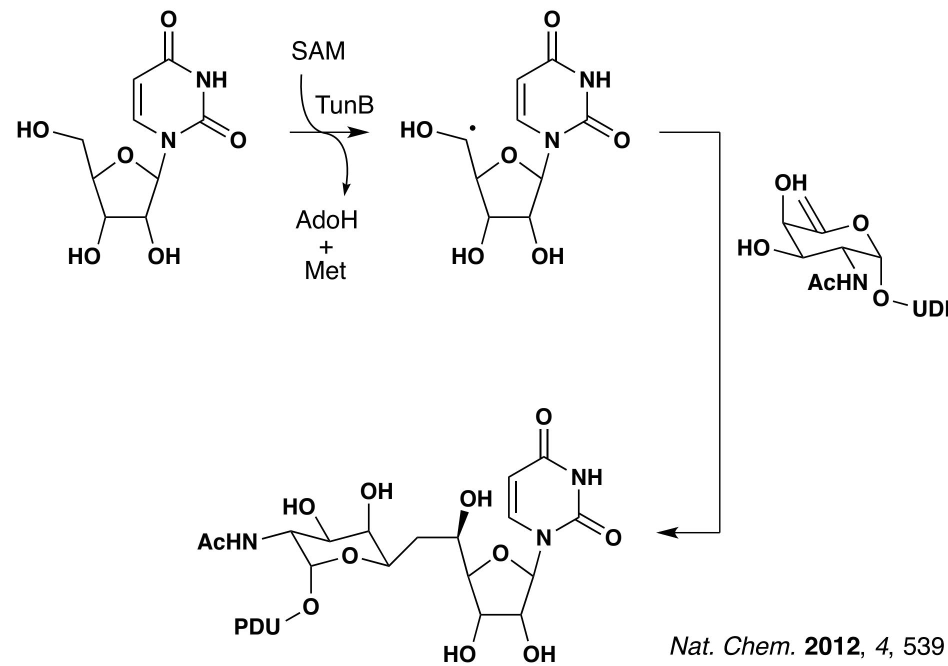
Biochim. Biophys. Acta **2012**, *1824*, 1213

Similar reactivity in the biosynthesis of lipoic acid



PNAS **2016**, *113*, 9446

C–C coupling in tunicamycin biosynthesis



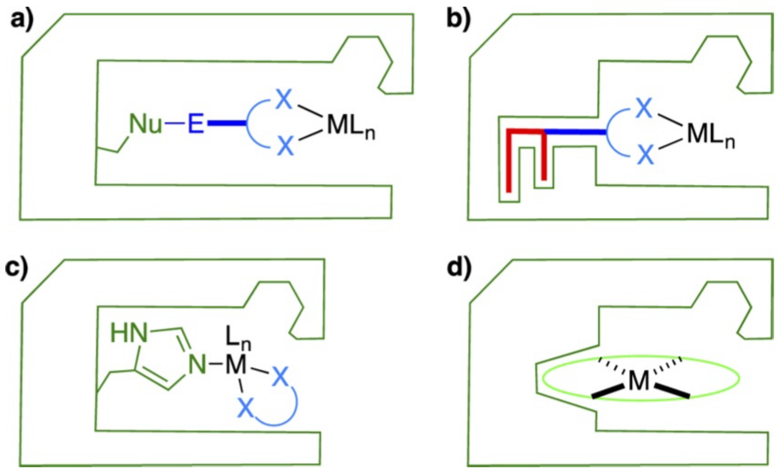
Nat. Chem. **2012**, *4*, 539

Natural and Artificial Metalloenzymes

Artificial metalloenzymes (ArMs)

Definition:
An ArM is an unnatural enzyme derived from insertion of a catalytically competent metal cofactor into a protein scaffold

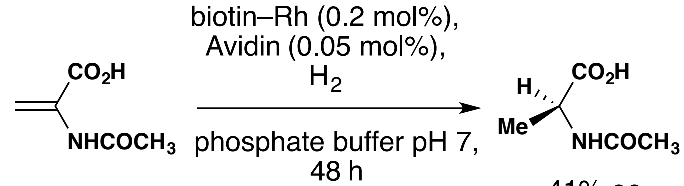
Current strategies for incorporation:



a: via covalent bond (with residues within the scaffold
b: supramolecular anchoring (exploits high affinity of certain scaffolds for particular substrates
c: dative bonding
d: metal substitution

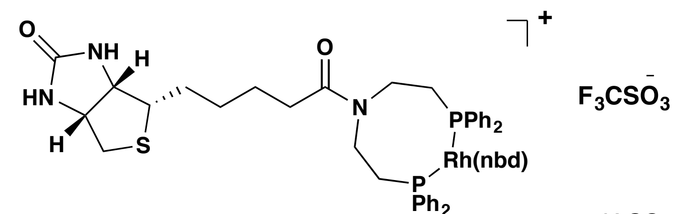
Some reviews:
Chem. Rev. **2018**, *118*, 142
Acc. Chem. Res. **2019**, *52*, issue 3 (special issue on ArMs)
Curr. Opin. Chem. Biol. **2017**, *37*, 48
Curr. Opin. Chem. Biol. **2015**, *25*, 27
Curr. Opin. Chem. Biol. **2014**, *19*, 99
Curr. Opin. Chem. Biol. **2010**, *14*, 184

First demonstration of ArM catalysis using avidin/biotin technology



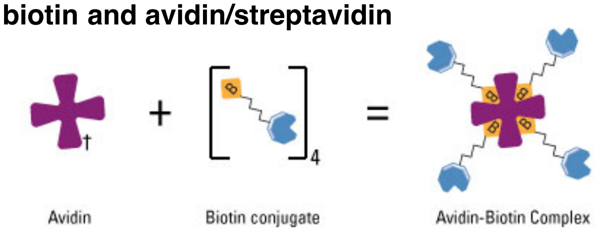
41% ee
500 TON

biotin–Rh:

