

XU GROUP  
*Department of Chemistry, Peking University*

# *Selected Weekly Literature Presentations*

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# Chemical Ligation in Peptides and Protein Synthesis

## Quiz

- Among the chemical ligation methods described above, which one(s) are not based on an acyl transfer process?
- Which C-terminal residues lead to poor efficiency in NCL reactions? Why?
- List several peptide thioester precursors.

# Outline

- Introduction
- Native Chemical Ligation(NCL)
  - Mechanism
  - Precursor of Thioester
  - Alternative of Cys
    - Auxiliary-based Methods
    - Ligation-desulfurization
    - Diselenide-Selenoester Ligation(DSL)
- Serine/Threonine Ligation(STL) & Cysteine/Penicillamine Ligation(CPL)
- $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation
- Staudinger Ligation
- Summary and Outlook
- Acknowledgement

# Outline

## ■ Introduction

## ■ Native Chemical Ligation(NCL)

- Mechanism

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## ■ Acknowledgement

# Introduction

## Natural Extraction

*The Journal of  
Laboratory and Clinical  
Medicine*

VOL. VII ST. LOUIS, FEBRUARY, 1922 No. 5

### ORIGINAL ARTICLES

THE INTERNAL SECRETION OF THE PANCREAS\*

By F. G. BANTING, M.B., AND C. H. BEST, B.A.

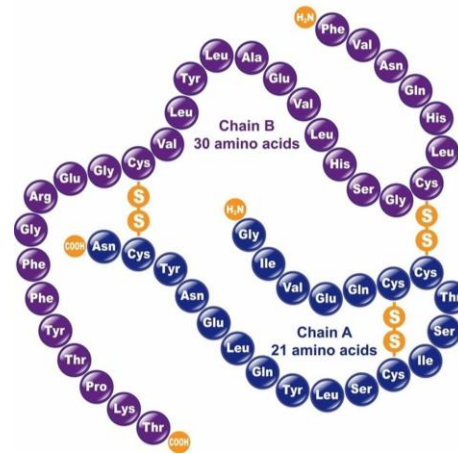
THE hypothesis underlying this series of experiments was first formulated by one of us in November, 1920,<sup>1</sup> while reading an article dealing with the relation of the isles of Langerhans to diabetes.<sup>1</sup> From the passage in this article, which gives a résumé of degenerative changes in the acini of the pancreas following ligation of the ducts, the idea presented itself that since the acinous, but not the islet tissue, degenerates after this operation, advantage might be taken of this fact to prepare an active extract of islet tissue. The subsidiary hypothesis was that trypsinogen or its derivatives was antagonistic to the internal secretion of the gland. The failures of other investigators in this much-worked field were thus accounted for.

