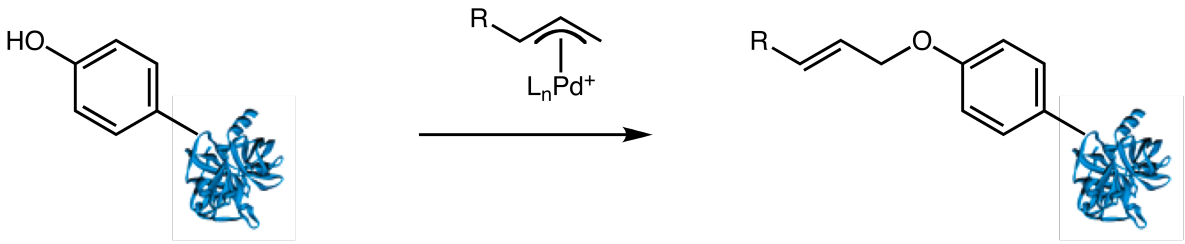
Bioconjugation: Chemical Approaches to Protein Modification



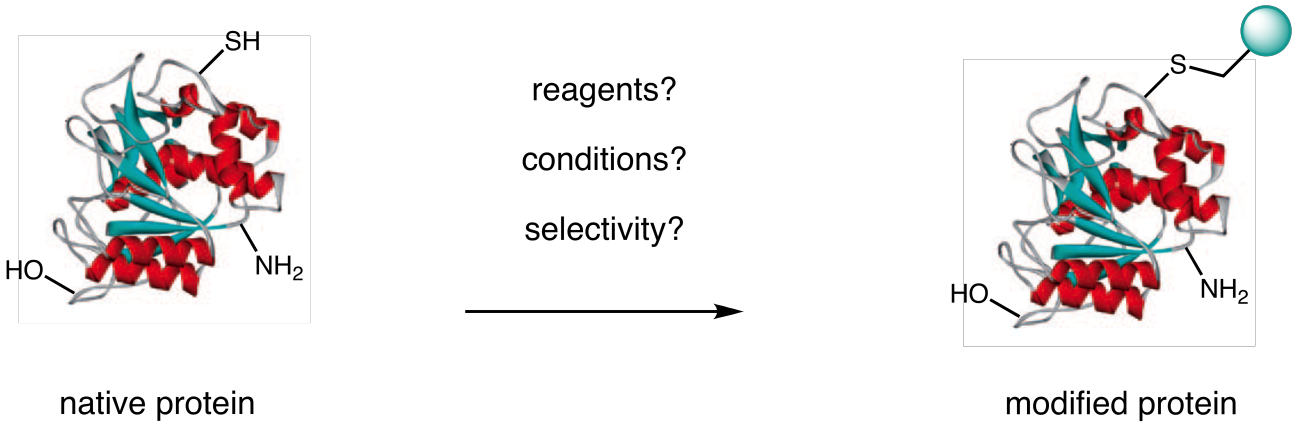
Chris Prier

MacMillan Group Meeting

November 8, 2011

What is Bioconjugation?

- The covalent modification of proteins using chemical reagents to create adducts with desired properties



native protein

modified protein

Why Synthesize Bioconjugates?

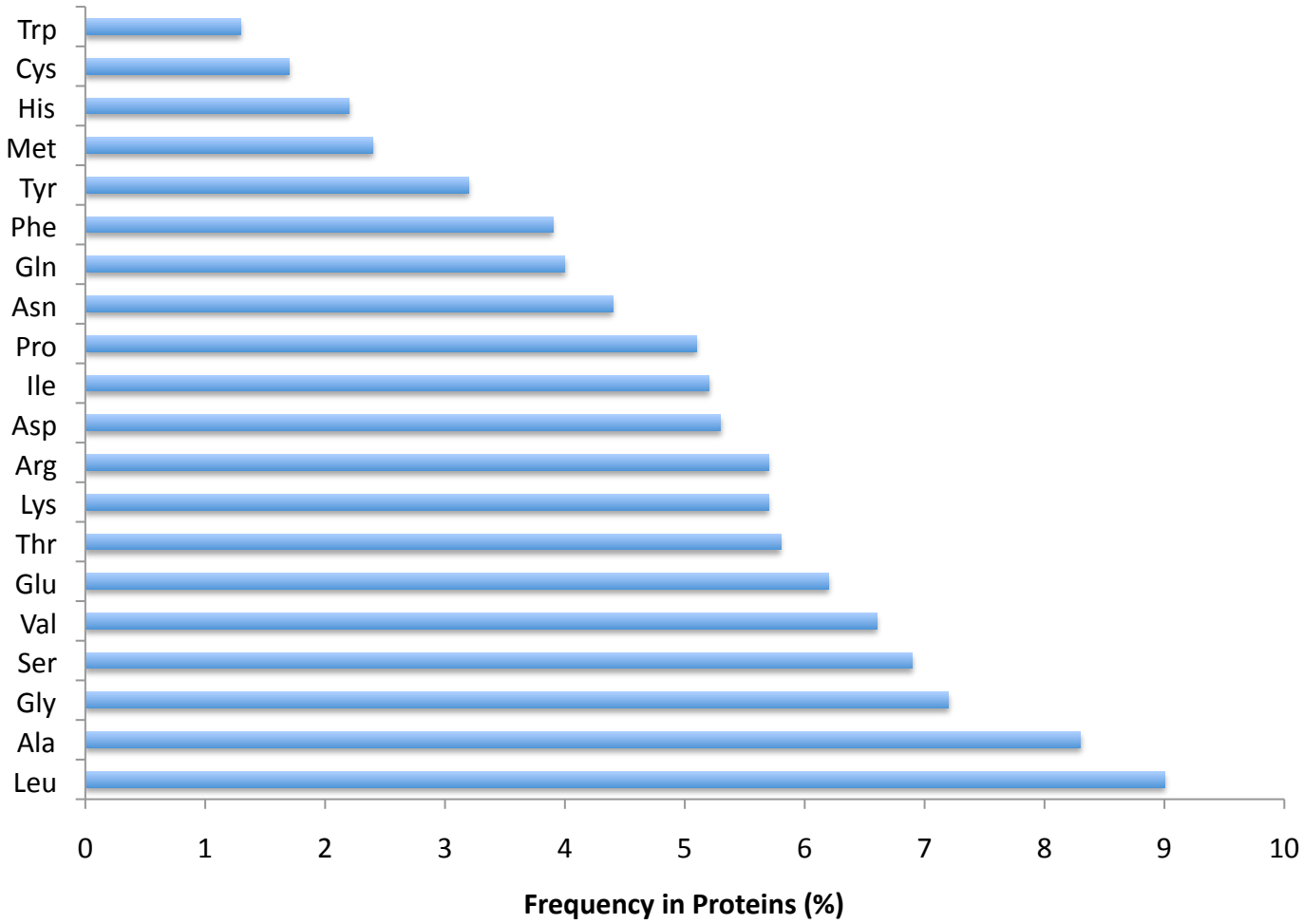
- attach probes for the elucidation of biological function
- access pure functionalized proteins to study effects of post-translational modifications
- improve efficacy and pharmacokinetic properties of protein therapeutics
- create new protein-based materials

Requirements for a Bioconjugation Reaction

A bioconjugation reaction must...

- proceed in aqueous solution (or solvent systems in which protein does not denature)
- proceed at mild pH and temperature (preferably room temp.)
- proceed at low substrate concentration (at or below 100 μM)
- have a short reaction time
- create precise, well-defined, and stable linkages
- tolerate functionality of amino acid side chains
- tolerate salts and surfactants often required for protein stability
- be selective for one amino acid over all other functionality in a protein

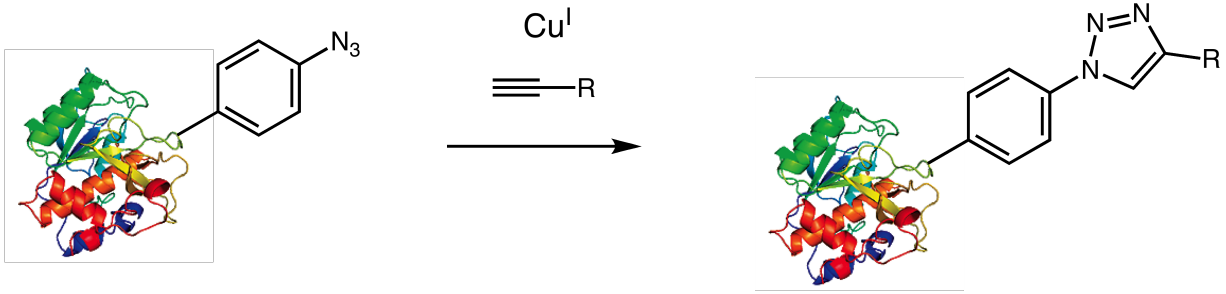
Relative Abundance of Amino Acids in Proteins



McCaldon, P.; Argos, P. *Proteins.* **1988**, *4,* 99-122.

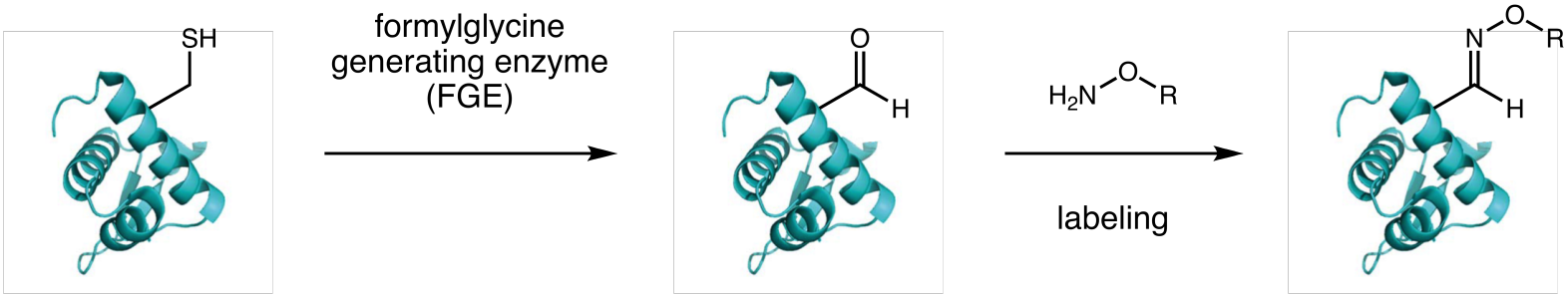
Approaches Not Covered in This Talk

- Bioorthogonal chemistry: non-biological functional groups enable selective functionalization



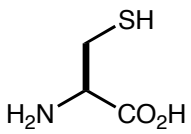
Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.*. **2003**, *125,* 3192-3193.

- Enzymatic modification: enzymes enable unique reactivity and selectivity

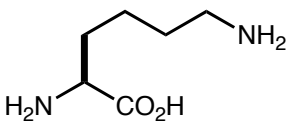


Wu, P.; Shui, W.; Carlson, B. L.; Hu, N.; Rabuka, D.; Lee, J.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106,* 3000-3005.

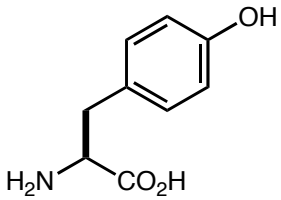
Bioconjugation: Chemical Modification of Proteins



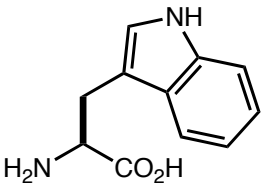
Cys



Lys

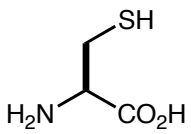


Tyr

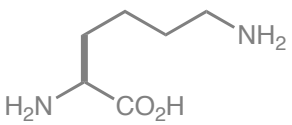


Trp

Bioconjugation: Chemical Modification of Proteins



Cys



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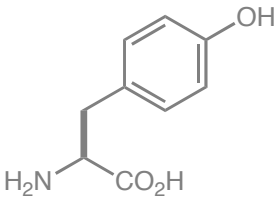

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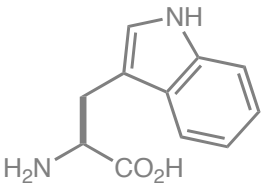
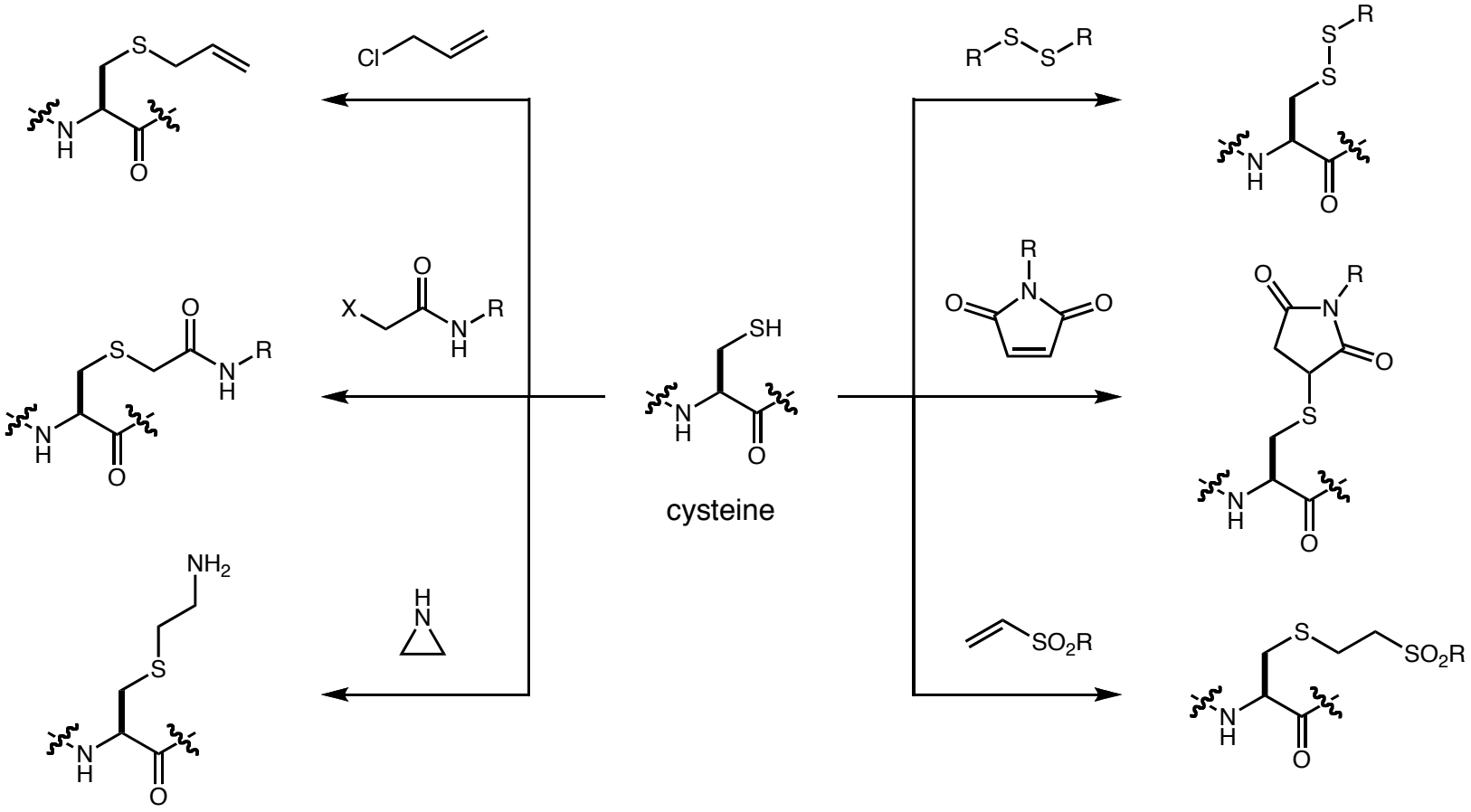