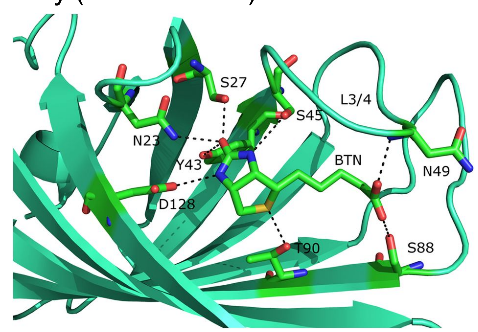


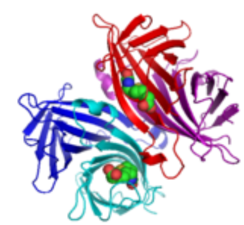
JACS **1978**, *100*, 306

Primer on biotin and avidin/streptavidin



Avidin/streptavidin: tetrameric protein capable of binding biotin with high affinity (Kd ~ 10^{-14} M)

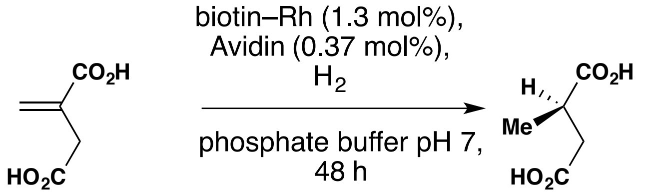




Tetrameric streptavidin with 2 bound biotin molecules

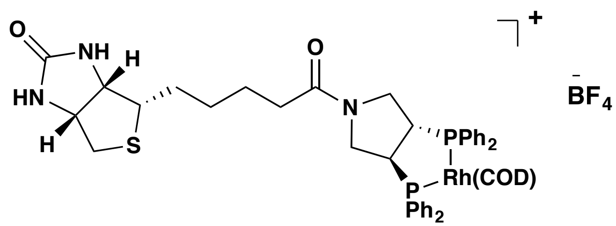
Natural and Artificial Metalloenzymes

Revisiting of the system in the late 90s...

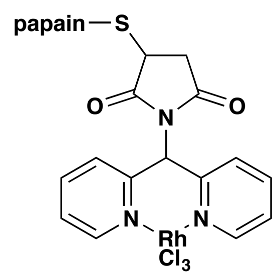


48% ee
31 TON

biotin–Rh:



Reetz's papain system

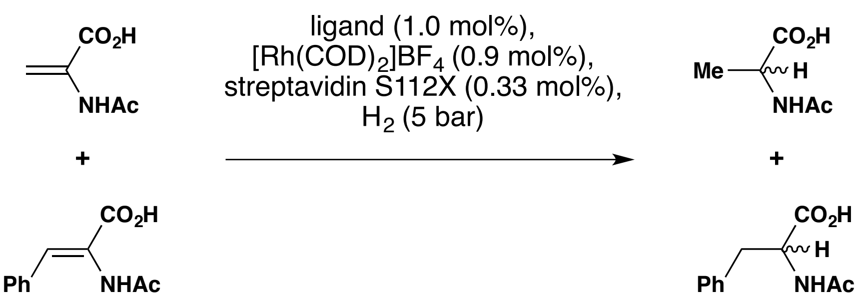


"Preliminary experiments concerning catalysis show that... are hydrogenation catalysts, ... although the ee values turned out to be less than 10%, which is no surprise."

Chimia **2002**, *56*, 721

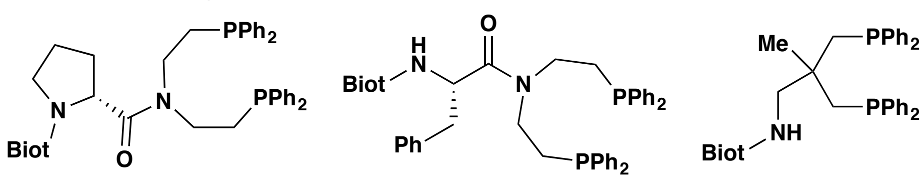
Tetrahedron: Asymmetry **1999**, *10*, 1887

Systematic study by Ward + protein engineering



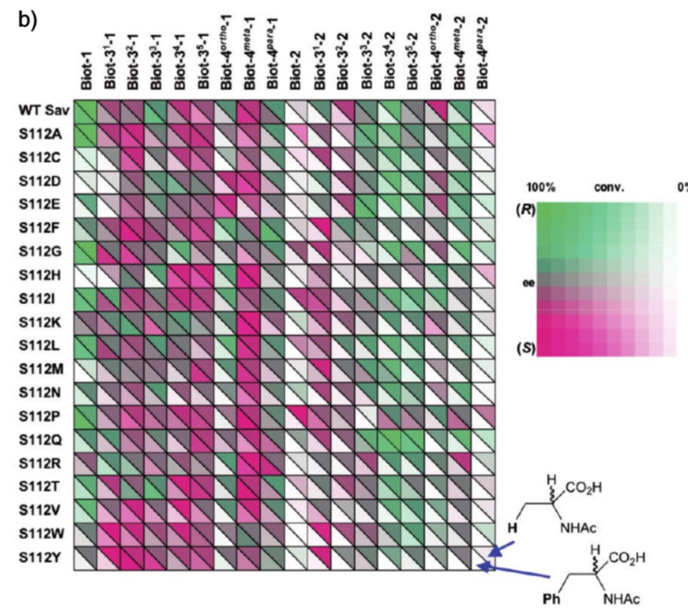
able to obtain quant. conversion with more than 90% ee for R or S enantiomer depending on ligand used

representative ligands:



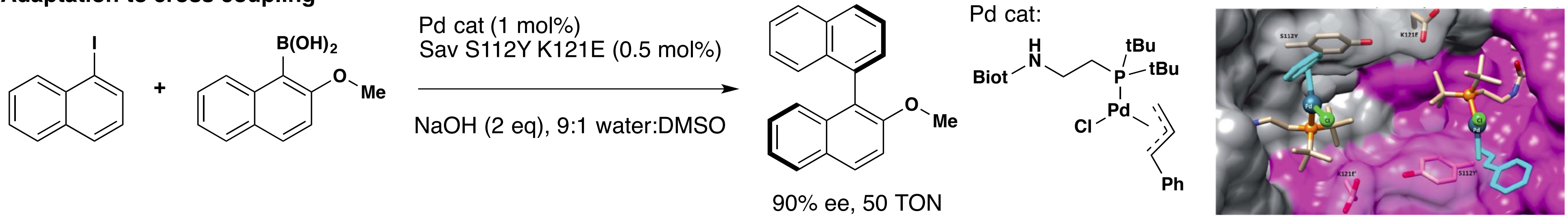
JACS **2003**, *125*, 9030
JACS **2004**, *126*, 14411
ACIE **2005**, *44*, 7764

b)



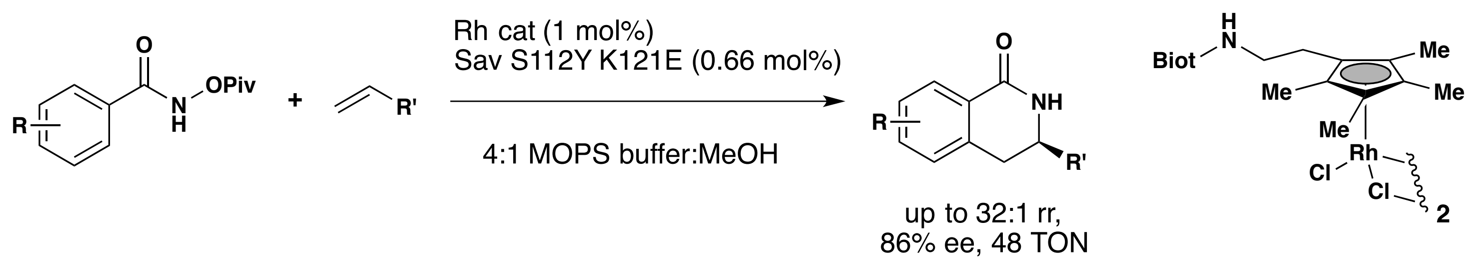
Natural and Artificial Metalloenzymes

Adaptation to cross coupling



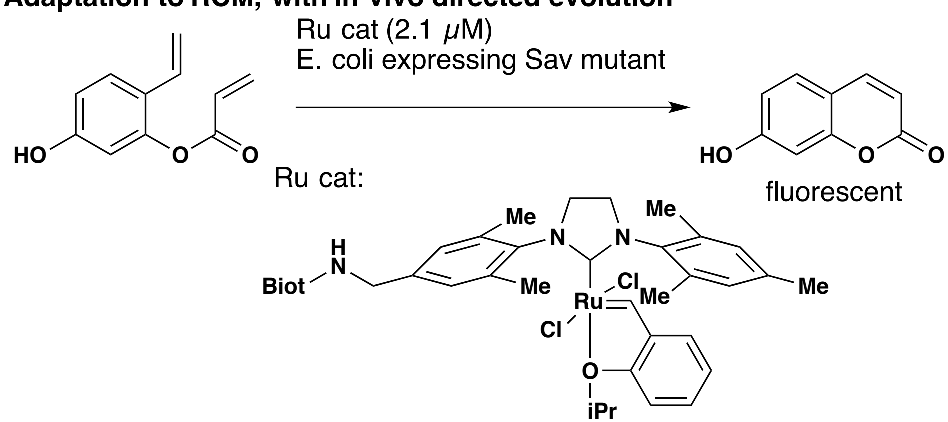
Chem. Sci. **2016**, *7*, 673

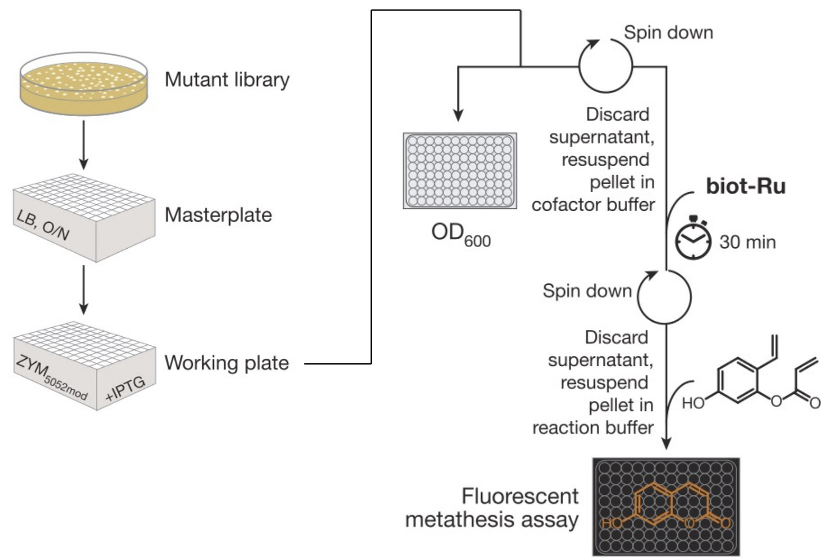
Adaptation to C–H activation



Science **2012**, *338*, 500

Adaptation to RCM, with in vivo directed evolution



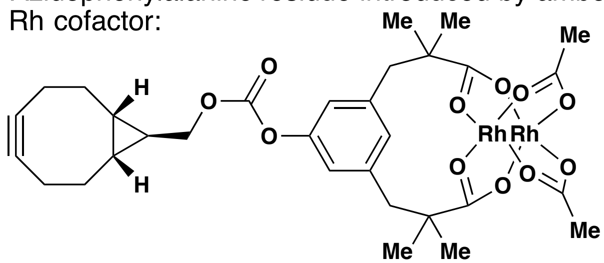


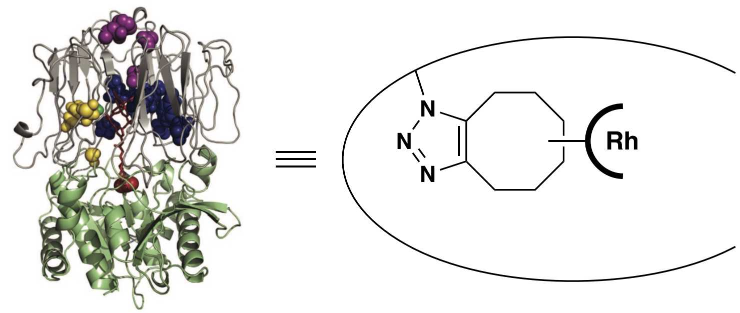
Nature **2016**, *537*, 661

Natural and Artificial Metalloenzymes

Prolyl oligopeptidase scaffold for ArM construction (Lewis)