Trp

Classic Approaches to Cysteine Modification

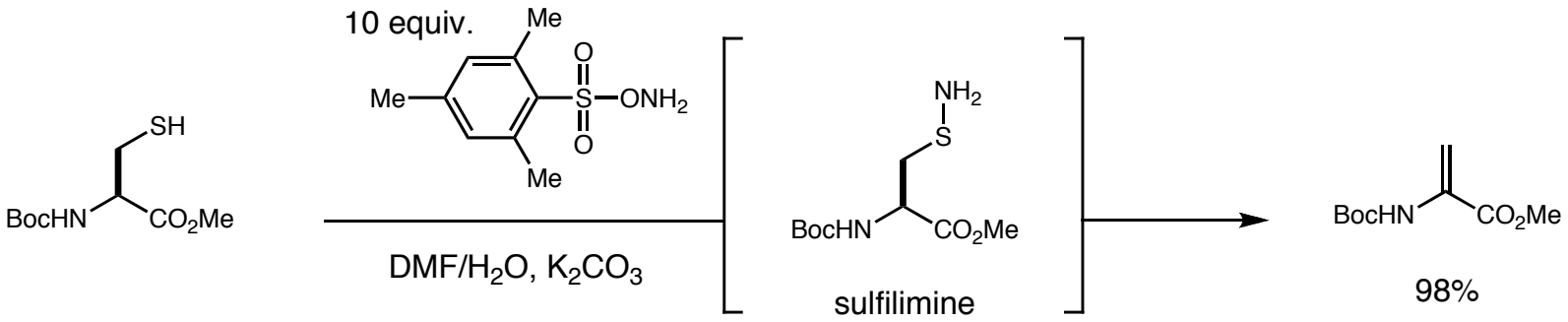
- Cysteine reacts selectively with a range of alkylating reagents and Michael acceptors



Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem. Asian J.* **2009**, *4,* 630-640.

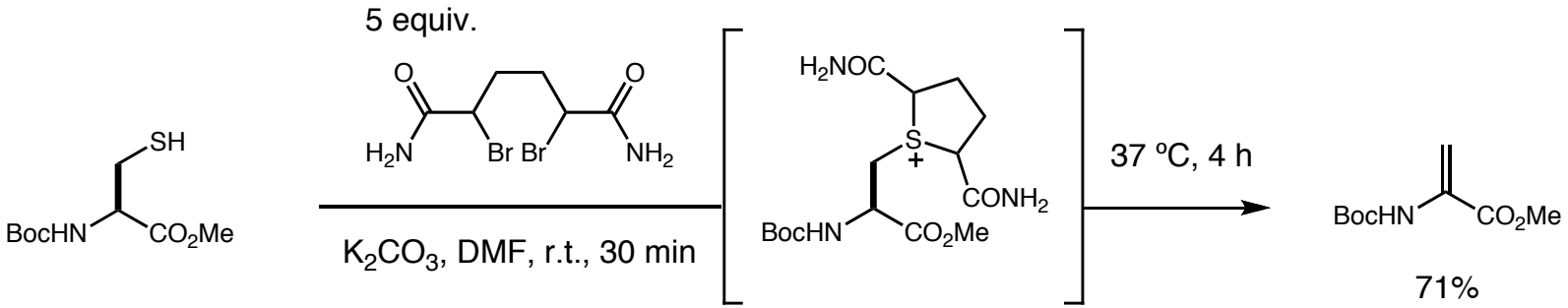
Conversion of Cysteine to Dehydroalanine

- Cys can be converted to dehydroalanine (Dha) by *o*-mesitylenesulfonylhydroxylamine (MSH)



Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130,* 5052-5053.

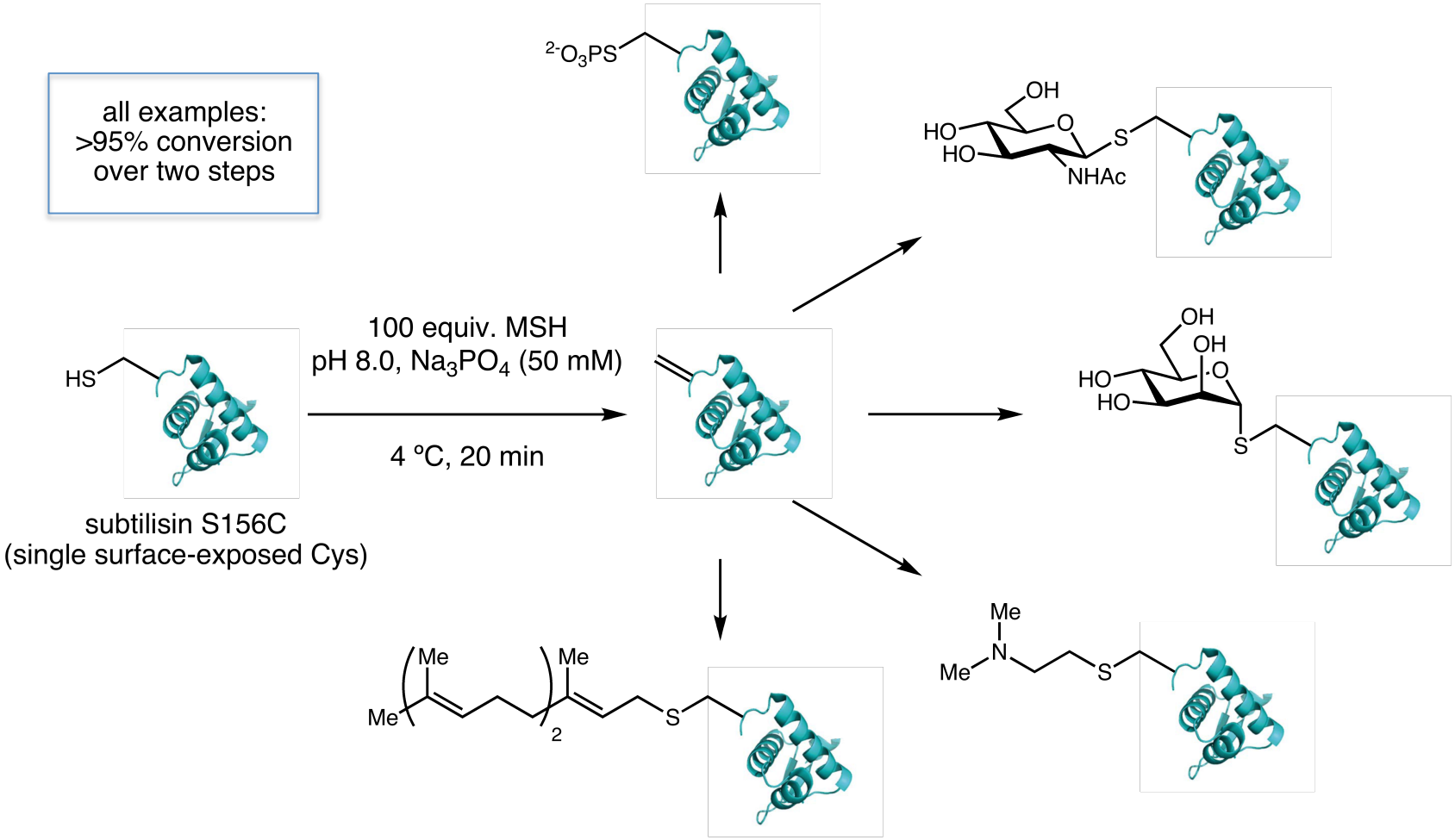
- Dha can also be generated by bis-alkylation/elimination using water-soluble dihalides



Chalker, J. M. et al. *Chem. Sci.* **2011**, *2,* 1666-1676.

Dehydroalanine Functionalization

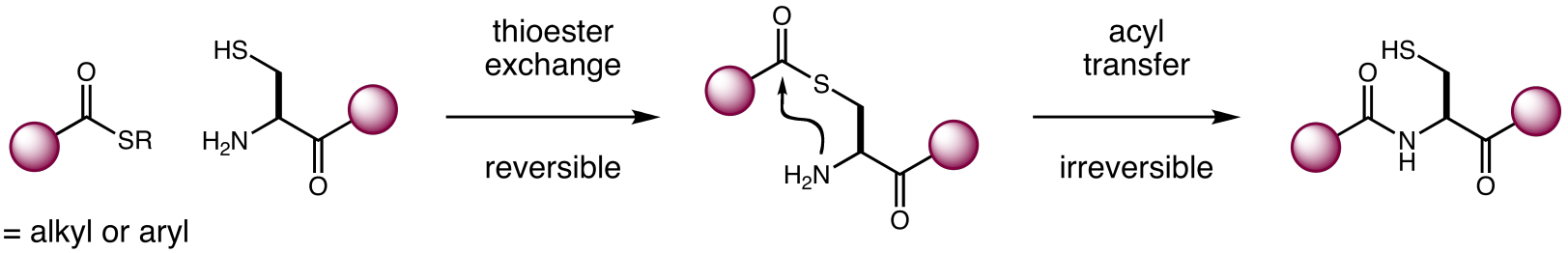
- Addition of functionalized thiols to Dha generates phosphorylated, glycosylated, and farnesylated proteins



Bernardes, G. J. L.; Chalker, J. M.; Errey, J. C.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130,* 5052-5053.

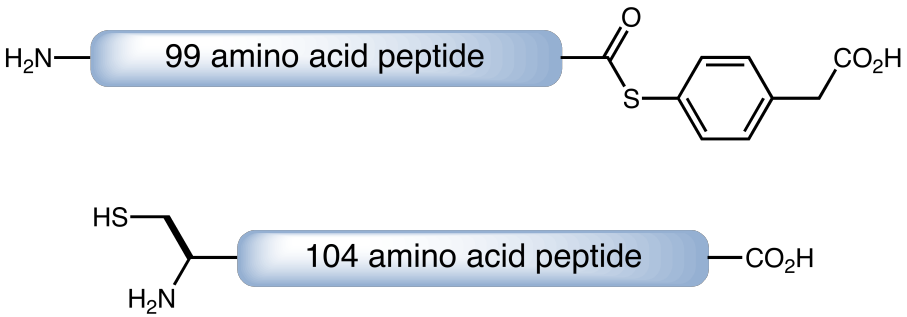
Native Chemical Ligation (NCL)

- Native chemical ligation enables union of two unprotected peptides at a cysteine residue



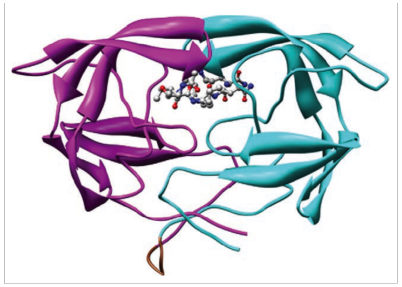
R = alkyl or aryl

- Powerful methodology for protein synthesis, enabled total chemical synthesis of HIV-1 protease



N-terminal Cys

NCL

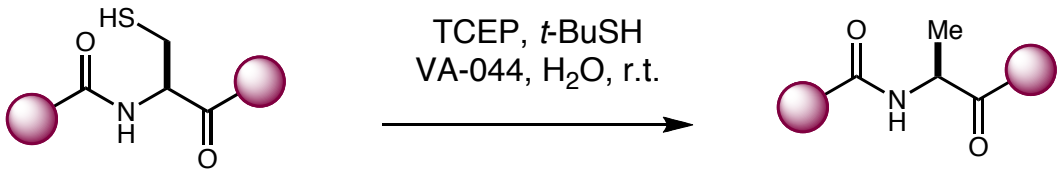


203 amino acid HIV-1 protease

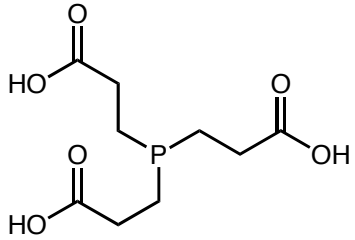
Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science.* **1994**, *266,* 776-779.
Torbeev, V. Y.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2007**, *46*, 1667-1670.