- chosen due to its cyclindrical shape
- large internal volume for cofactor anchoring
- cofactor anchoring by strain promoted azide alkyne cycloaddition
- Azidophenylalanine residue introduced by amber suppression
- Rh cofactor:

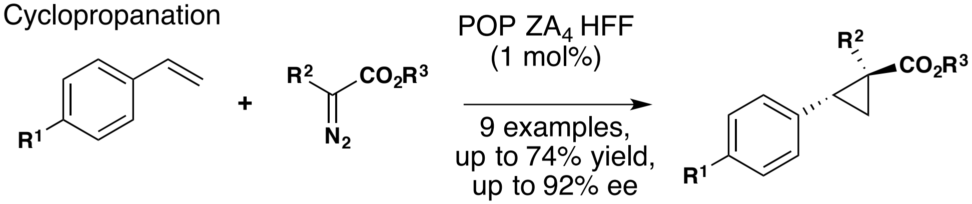




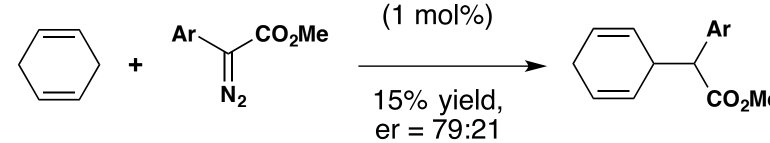
Nature Commun. **2015**, *6*, 7789

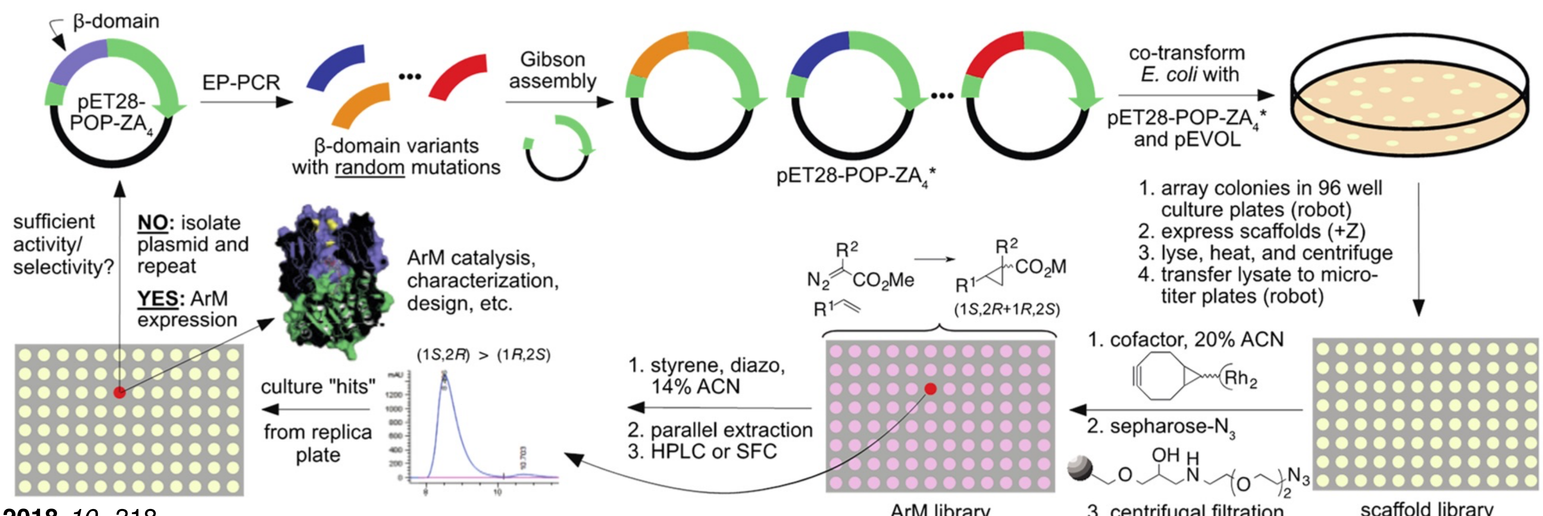
Reaction scope

Cyclopropanation



C–H insertion





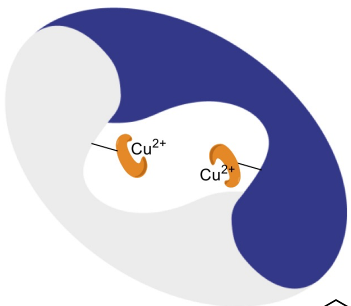
Nature Chem. **2018**, *10*, 318

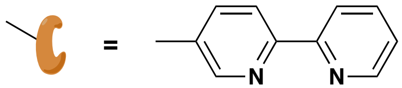
Natural and Artificial Metalloenzymes

Other protein scaffolds for ArM creation

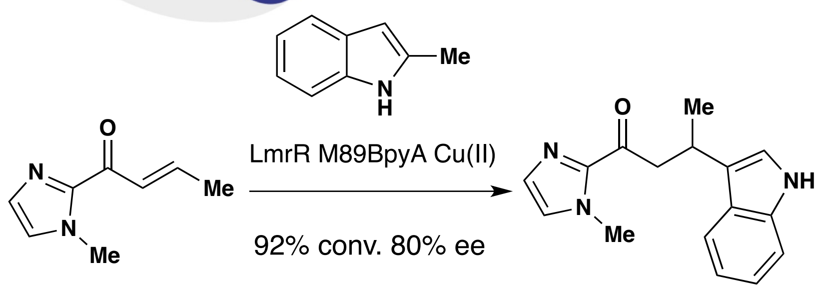
- LmrR : lactococcal multidrug resistance regulator
- homodimeric protein with a large hydrophobic pore

Chem. Sci. **2015**, *6*, 770

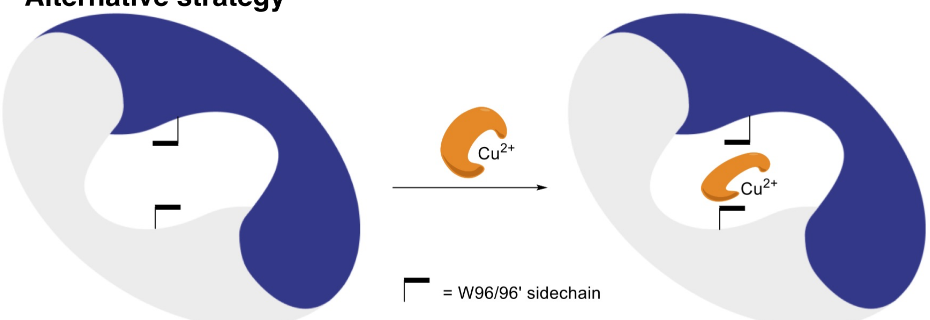


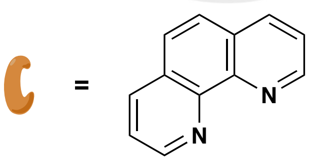


(obtained via amber suppression)



Alternative strategy



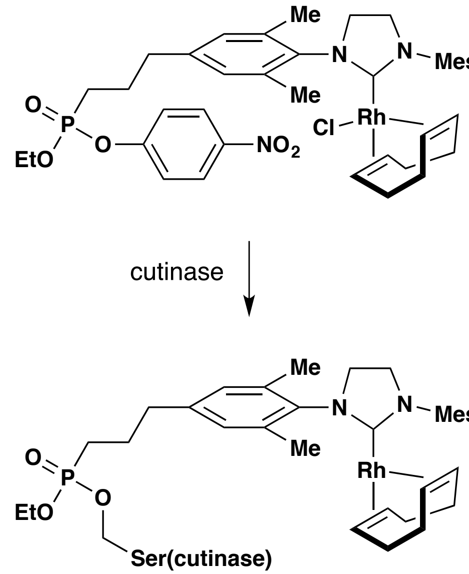


- Bypasses amber suppression protocol
- Relies on hydrophobic interaction between phen and the 2 Trp residues

JACS **2015**, *137*, 9796

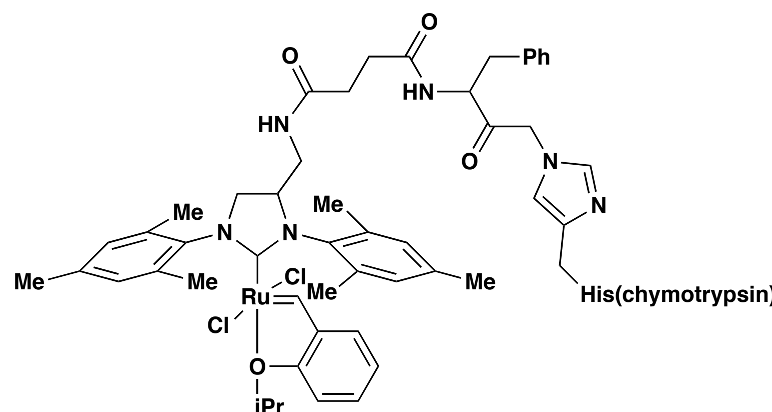
Miscellaneous strategies

- Anchoring onto serine hydrolase



Chem. Comm. **2015**, *51*, 6792

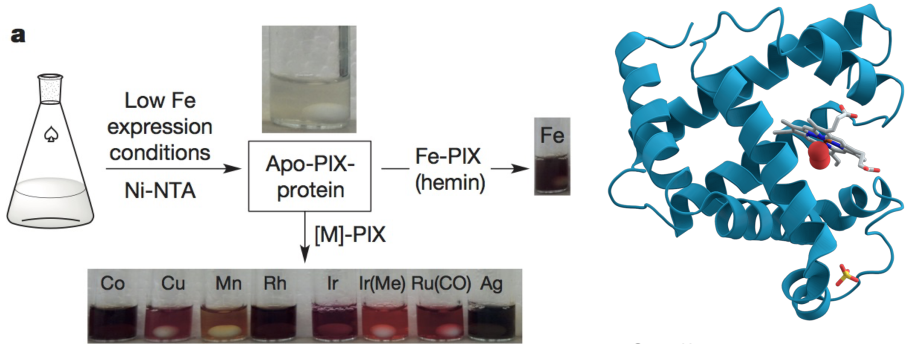
- Anchoring onto chymotrypsin



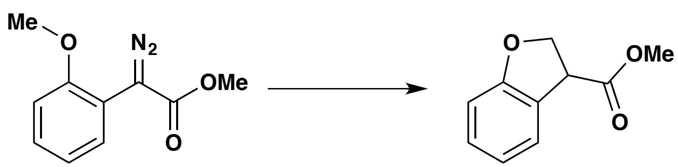
Chem. Comm. **2012**, *48*, 1662

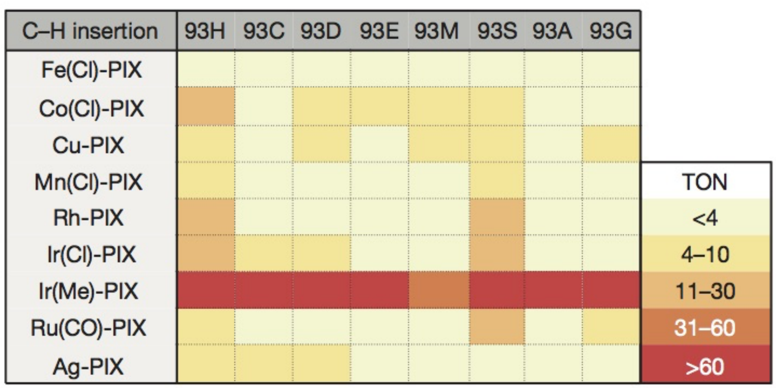
Natural and Artificial Metalloenzymes

Metal substitution strategy for ArM creation (Hartwig)