Desulfurization Expands Utility of NCL

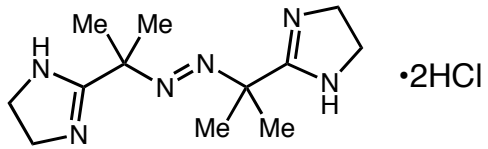
- Desulfurization converts the cysteine residue at the NCL ligation site to the more abundant alanine



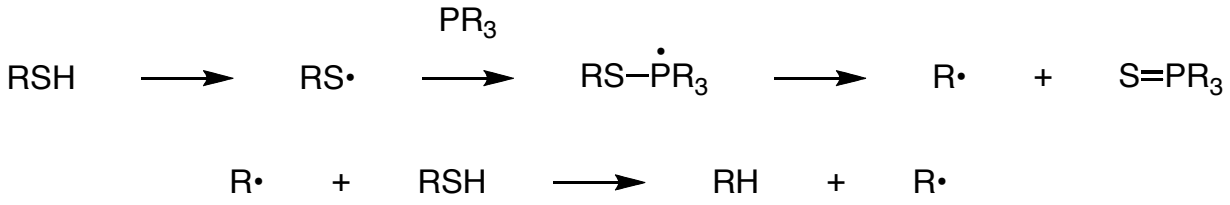
cysteine alanine



TCEP = tris(2-carboxyethyl)phosphine



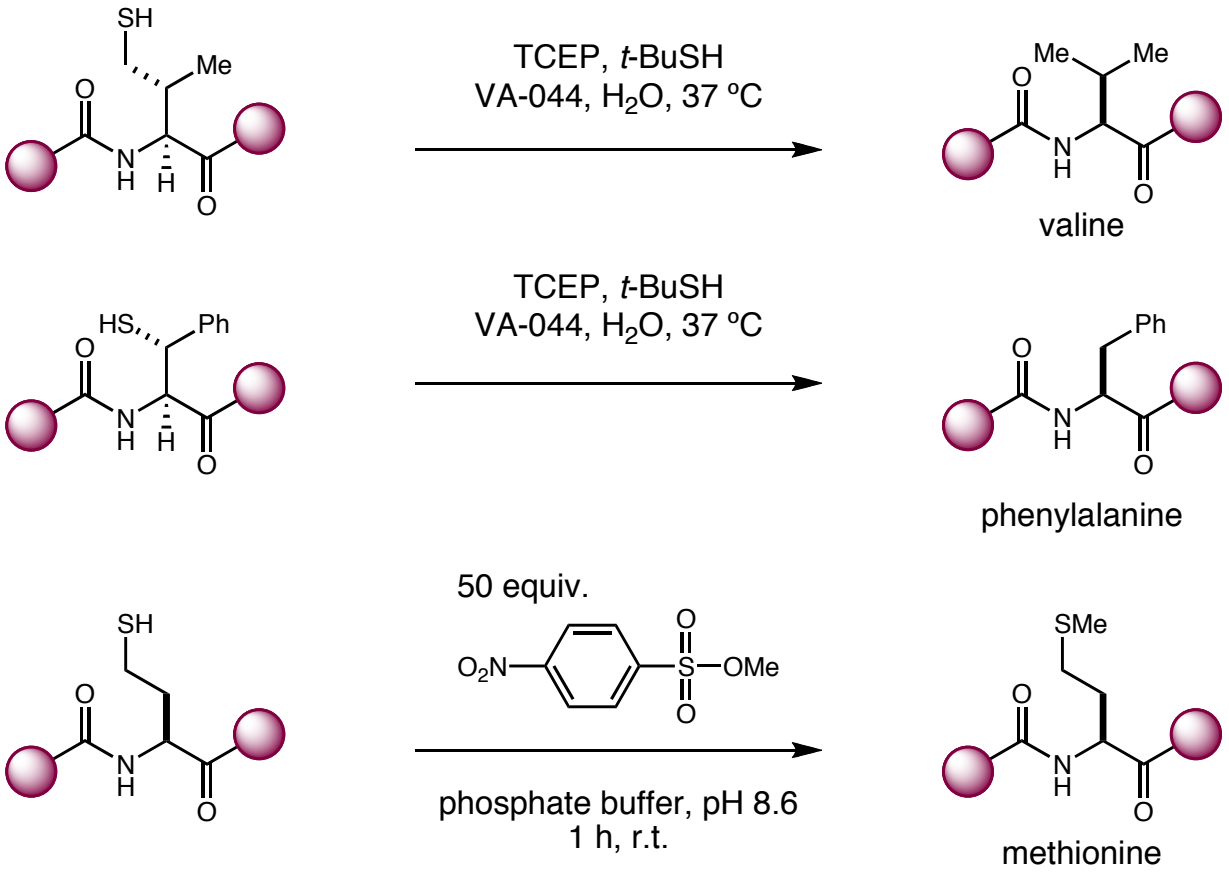
VA-044 = 2,2'-azobis[2-(2-imidazolin-2-yl) propane]dihydrochloride



Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123,* 526-533.
Wan, Q.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2007**, *46,* 9248-9252.

Use of Cysteine Analogs in the NCL

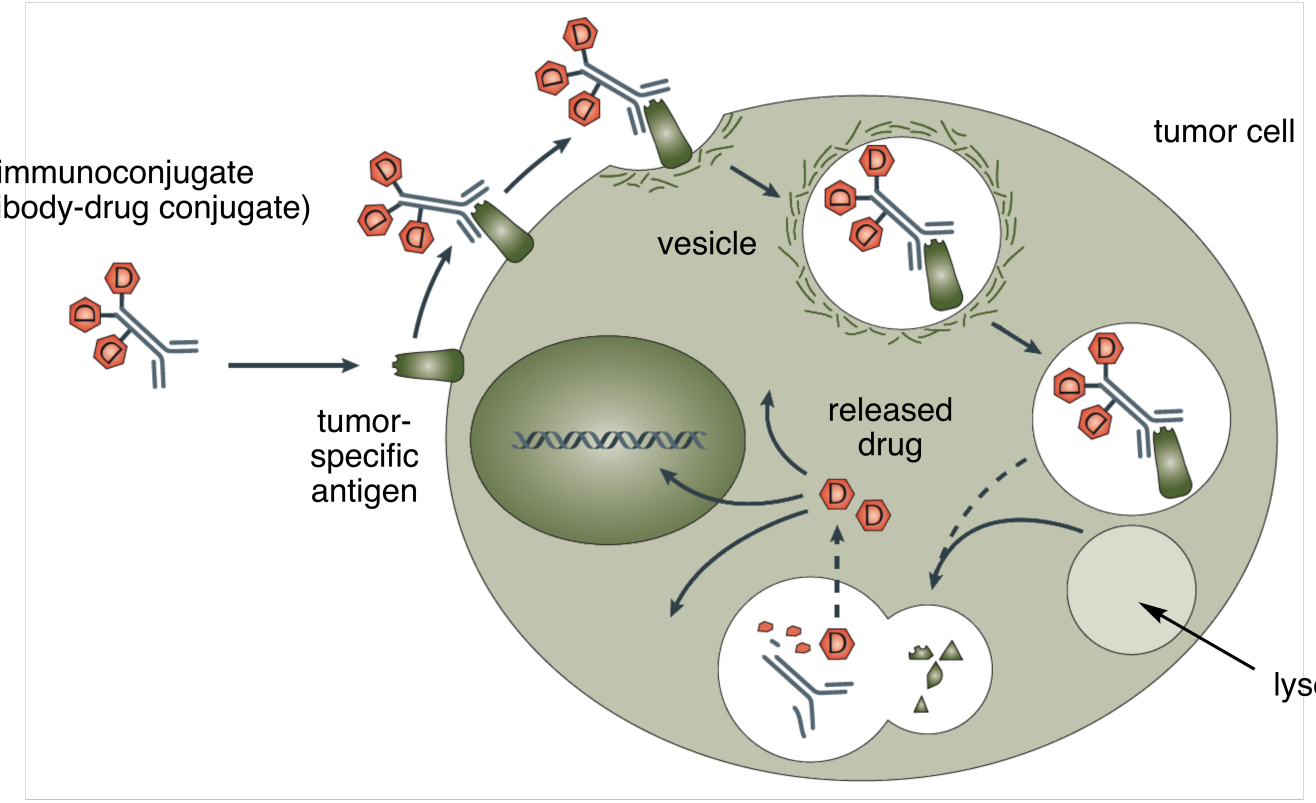
- Various cysteine analogs are competent substrates in the NCL and can be converted to natural amino acids



Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2008**, *47*, 8521-8524.
Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064-10065.
Tam, J. P.; Yu, Q. *Biopolymers*. **1998**, *46*, 319-327.

Application to Preparation of Immunoconjugates

- Immunoconjugates are monoclonal antibodies (mAbs) coupled to highly toxic agents
- Antibodies target antigens which are overexpressed on the surface of cancer cells, delivering the drug specifically to those cells, reducing systemic toxicity

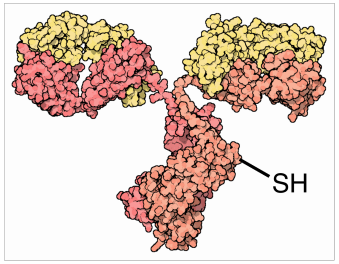


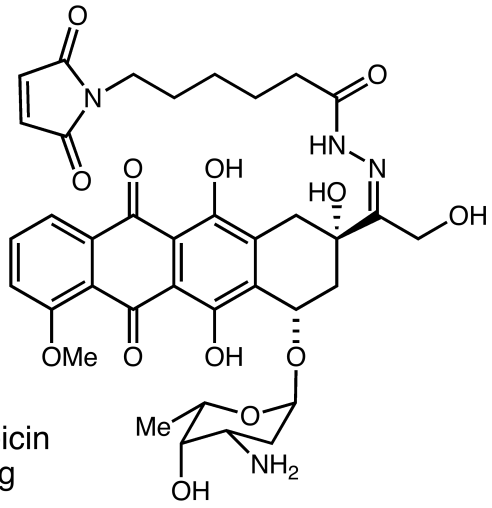
Schrama, D.; Reisfeld, R. A.; Becker, J. C. *Nature Reviews Drug Discovery.* **2006**, *5,* 147-159.

SGN-15: A Small Molecule-Linked Immunoconjugate

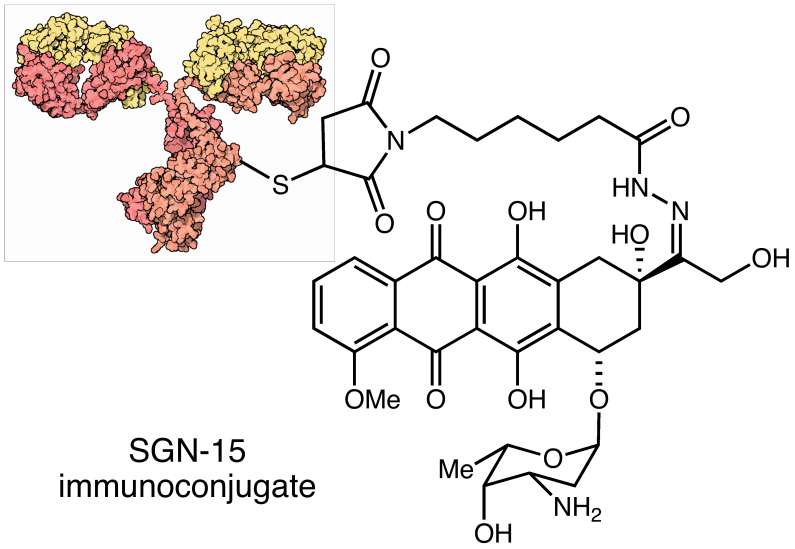
- mAb BR96 is a monoclonal antibody which targets human carcinomas
- Conjugation to the toxic small molecule doxorubicin is achieved by conjugate addition of Cys to maleimide

BR96





doxorubicin analog

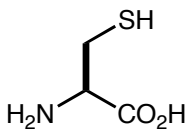


SGN-15 immunoconjugate

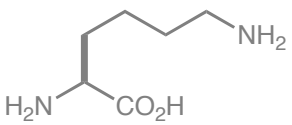
- BR96 has 8 reactive cysteines, each conjugate bears 8 doxorubicin molecules
- Acidic conditions of lysosome hydrolyze hydrazone, releasing cytotoxic drug

Wu, A. M.; Senter, P. D. *Nature Biotechnology.* **2005**, *23,* 1137-1146.

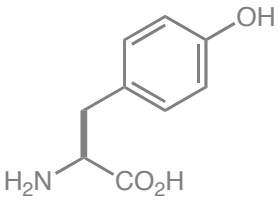
Bioconjugation: Chemical Modification of Proteins



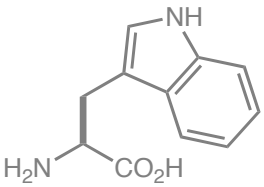
Cys



Lys



Tyr



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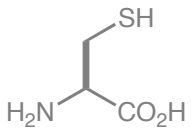
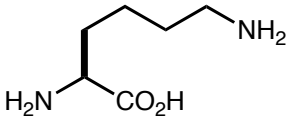
Bioconjugation: Chemical Modification of Proteins



Cys



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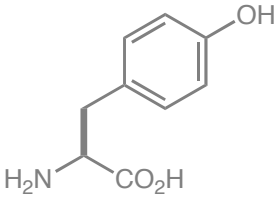

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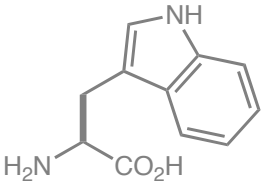

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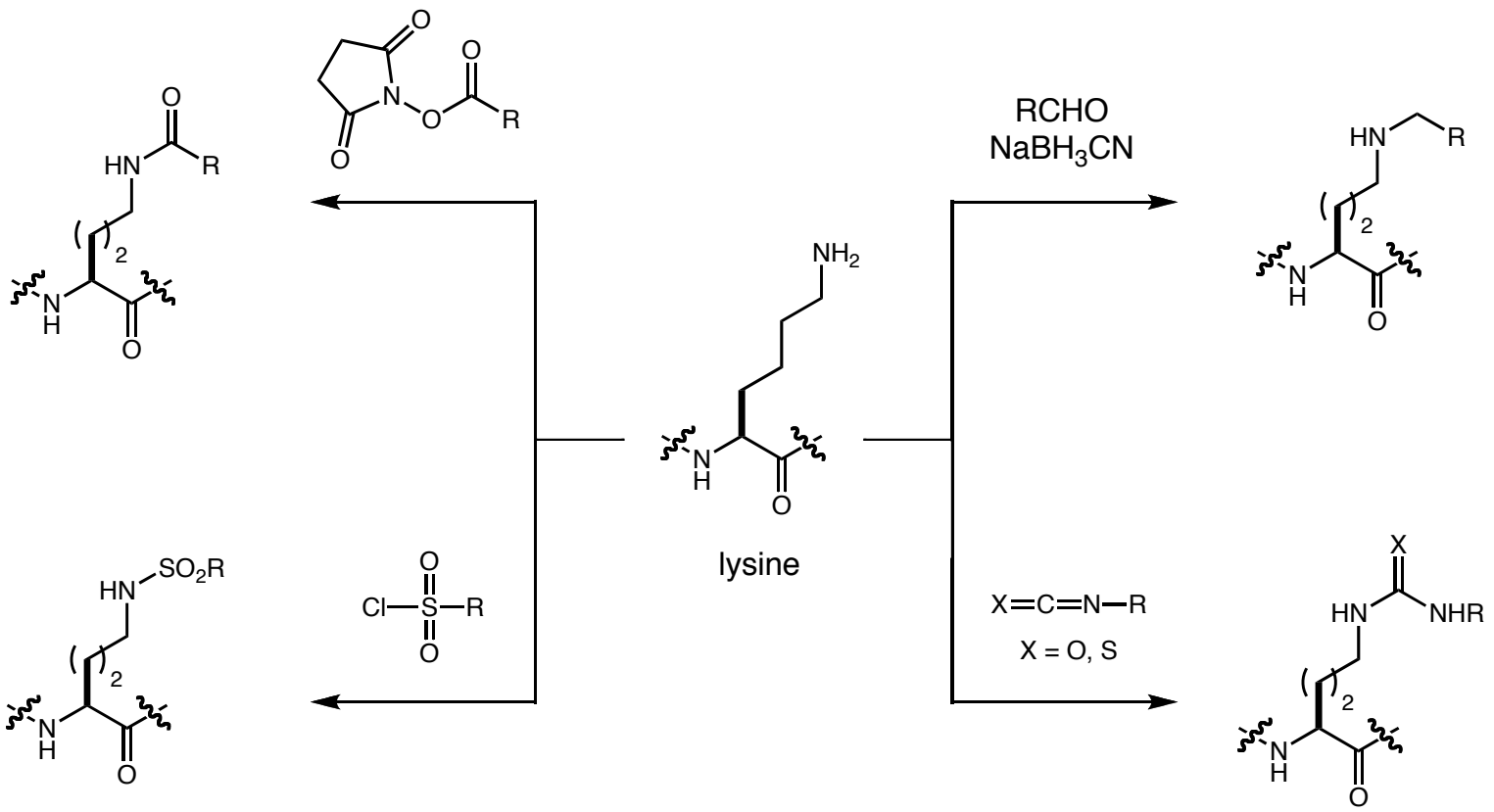
Classic Approaches to Lysine Modification