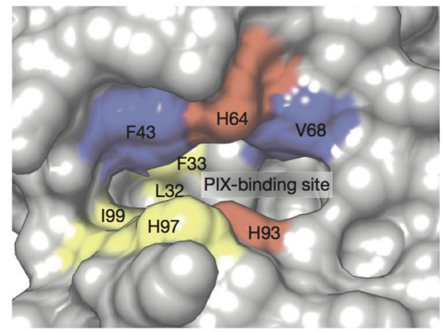
Scaffold = myoglobin

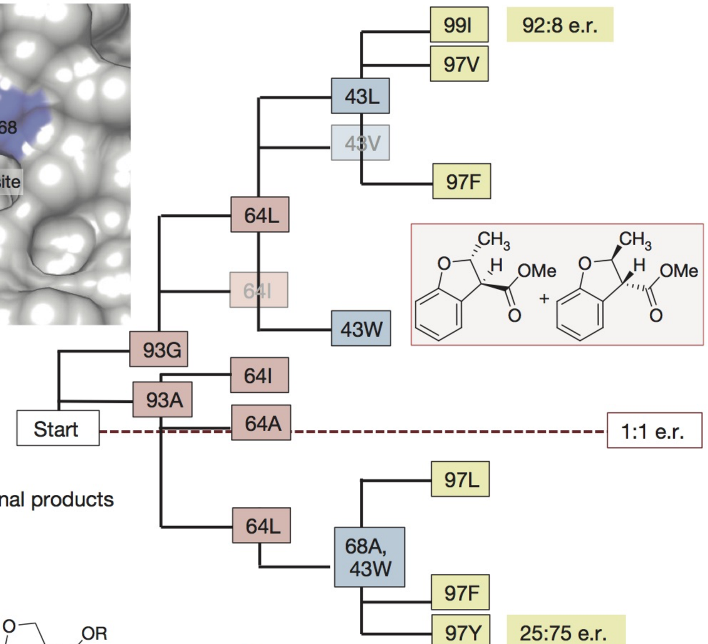




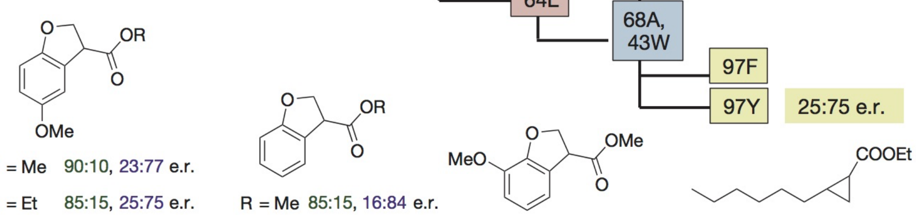
Nature **2016**, *534*, 534

Tuning selectivity by mutagenesis





Selectivities obtained for additional products



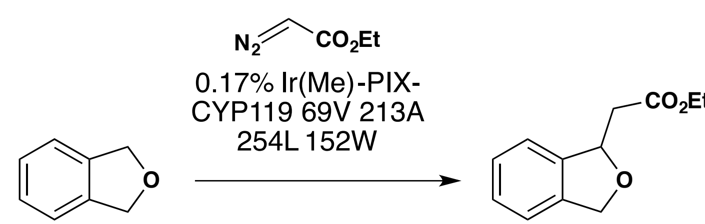
R = Me 90:10, 23:77 e.r.
R = Et 85:15, 25:75 e.r.
R = Bn 77:23, 17:83 e.r.

R = Me 85:15, 16:84 e.r.
R = Et 81:19, 20:80 e.r.

90:10, 13:87 e.r.

91:9 e.r., 40:1 d.r.

Improvement of kinetics and reaction scope by using different scaffold



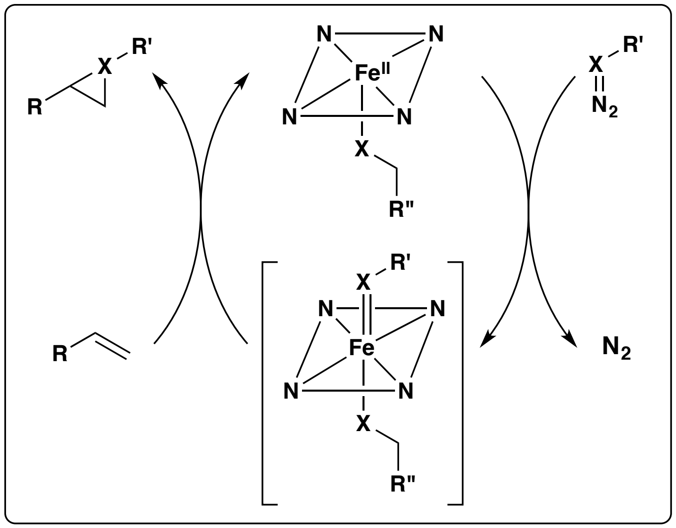
55% yield, 68% ee

CYP119 = thermostable P450 from *S. solfataricus*

Science **2016**, *354*, 102

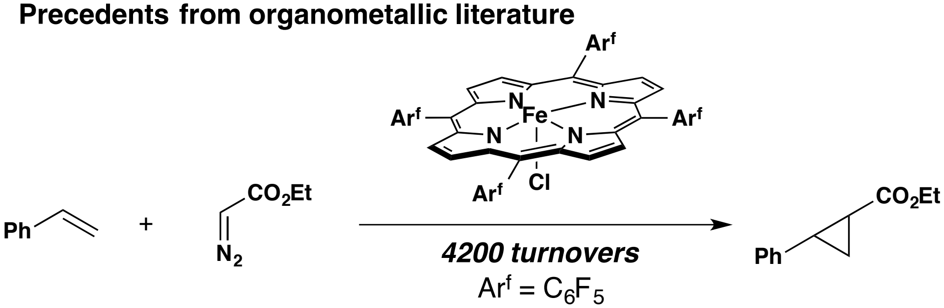
Natural and Artificial Metalloenzymes

Repurposing hemeproteins for carbene/nitrene transfer (without metal substitution)