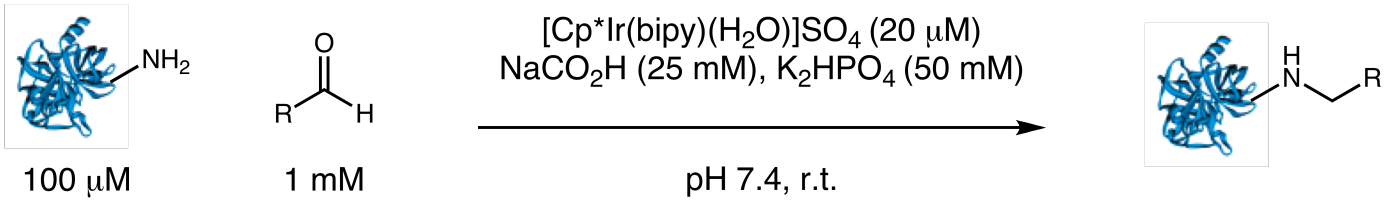
- Lysine reacts with a range of alkylation, acylation, or sulfonylation reagents

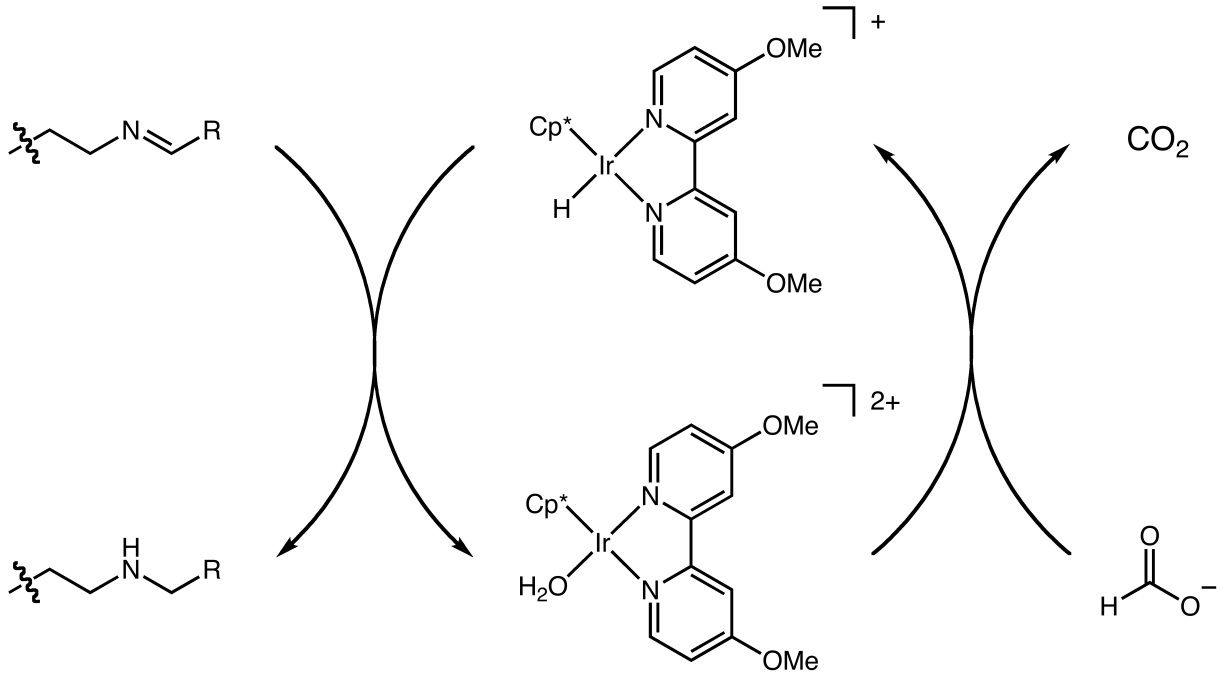


Sletten, E. M.; Bertozzi, C. R. *Angew. Chem. Int. Ed.* **2009**, *48*, 6974-6998.

Iridium-Catalyzed Reductive Alkylation of Lysine Residues

- Water-stable iridium catalyst reductively alkylates lysine under mild, non-denaturing conditions

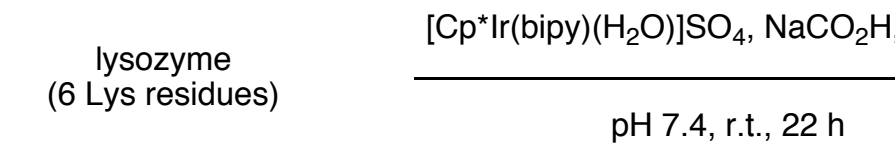




McFarland, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127,* 13490-13491.

Iridium-Catalyzed Reductive Alkylation of Lysine Residues

- A range of alkyl- and aryl-substituted aldehydes are competent reaction partners



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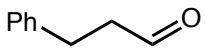
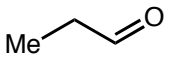
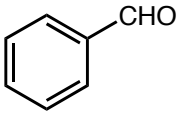
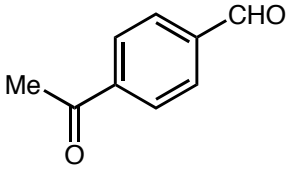
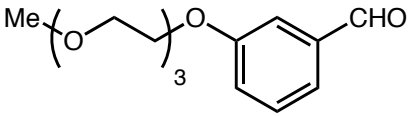
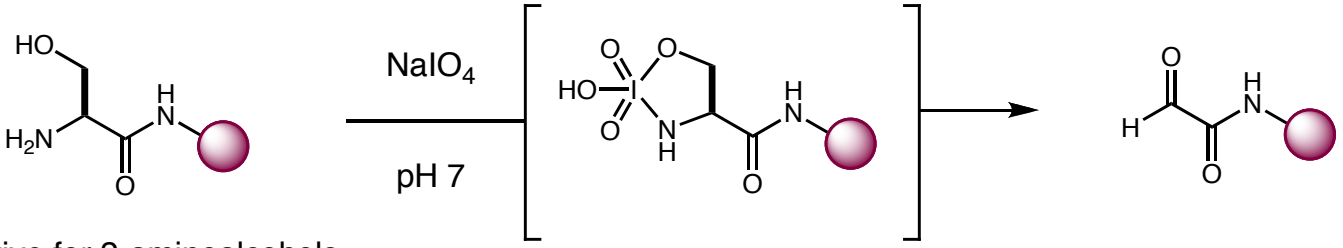
	6	21	37	25	11
	72	28	0	0	0
	41	43	16	0	0
	18	46	29	7	0
	6	26	40	23	5

McFarland, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127,* 13490-13491.

Approaches to N-Terminus Modification

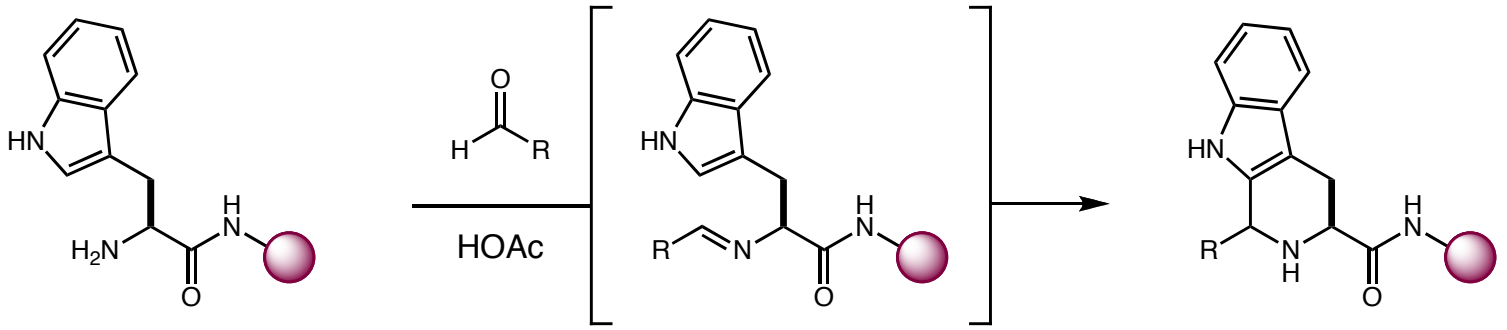
- Oxidation of *N*-terminal serine and threonine residues with periodate gives glyoxamides



selective for 2-aminoalcohols

Geoghegan, K. F.; Stroh, J. G. *Bioconjugate Chem.* **1992**, *3,* 138-146.

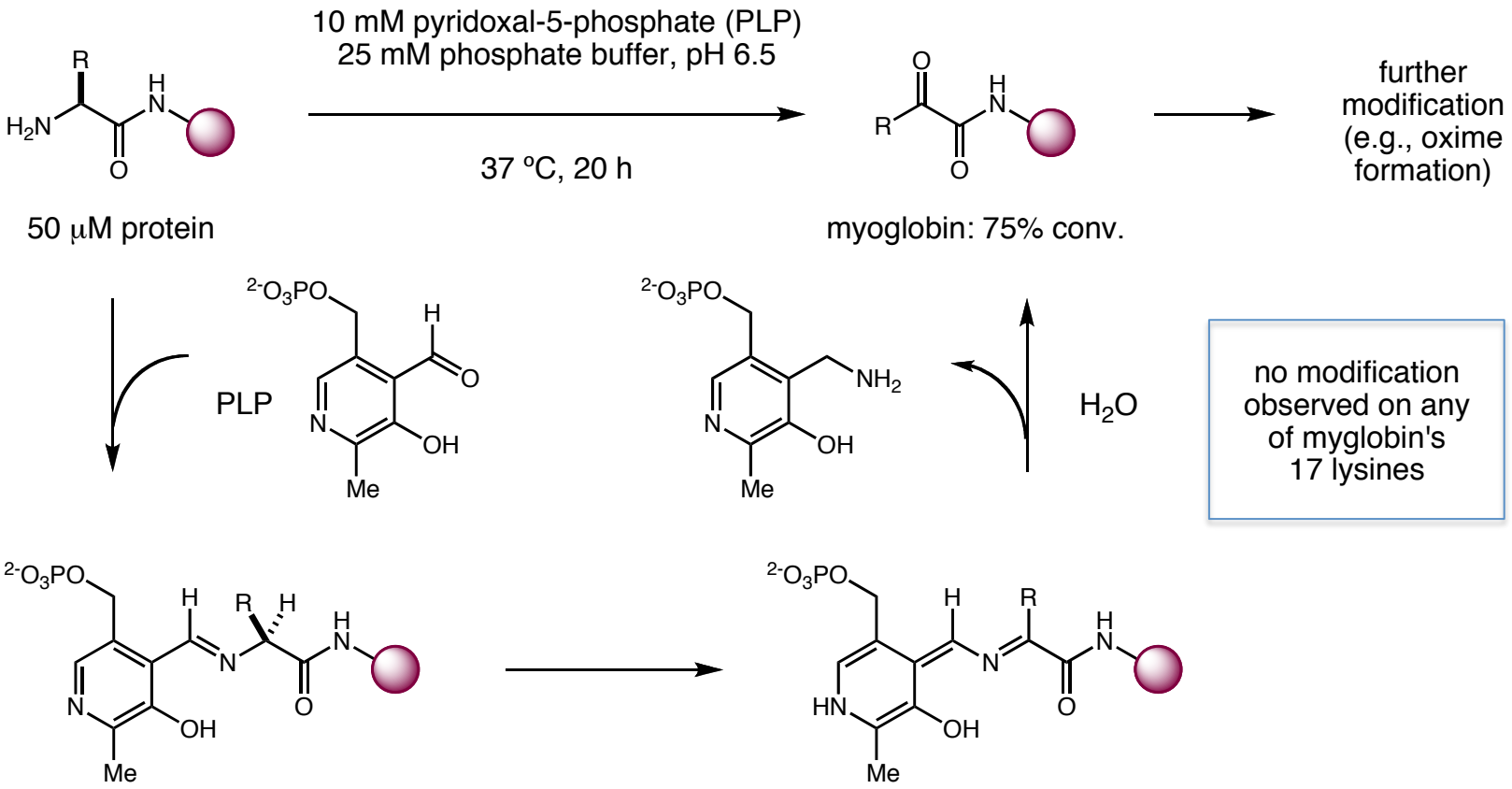
- Modification of *N*-terminal tryptophan residues through Pictet-Spengler reaction



Li, X.; Zhang, L.; Hall, S. E.; Tam, J. P. *Tetrahedron Lett.* **2000**, *41,* 4069-4073.

N-Terminus Modification via Transamination

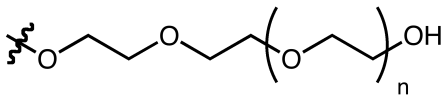
- Pyridoxal-5-phosphate (PLP) converts *N*-terminal residues to α-ketoamides, enabling further modification



Gilmore, J. M.; Scheck, R. A.; Esser-Kahn, A. P.; Joshi, N. S.; Francis, M. B. *Angew. Chem. Int Ed.* **2006**, *45,* 5307-5311.

PEGylation

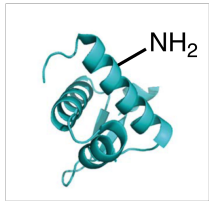
- Conjugation of poly(ethylene glycol) (PEG) chains to lysine improves properties of therapeutic proteins

PEG = 

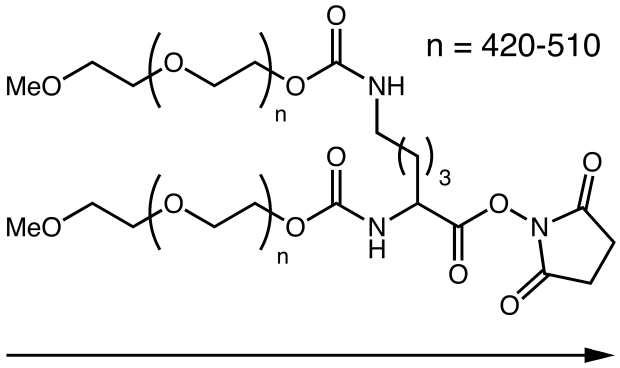
branched or linear, mixture of molecular weights

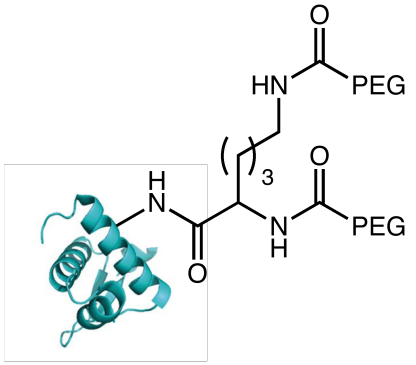
- Higher solubility
- Longer *in vivo* half lives
- Reduced immunogenicity
- Resistance to proteolysis

- Pegasys® is a 40 kDa PEG-conjugated interferon-α-2a, approved in 2002 for treatment of hepatitis C



interferon-α-2a
half life = 0.7 h

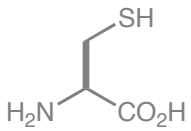




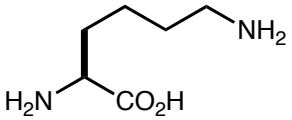
Pegasys® PEG-drug conjugate
half life = 51 h

Veronese, F. M.; Pasut, G. *Drug Discovery Today*. **2005**, *10,* 1451-1458.
Bailon, P. et al. *Bioconjugate Chem*. **2001**, *12*, 195-202.

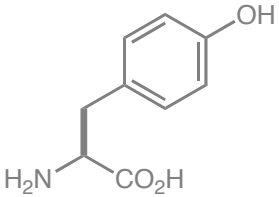
Bioconjugation: Chemical Modification of Proteins



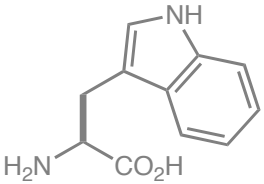
Cys



Lys

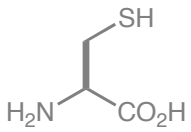


Tyr

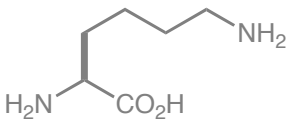


Trp

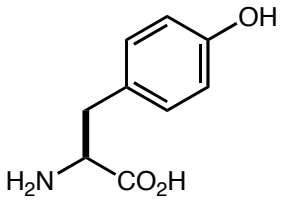
Bioconjugation: Chemical Modification of Proteins



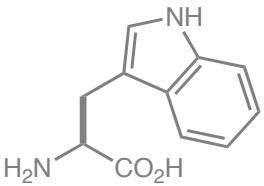
Cys



Lys



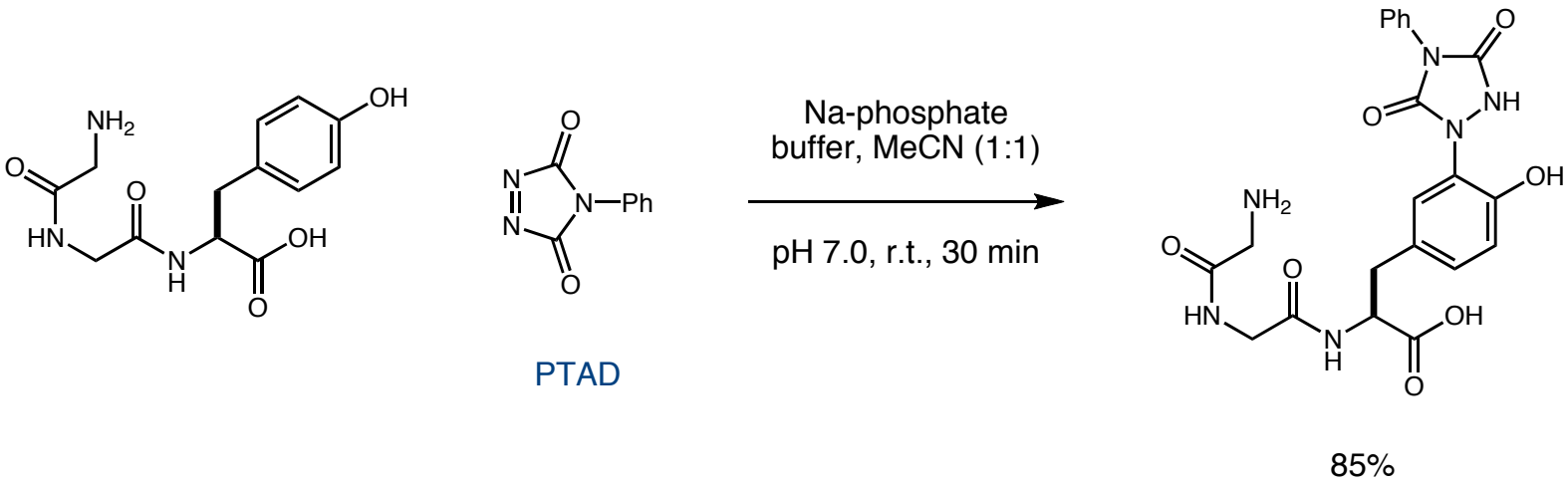
Tyr



Trp

Tyrosine Modification with PTAD

- 4-phenyl-3*H*-1,2,4-triazole-3,5(4*H*)-dione (PTAD) engages in an ene reaction with tyrosine

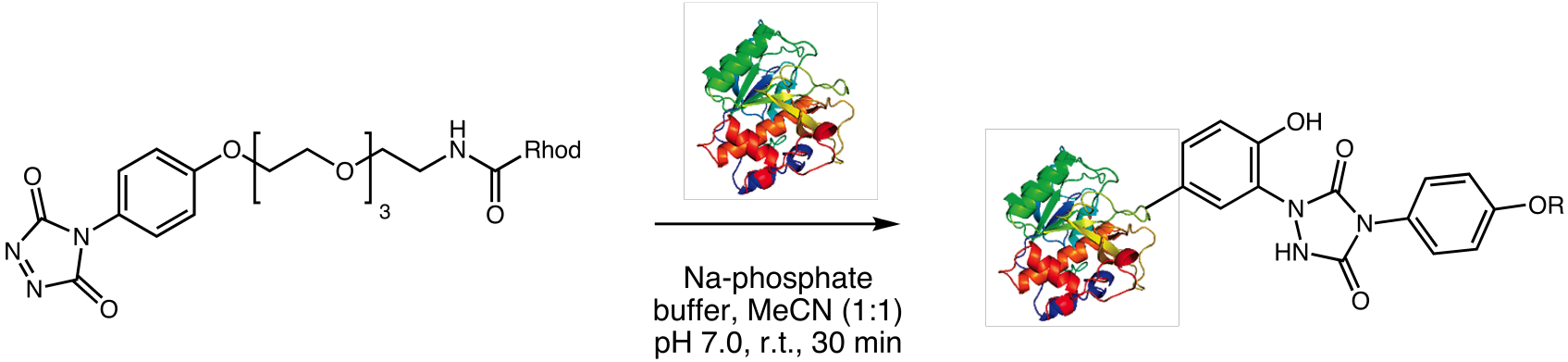


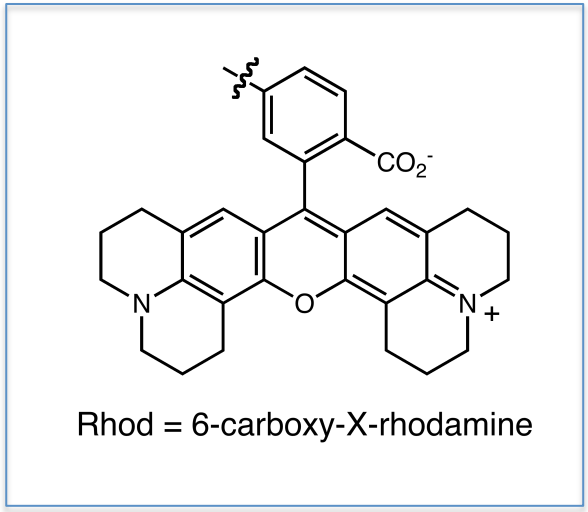
- Reaction is selective for tyrosine (although Trp and Lys give trace product in absence of Tyr)
- Adducts are stable from pH 2 to pH 10 and do not decompose at high temperature (120 °C)
- No bis-addition products are observed

Ban, H.; Gavrilyuk, J.; Barbas, C. F. III. *J. Am. Chem. Soc.* **2010**, *132,* 1523-1525.

Tyrosine Modification with PTAD

- Utility demonstrated by linking a rhodamine dye to tyrosine-containing proteins



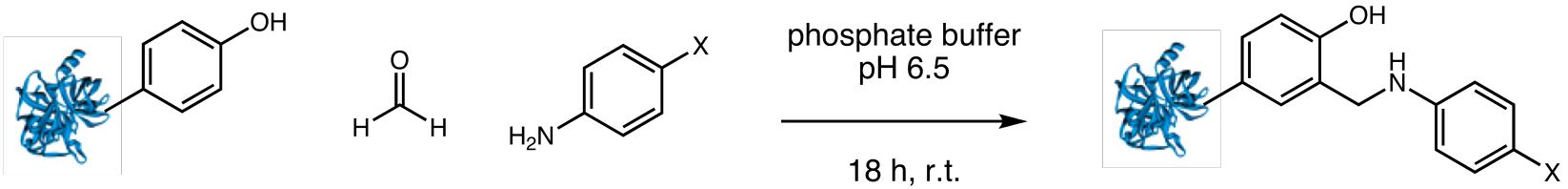


	conversion by UV-vis
bovine serum albumin	96%
chymotrypsinogen A	81%
myoglobin	8%

Ban, H.; Gavrilyuk, J.; Barbas, C. F. III. *J. Am. Chem. Soc.* **2010**, *132,* 1523-1525.

Tyrosine Alkylation via Three-Component Mannich Reaction

- Iminium ions generated from simple aldehydes and anilines react selectively with tyrosine

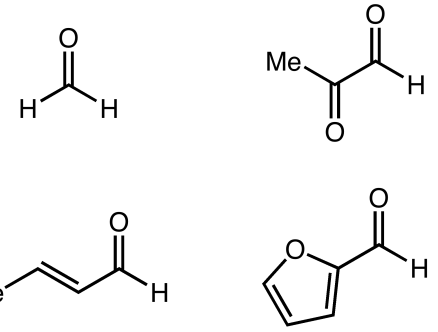


chymotrypsinogen A (200 μM) | 25 mM | 25 mM

X substituent	+1 mod. (%)	+2 mod. (%)
$-CH_2CH_2OH$	45	35
$-CH_2CH_2NH_2$	47	26
$-CH_3$	46	16
$-OCH_3$	43	0
$-Cl$	36	11
$-CO_2H$	33	10
$-F$	9	0
$-NO_2$	0	0
–rhodamine dye	66	0

2 262

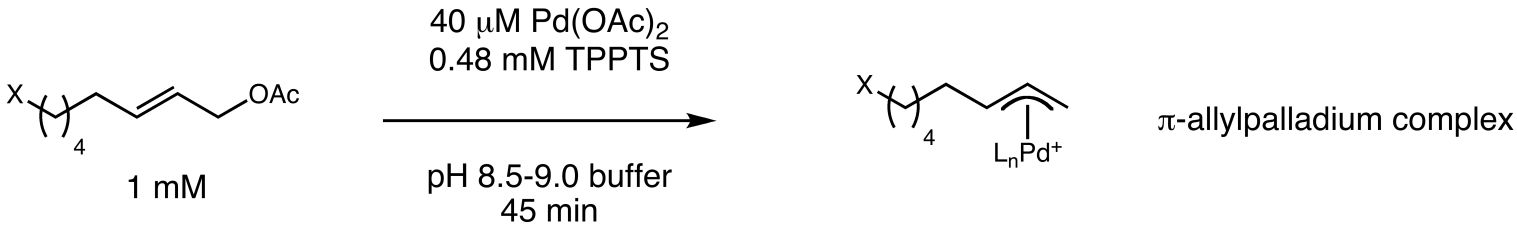
Aldehyde Scope



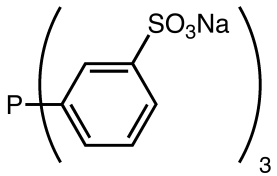
Joshi, N. S.; Whitaker, L. R.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126,* 15942-15943.

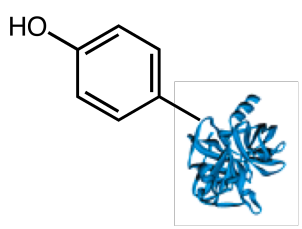
Tyrosine Allylation with π-Allylpalladium Complexes

- π-Allylpalladium complexes generated from allylic acetates selectively modify tyrosine residues



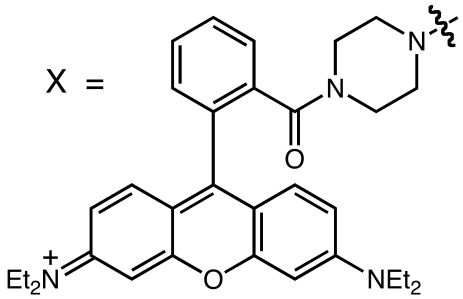
TPPTS = triphenylphosphine tris(sulfonate)

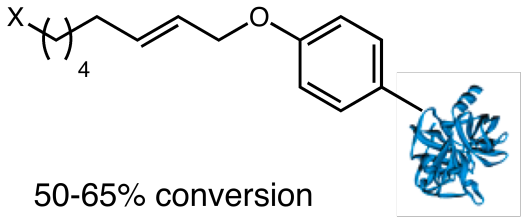




chymotrypsinogen A (200 μM)