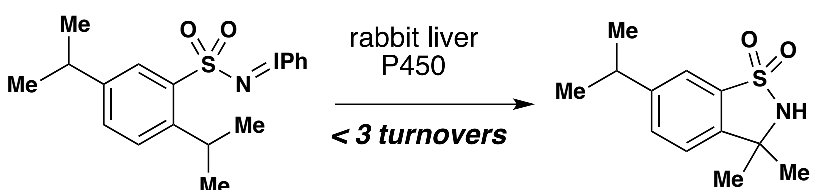


For carbene, X = C, R' = CO_2Et
For nitrene, X = N, R' = SO_2Ar

Precedents from organometallic literature

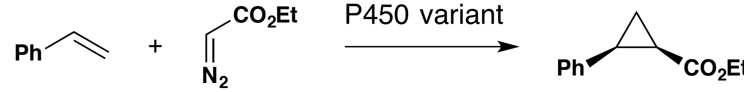


JACS **1995**, *117*, 9194



JACS **1985**, *105*, 6728

Enantioselective cyclopropanation

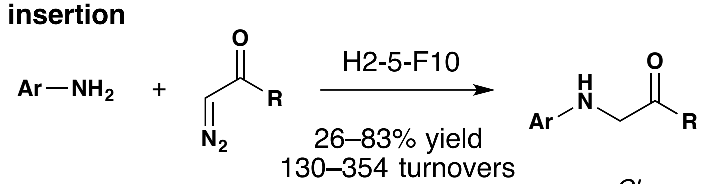


P450$_{BM3}$-CIS T438S: cis:trans = 92:8
ee$_{cis}$ = 97%
P411$_{BM3}$-CIS: cis:trans = 90:10
ee$_{cis}$ = 99%

Science **2013**, *339*, 307
NCB **2013**, *9*, 485

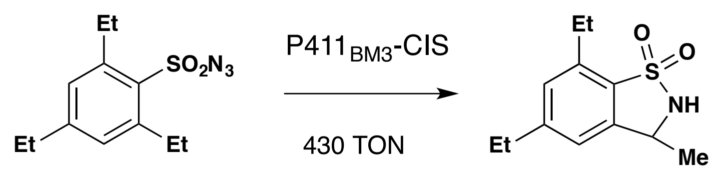
Note: P411$_{BM3}$ = P450$_{BM3}$ with Cys to Ser axial substitution

N-H insertion

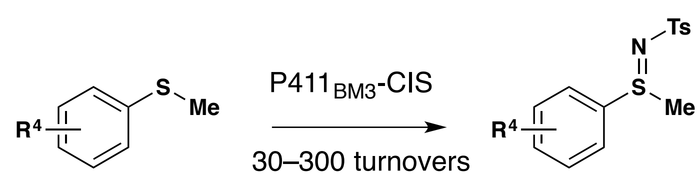


Chem. Sci. **2013**, *5*, 598

Enantioselective amination and sulfimidation



ACIE **2013**, *52*, 9309

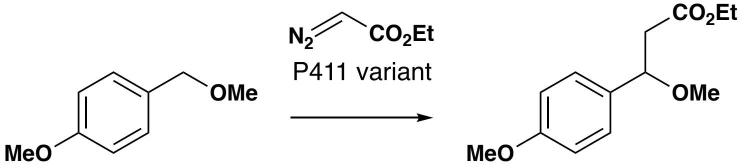


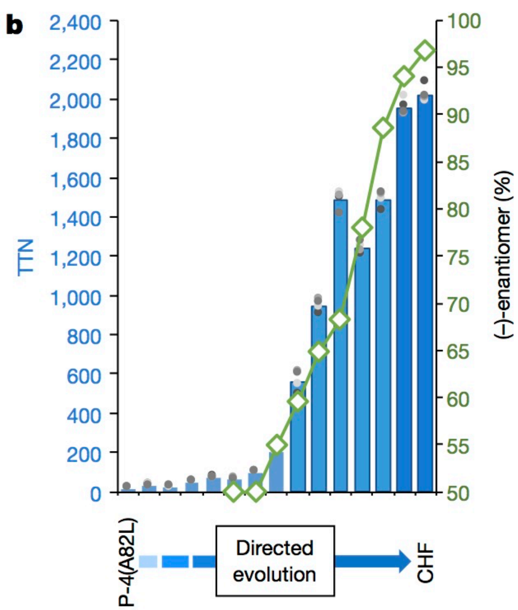
JACS **2014**, *136*, 8766

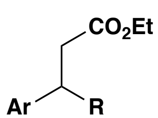
For related studies by Fasan:
ACIE **2015**, *54*, 1744; *Chem. Comm.* **2015**, *15*, 1532; *Chem. Sci.* **2015**, *6*, 2488; *ACIE* **2016**, *55*, 16110; *JACS* **2017**, *139*, 5293

Natural and Artificial Metalloenzymes

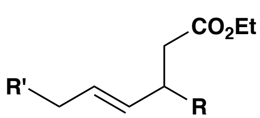
Enantioselective C-H insertion



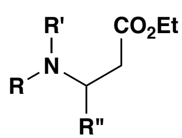




12 examples,
up to 2150 TON,
up to >99% ee



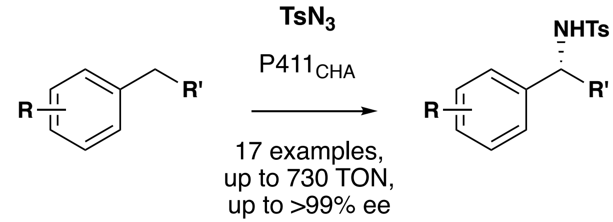
5 examples,
up to 3750 TON,
up to 98% ee