X =



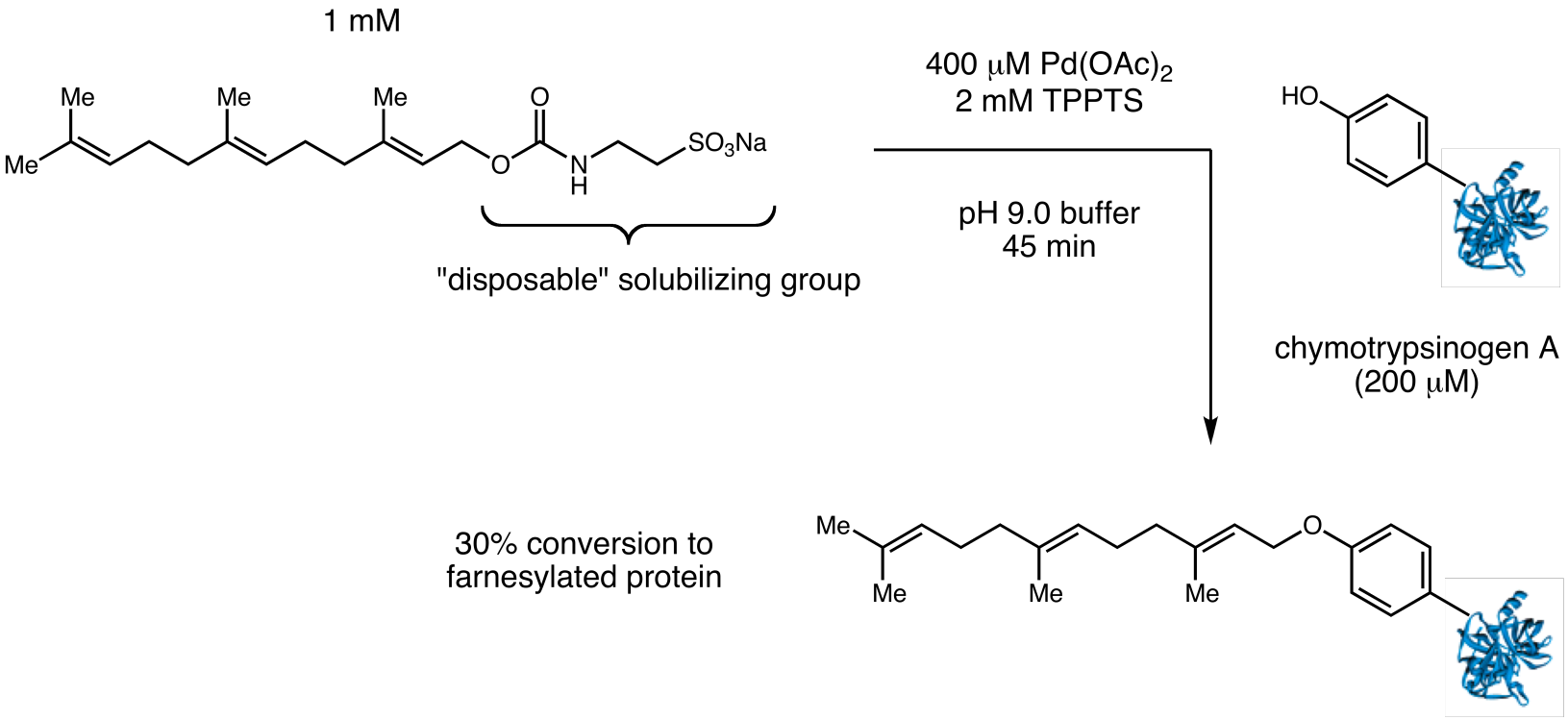


50-65% conversion

Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128,* 1080-1081.

Tyrosine Allylation with π-Allylpalladium Complexes

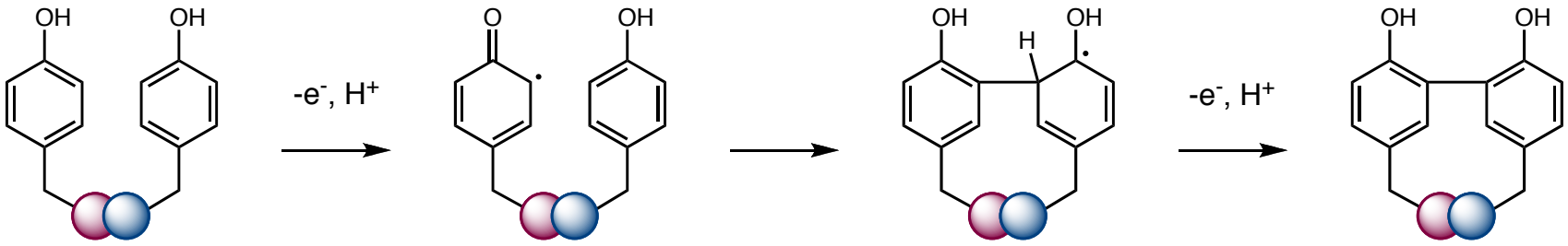
- Highly polar leaving groups solubilize allyl carbamate reagent and enable transfer of hydrophobic groups



Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128,* 1080-1081.

Protein Cross-Linking via Tyrosine Oxidation

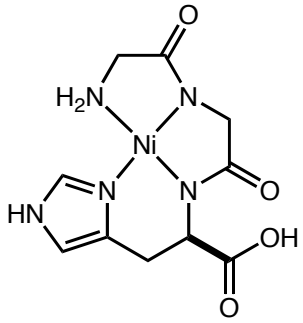
- Metal oxidants induce coupling of tyrosine residues brought into proximity by protein-protein interactions



protein-protein interaction

tyrosyl radical

cross-linked proteins



Ni(II)(Gly-Gly-His) (**A**)

suitable oxidants:
magnesium monoperoxyphthalate (MMPP) or oxone ($KHSO_5$)

UvsX gp32

proteins known to bind each other

excess **A**, MMPP →

covalent UvsX-gp32 dimer

ubiquitin BSA

proteins known to be monomeric

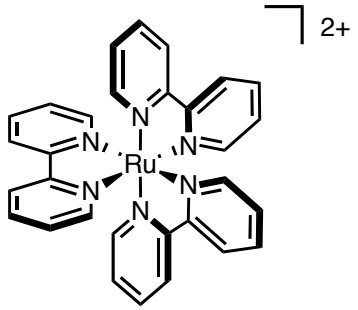
excess **A**, MMPP →

no cross-linking observed (even at 100 μM protein)

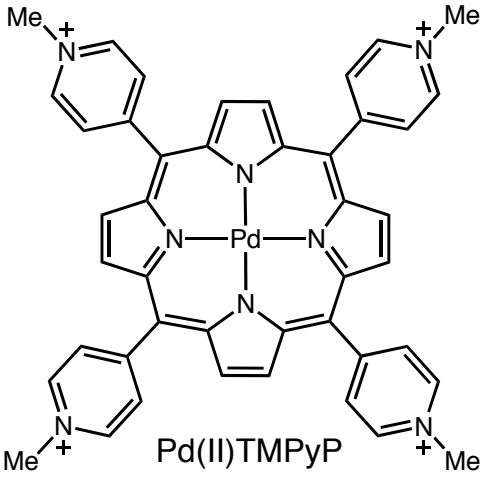
Brown, K. C.; Yang, S.-H.; Kodadek, T. *Biochemistry*. **1995**, *34*, 4733-4739.

Photoinitiated Protein Cross-Linking

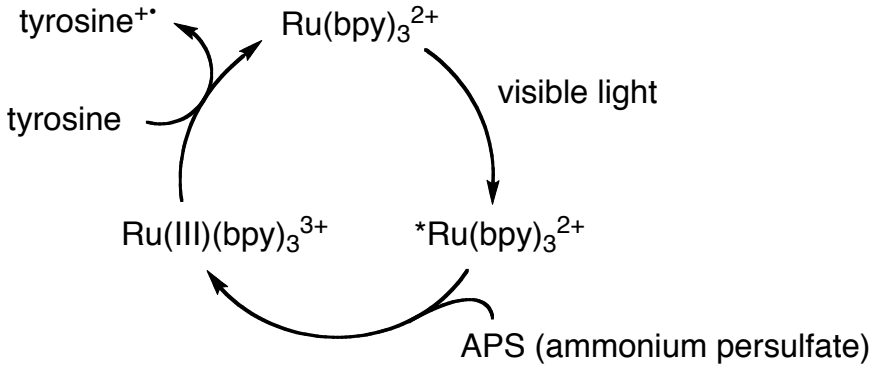
- $Ru(bpy)_3^{2+}$ and palladium porphyrins initiate oxidative cross-linking upon irradiation with visible light

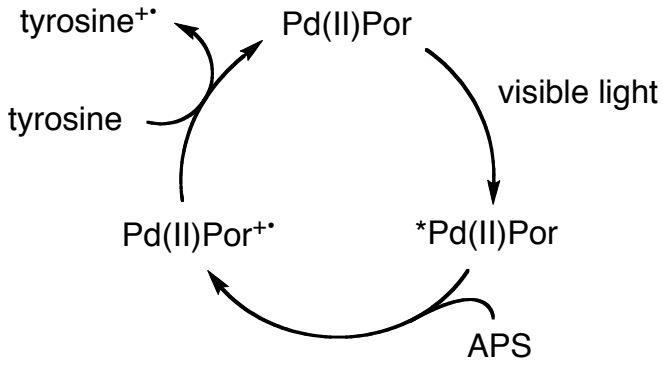


$Ru(bpy)_3Cl_2$



Pd(II)TMPyP

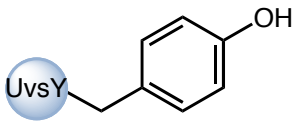




Fancy, D. A.; Kodadek, T. *Proc. Natl. Acad. Sci.* **1999**, *96,* 6020-6024.
Kim, K.; Fancy, D. A.; Carney, D.; Kodadek, T. *J. Am. Chem. Soc.* **1999**, *121*, 11896-11897.

Photoinitiated Protein Cross-Linking

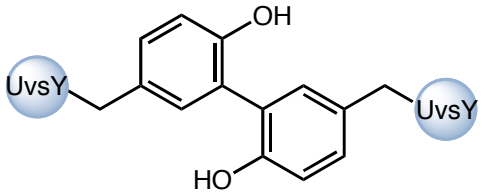
- $Ru(bpy)_3^{2+}$ and palladium porphyrins perform oxidative cross-linking chemistry very rapidly



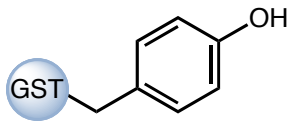
UvsY exists as a hexamer

$Ru(bpy)_3^{2+}$, APS

0.5 s, visible light (150 W)



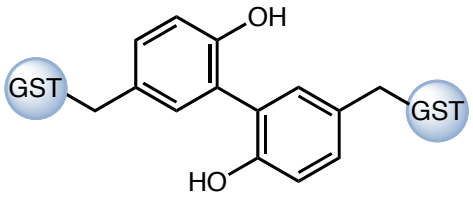
60% yield of cross-linked products



glutathione S-transferase (GST) exists as a homodimer

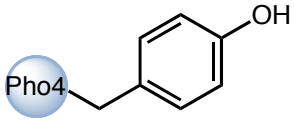
Pd(II)TMPyP, APS

10 s, visible light (150 W)



50% yield of cross-linked products

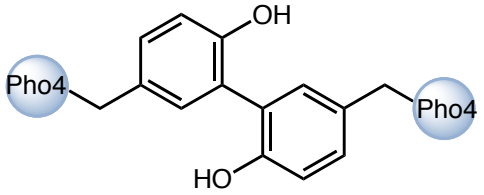
- Reaction proceeds in cell lysate: reveals interactions of one protein in the presence of thousands of others



dimeric yeast transcription factor

$Ru(bpy)_3^{2+}$, APS

1 s, visible light (150 W)
E. coli cell lysate



Fancy, D. A.; Kodadek, T. *Proc. Natl. Acad. Sci.* **1999**, *96*, 6020-6024.
Kim, K.; Fancy, D. A.; Carney, D.; Kodadek, T. *J. Am. Chem. Soc.* **1999**, *121*, 11896-11897.
Fancy, D. A.; Denison, C.; Kim, K.; Xie, Y.; Holdeman, T.; Amini, F.; Kodadek, T. *Chem. Biol.* **2000**, *7*, 697-708.

Application to Mapping Protein-Protein Interactions

- Tyrosine cross-linking employed to map subunit interactions in the yeast 20S proteasome

20S proteasome: large multi-protein complex

↓ $Ru(bpy)_3^{2+}$, APS, visible light, 0.5 s

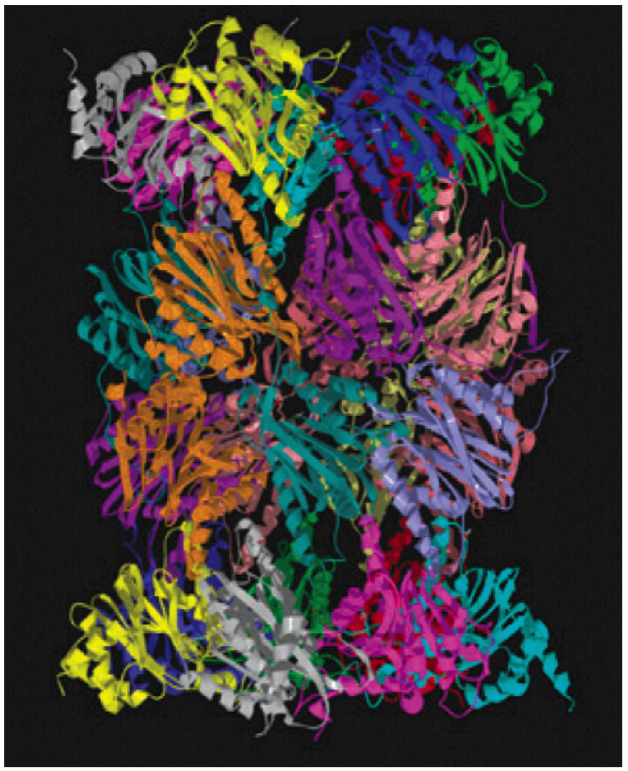
subunits which make contacts with each other are cross-linked

↓ separate fragments by electrophoresis

digest separated cross-linked fragments with trypsin

↓ identify proteins by mass spectrometry

use data to construct a "map" of the complex



20S proteasome
(2 copies each of 14 unique proteins)

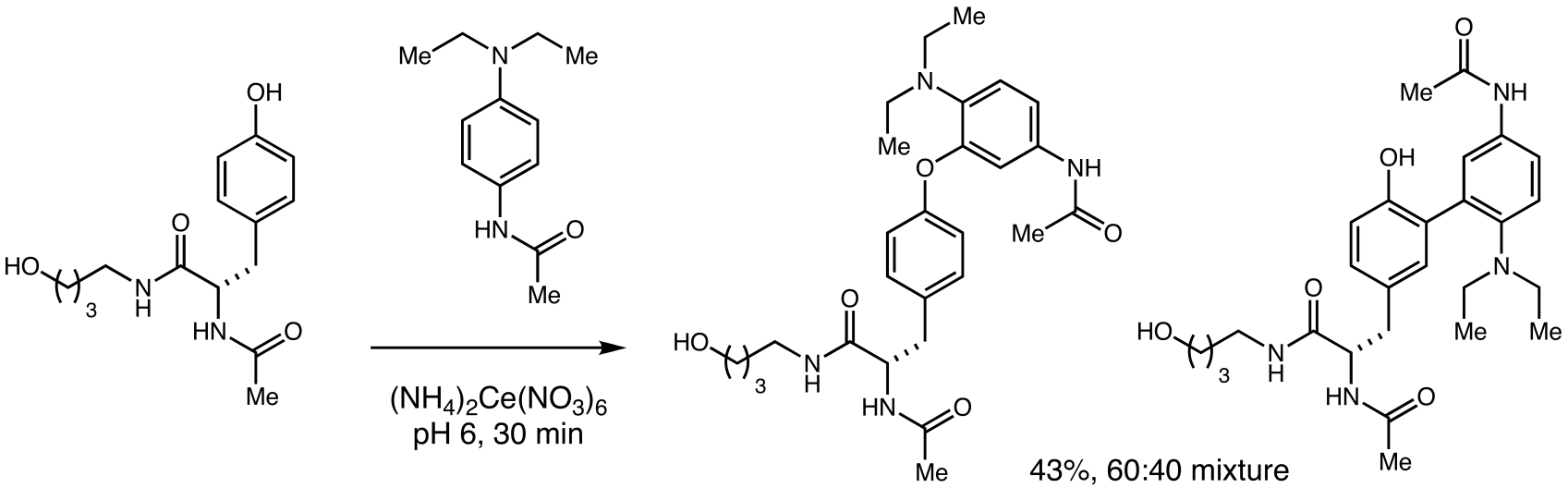
- Method successfully identified interactions between 12 of the 14 subunits, crystal structure confirmed that none are "false positives"

- Limitations: Depends on presence of Tyr residues at subunit interfaces; can be difficult to separate cross-linked dimers of similar molecular weight; does not identify which residues form interface

Denison, C.; Kodadek, T. *J. Proteome Res.* **2004**, *3,* 417-425.