5 examples,
up to 2330 TON,
up to 80% ee

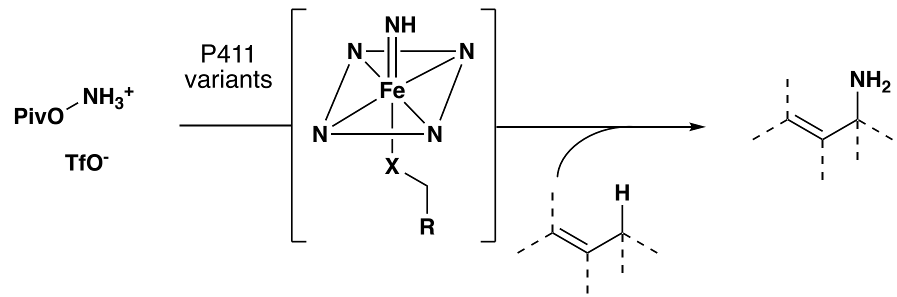
Nature **2019**, *565*, 67

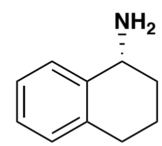
Enantioselective intermolecular C-H amination



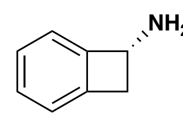
Nature Chem. **2017**, *9*, 629

Alternative nitrene source

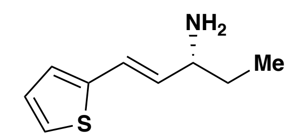




580 TTN,
93% ee



120 TTN,
95% ee

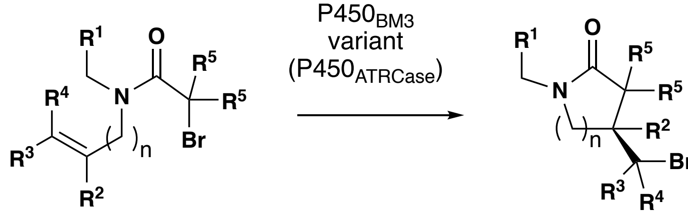


600 TTN,
89% ee

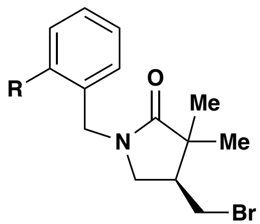
J. Am. Chem. Soc. **2020**, *142*, 10279

Natural and Artificial Metalloenzymes

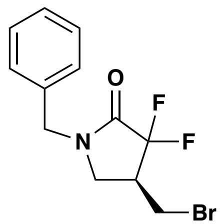
Atom Transfer Radical Cyclization with P450



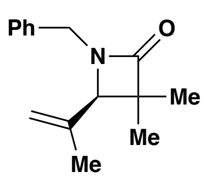
Selected product scope



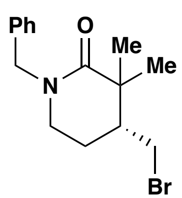
TTN = 890–8100
e.r. = 85:15–97:3



TTN = 330
e.r. = 78:22

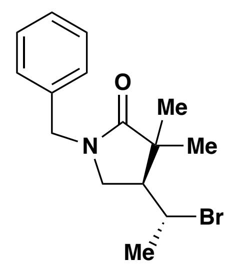


TTN = 750
e.r. = 91:9

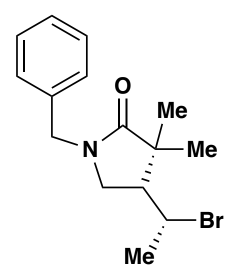


TTN = 470
e.r. = 68:32

diastereodivergent cyclization with different ATRCases

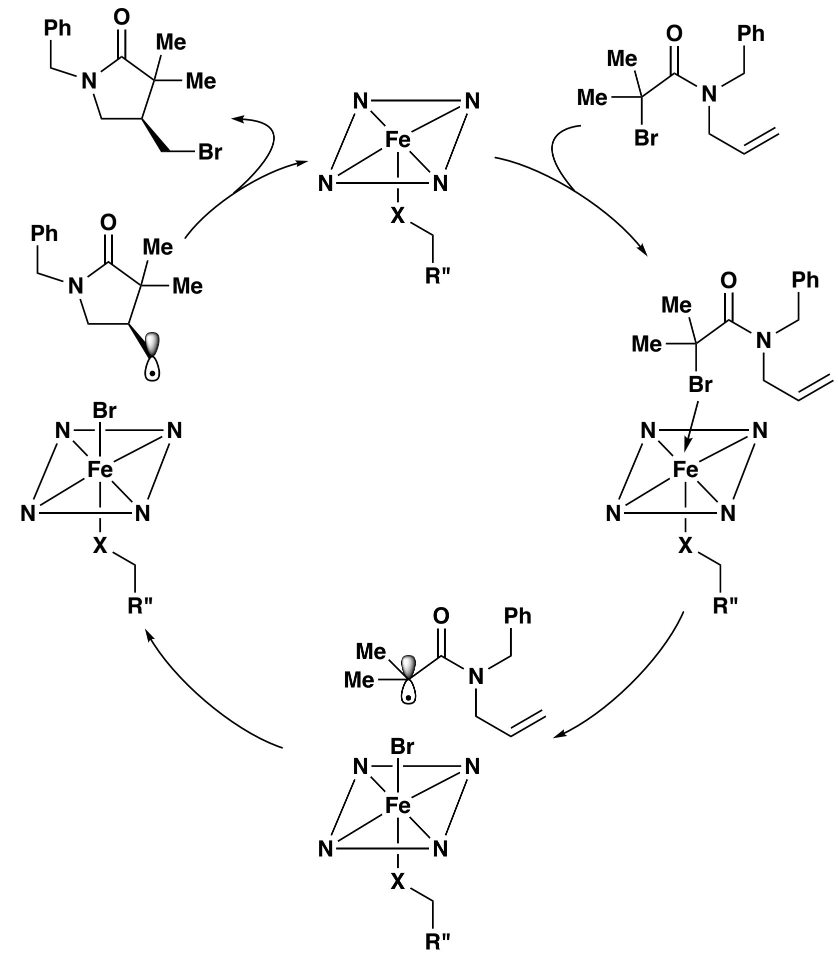


TTN = 1410
e.r. = 99:1, d.r. = 24:1



TTN = 245
e.r. = 55:45, d.r. = 1:4.7

Proposed mechanism (supported by DFT)



Science **2021**, *374*, 1612
For discussion on the role of hydrogen bonding in the active site in enantiocontrol: *JACS* **2022**, *144*, 13344