Oxidative Coupling of Tyrosine with Anilines

- Cerium(IV) ammonium nitrate (CAN) promotes oxidative coupling between tyrosine and anilines

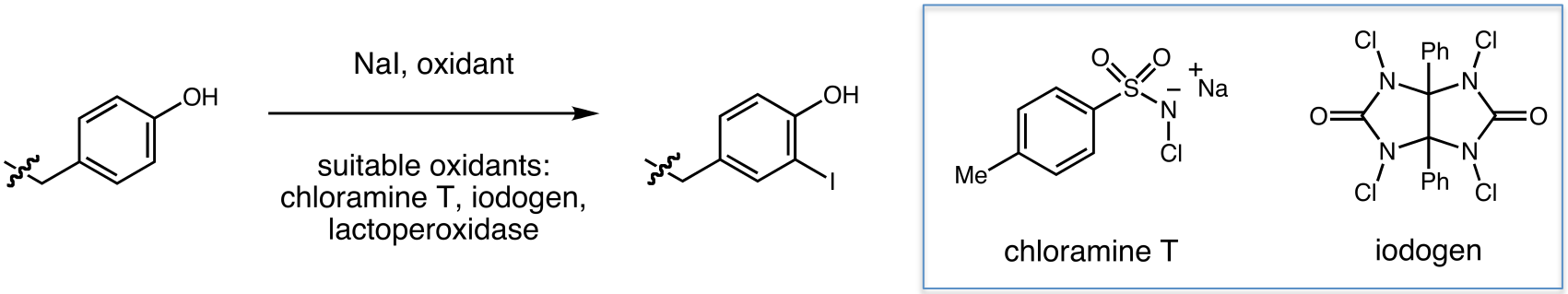


- Applied to the modification of the tyrosine residue of angiotensin

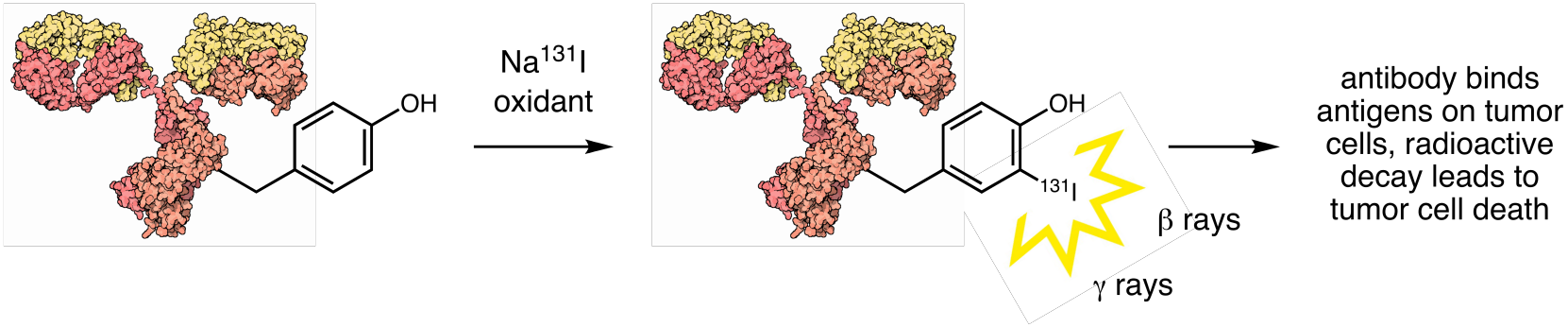
Seim, K. L.; Obermeyer, A. C.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133,* 16970-16976.

Radioiodination of Tyrosine Residues

- Iodination of proteins selectively installs iodine at ortho position of tyrosine



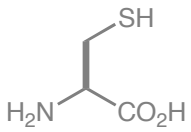
- Radioimmunotherapy: "armed" antibodies are generated by iodination with radioactive isotope ^{131}I



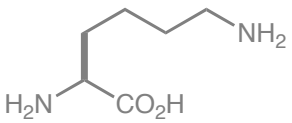
- Tositumomab (Bexxar) is a ^{131}I-containing monoclonal antibody approved in 2003 for the treatment of non-Hodgkin's lymphoma

Milenic, D. E.; Brady, E. D.; Brechbiel, M. W. *Nature Reviews Drug Discovery.* **2004**, *3,* 488-498.
Nikula, T. K.; Bocchia, M.; Curcio, M. J.; Sgouros, G.; Ma, Y.; Finn, R. D.; Scheinberg, D. A. *Molecular Immunology.* **1995**, *32*, 865-872.

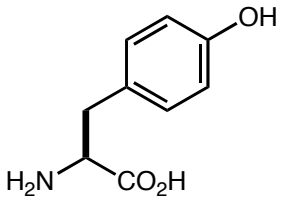
Bioconjugation: Chemical Modification of Proteins



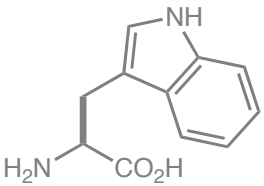
Cys



Lys

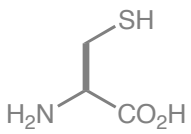


Tyr

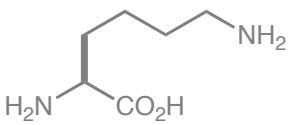


Trp

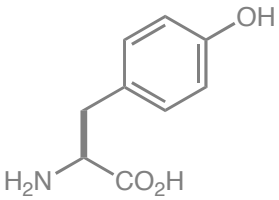
Bioconjugation: Chemical Modification of Proteins



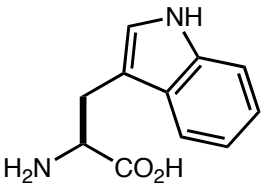
Cys



Lys



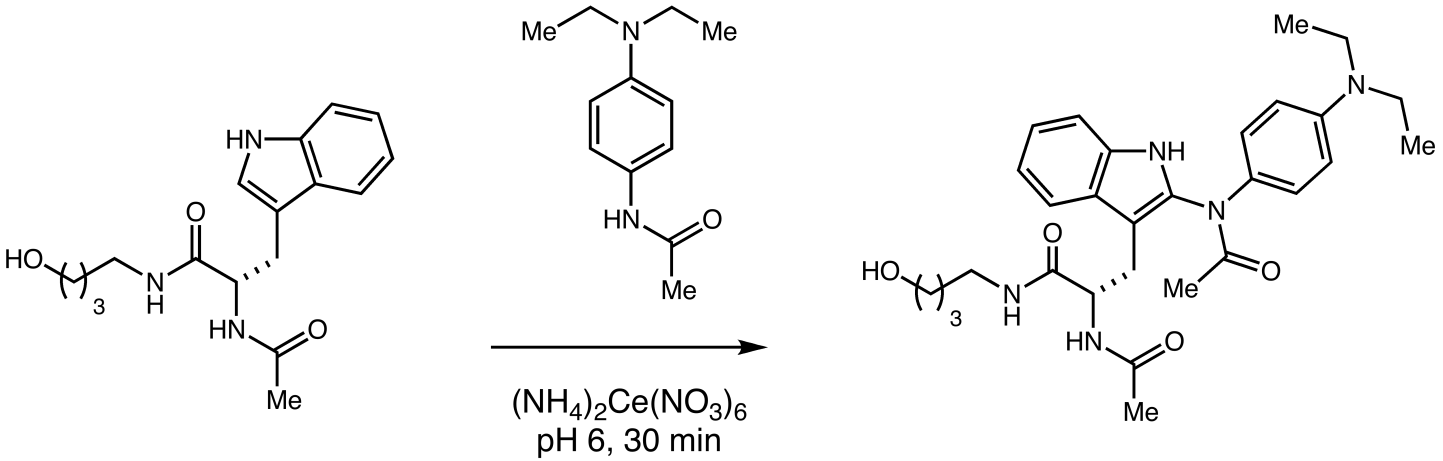
Tyr



Trp

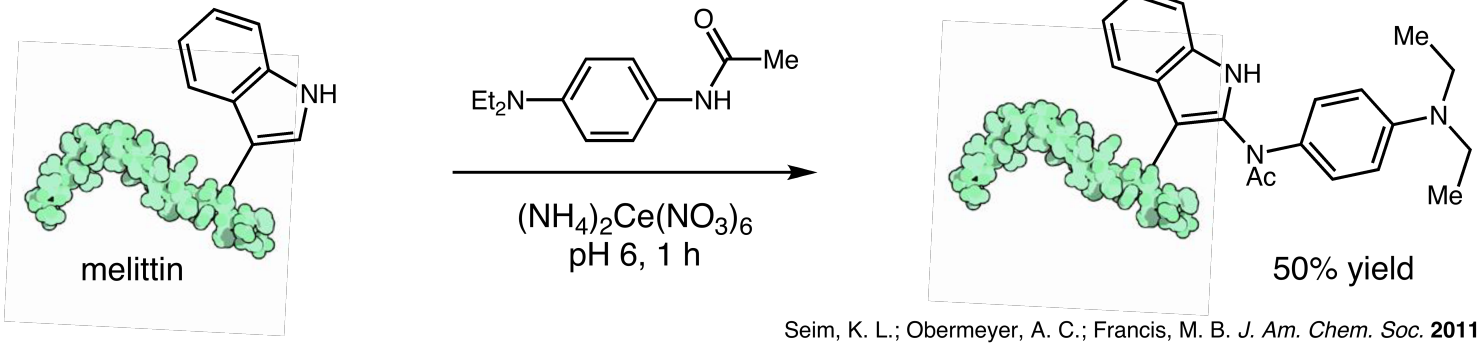
Oxidative Coupling of Tryptophan with Anilines

- Analogous to oxidative coupling of tyrosine residues, CAN also promotes oxidative coupling of tryptophan



43%, single product

- Applied to the modification of the tryptophan residue of melittin

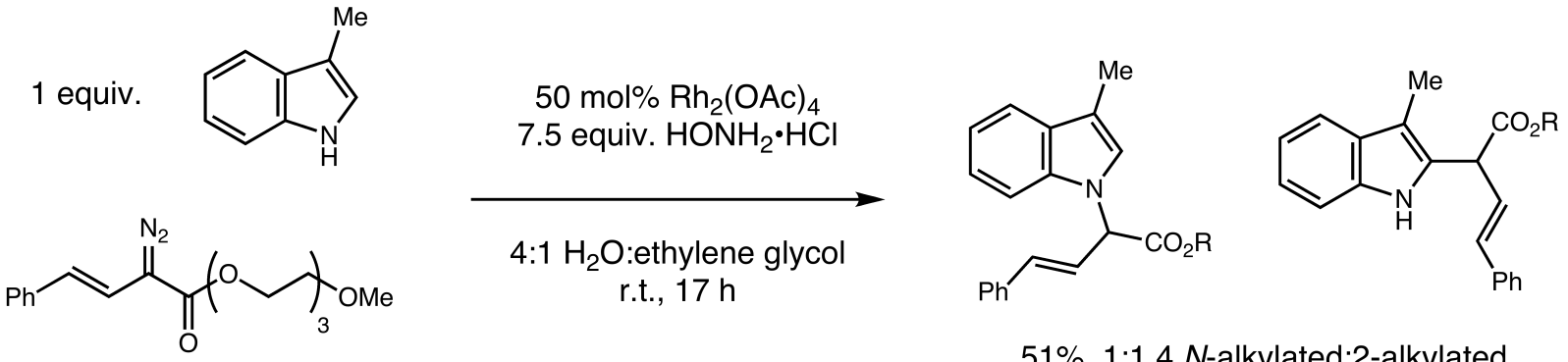


50% yield

Seim, K. L.; Obermeyer, A. C.; Francis, M. B. *J. Am. Chem. Soc.* **2011**, *133,* 16970-16976.

Tryptophan Modification with Rhodium Carbenoids

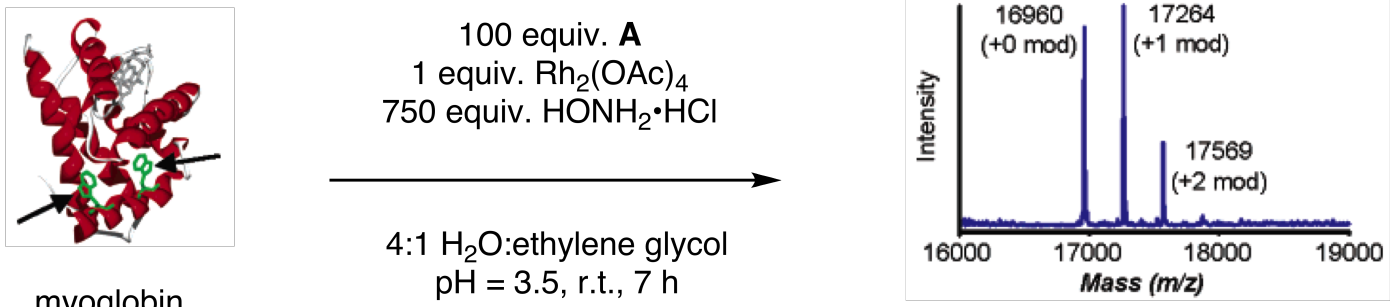
- Rhodium carbenoids efficiently functionalize 3-methylindole in aqueous solution



A, 4.0 equiv.

51%, 1:1.4 *N*-alkylated:2-alkylated

- Applicable to the modification of horse heart myoglobin



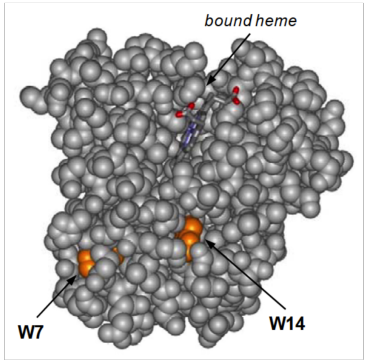
myoglobin

60% conversion to mono- and dimodified myoglobin

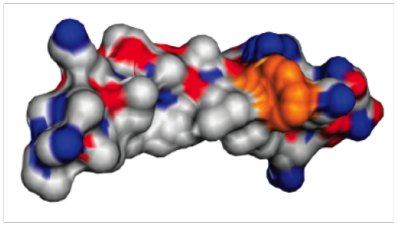
Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126,* 10256-10257.

Tryptophan Modification with Rhodium Carbenoids

- Major drawback: low pH is required to denature proteins; most Trp residues are not solvent accessible

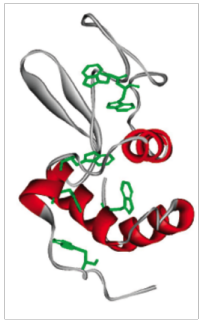


myoglobin: Trp residues buried



melittin: highly exposed tryptophan
successfully modified at pH 6.0 (50% conversion)

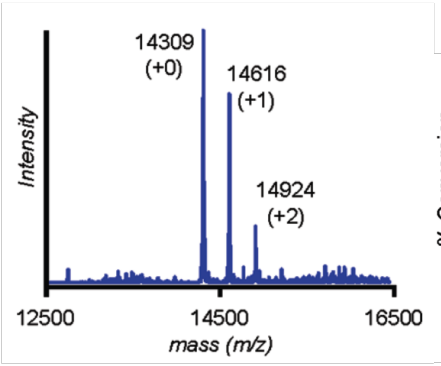
- Reactivity correlates with known thermal stability; lysozyme is known to denature above 74 ºC at pH 7

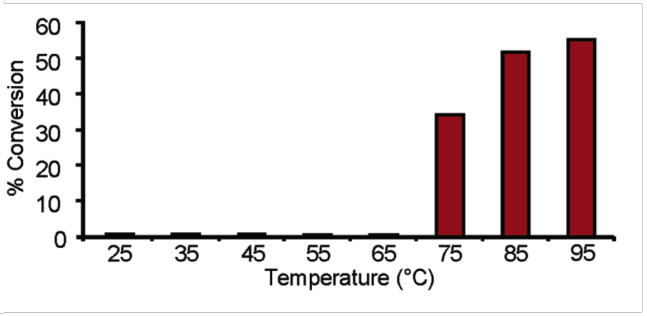


lysozyme
(6 Trp residues)

200 equiv. **A**
1 equiv. $Rh_2(OAc)_4$
750 equiv. *t*-BuNHOH

4:1 H_2O:ethylene glycol
pH = 6.0, r.t., 5 min

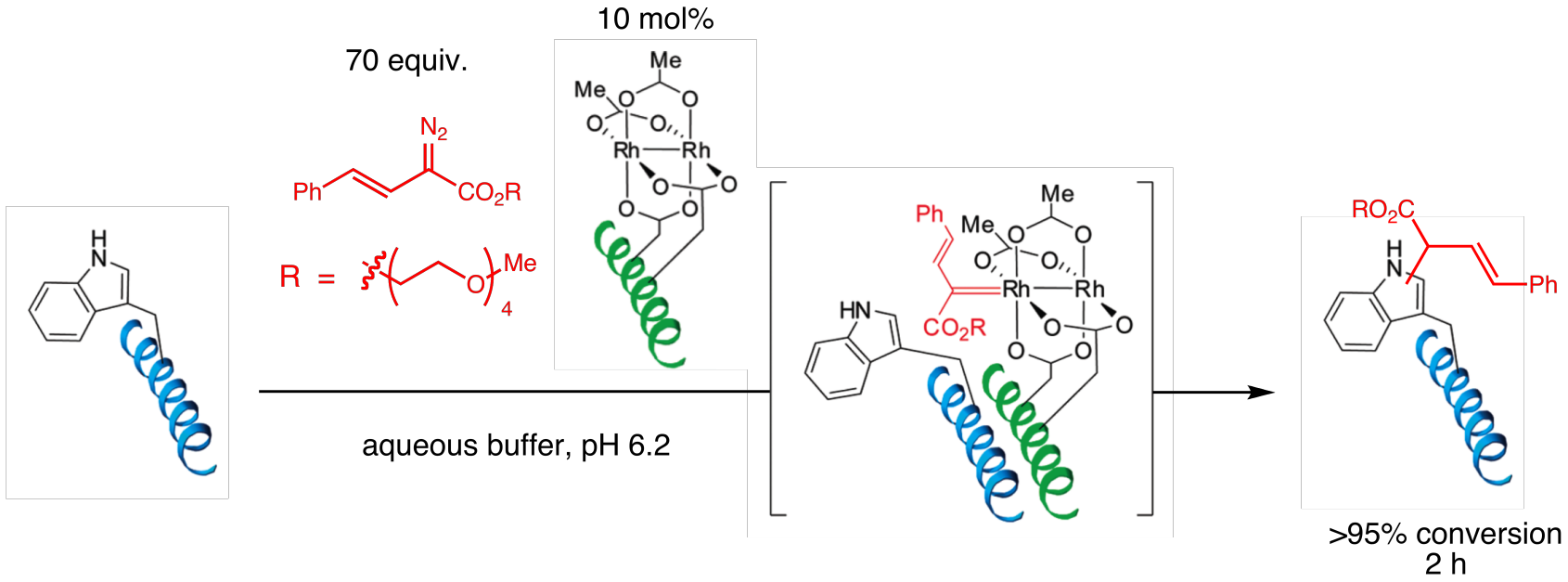


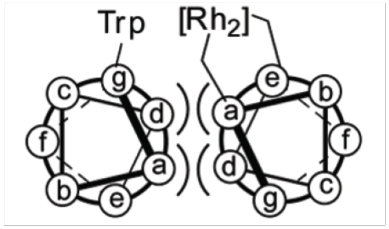


Antos, J. M.; McFarland, J. M.; Iavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2009**, *131*, 6301-6308.

Selective Tryptophan Modification via Molecular Recognition

- Binding of target peptide with complementary metallopeptide delivers dirhodium species to a specific Trp



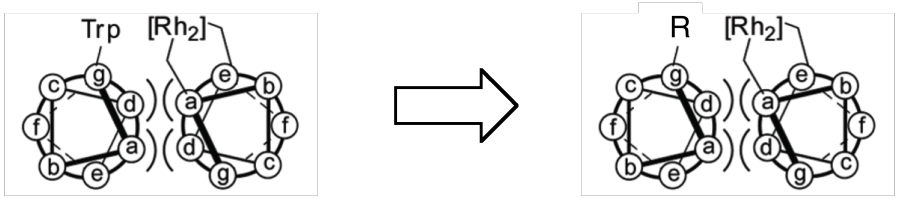


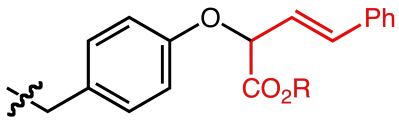
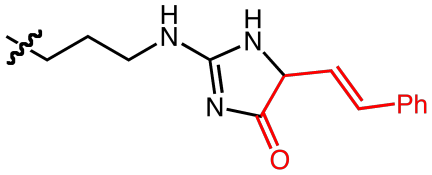
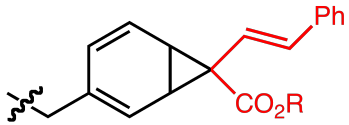
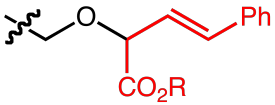
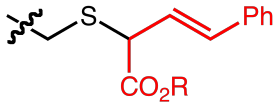
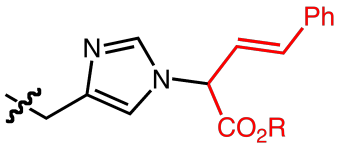
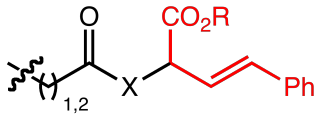
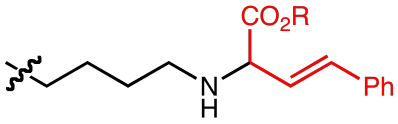
- 100 mol% $Rh_2(OAc)_4$ instead of metallopeptide: 8% conv., 2 h
- competition experiment with random Trp-containing peptide: >95% selectivity for Trp residue on complementary α-helix

Popp, B. V.; Ball, Z. T. *J. Am. Chem. Soc.* **2010**, *132*, 6660-6662.

Promiscuous Proximity-Driven Reactivity

- Dirhodium metallopeptides modify many natural amino acids when brought into proximity by substrate binding

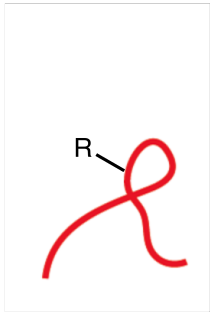


		conv.			conv.
Tyr		>95%	Arg		73%
Phe		>95%	Ser		28%
Cys		78%	His		17%
Gln, Asn Glu, Asp	 X = NH, O	53 to >95%	Lys		8%

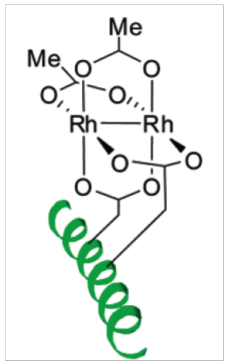
Popp, B. V.; Ball, Z. T. *Chem. Sci.* **2011**, *2,* 690-695.

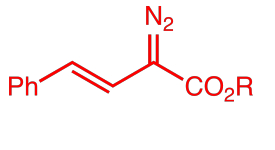
Promiscuous Proximity-Driven Reactivity

- No reactivity is observed for any residues except Trp in control experiments



random peptide

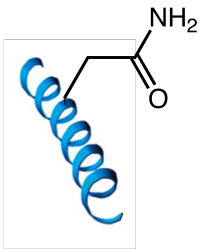




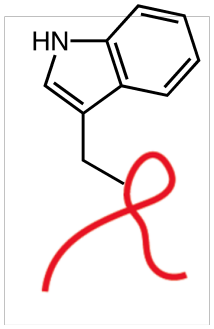
aqueous buffer
pH 6.2

no reactivity for any R ≠ W

- Molecular recognition overrides the inherently greater reactivity of Trp

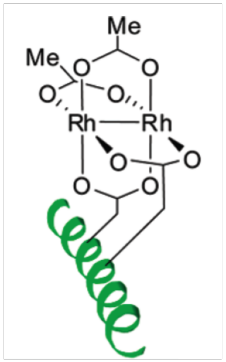


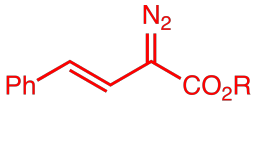
$E3_gQ$



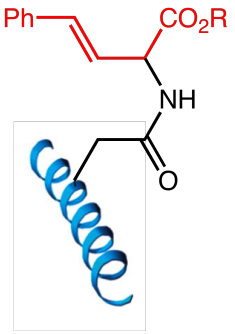
random peptide

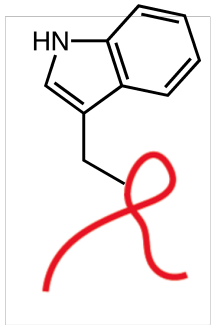
10 mol%





aqueous buffer
pH 6.2



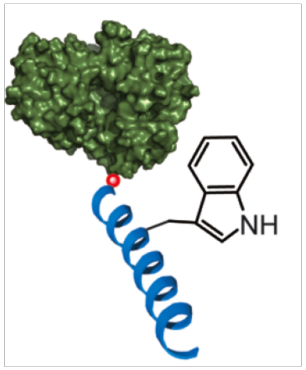


45:1 selectivity for $E3_gQ$

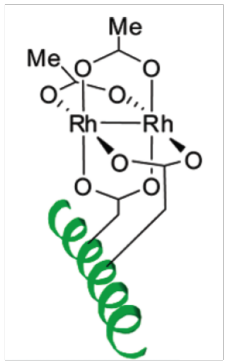
Popp, B. V.; Ball, Z. T. *Chem. Sci.* **2011**, *2,* 690-695.

Application to Protein Biotinylation

- Fusion protein bearing complementary E3 helix undergoes modification in cell lysate

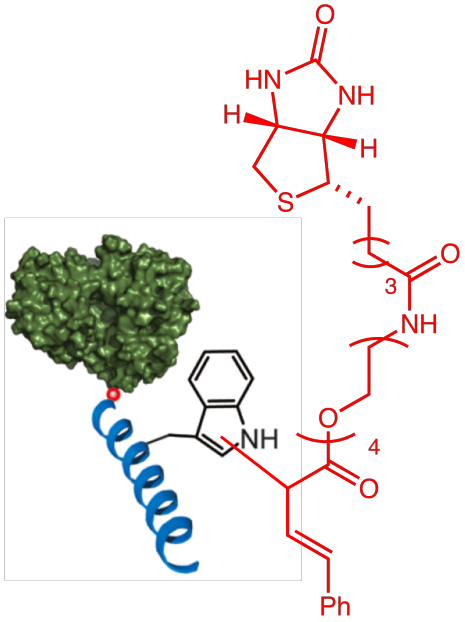


maltose binding protein-$E3_gW$ fusion

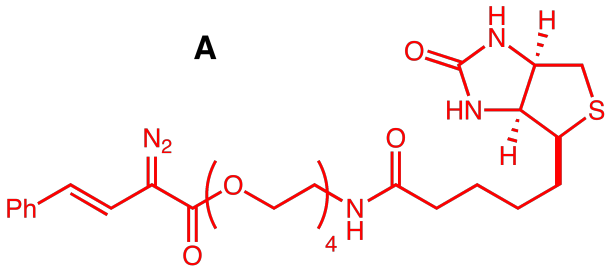


100 equiv. **A**

aqueous buffer
pH 7.2, 4 °C
E. coli cell lysate



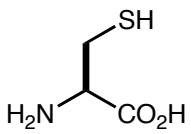
A



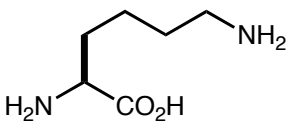
equiv. metallopeptide	conversion
0.2	5%
1.0	20%
5.0	70%

Chen, Z.; Popp, B. V.; Bovet, C. L.; Ball, Z. T. *ACS Chem. Biol.* **2011**, *6,* 920-925.

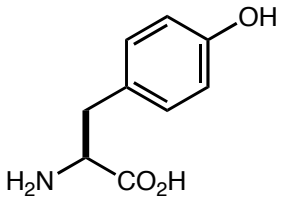
Bioconjugation: Chemical Modification of Proteins



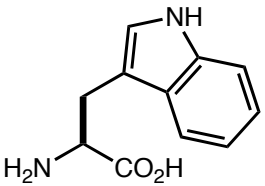
Cys



Lys



Tyr



Trp