## Animal insulin(1922)

Biologically native proteins

Initial discovery &  
characterization

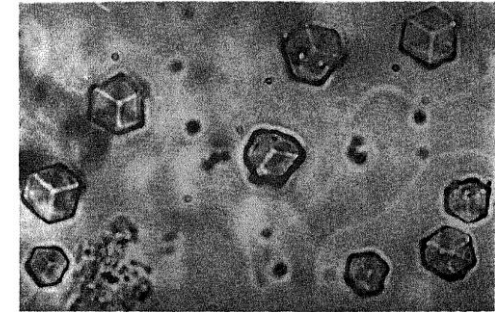
## Recombinant Expression



## Human insulin(1979)

Therapeutic proteins  
Genetic engineering  
Scalable production

## Chemical Synthesis



## Crystalline insulin(1966)

Any sequences  
Atomically precise  
Non-natural modifications

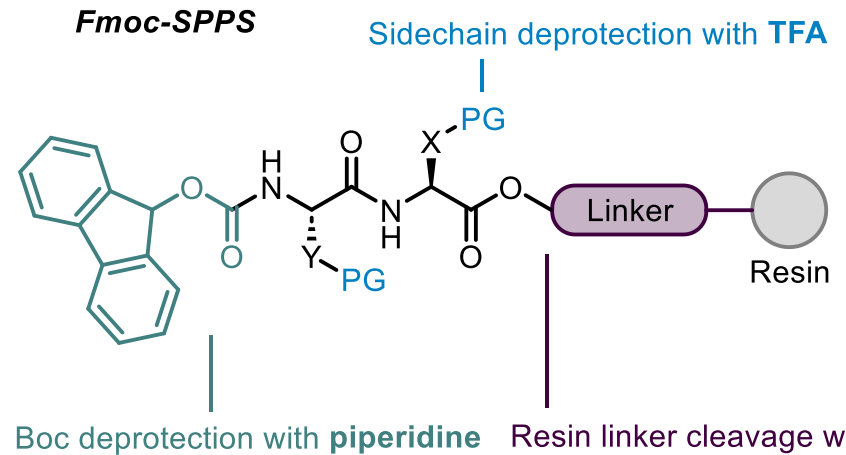
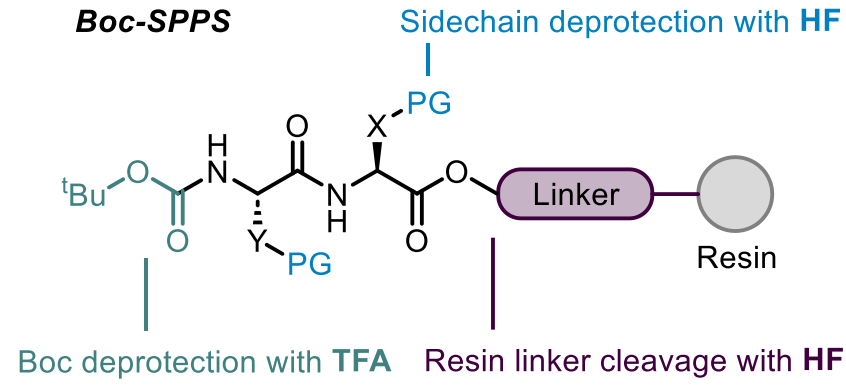
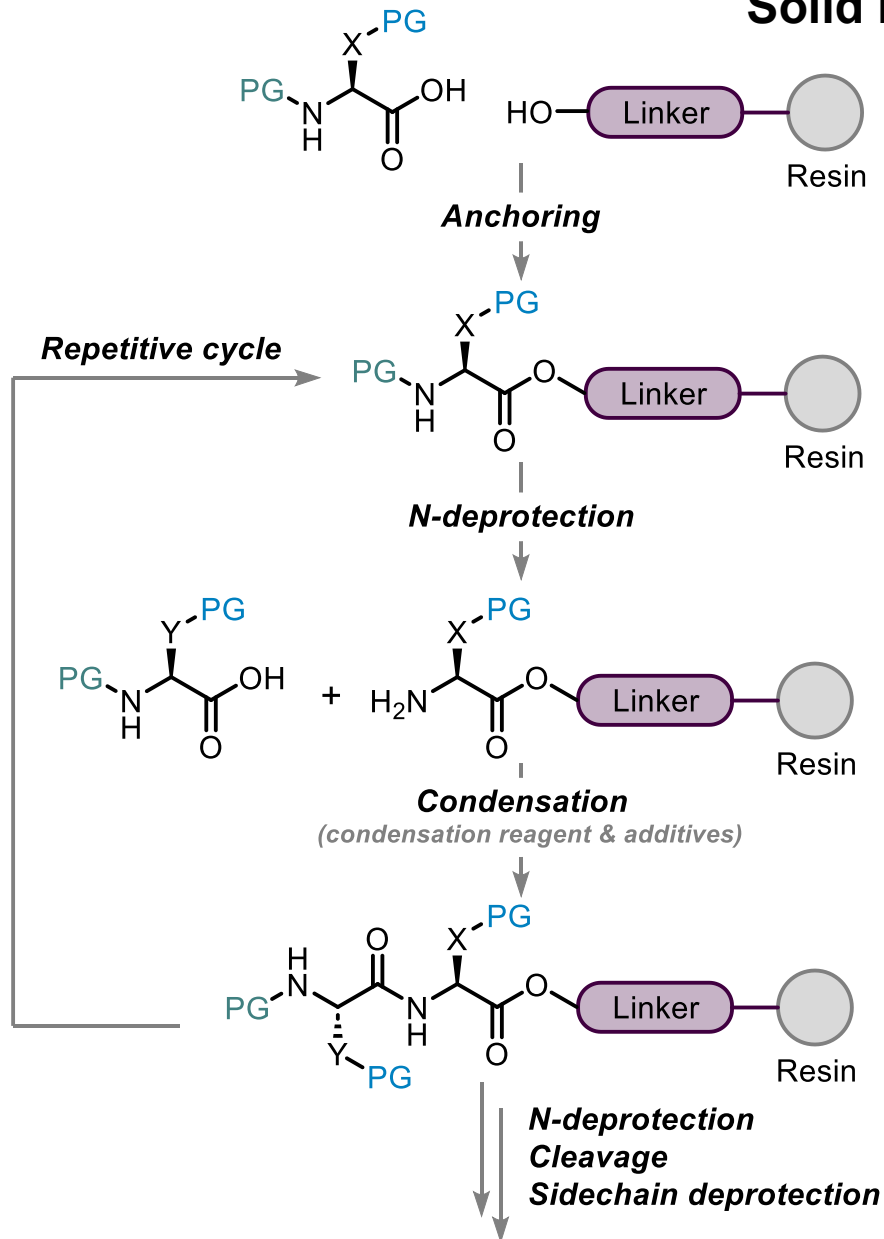
Banting F.G., Best C.H., *J. Lab. Clin. Med.*, **1922**. 12(5), 251-266.

Goeddel D.V., Kleid D.G., Bolivar F., et al. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, 76(1), 106-110.

Yuehting Kung, Yucang Du, Weihe Huang, et al., *Sci. Sin.*, **1966**, 15(4): 544-561.

# Introduction

## Solid Phase Peptides Synthesis, SPPS



Repetitive TFA deprotection causes peptides-resin cleavage

Peptides containing fragile sequences couldn't survive the HF conditions

Hazard HF used

TFA = TFA/DCM(1:1 mixture)

HF = anhydrous HF

piperidine = piperidine/DCM(1:4 mixture)

Orthogonal protection

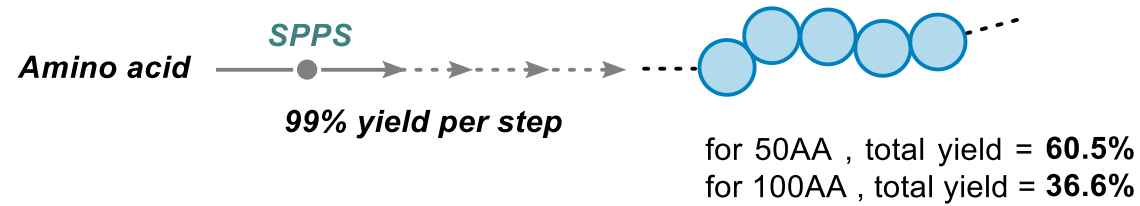
Mild conditions

### Limitations and challenges:

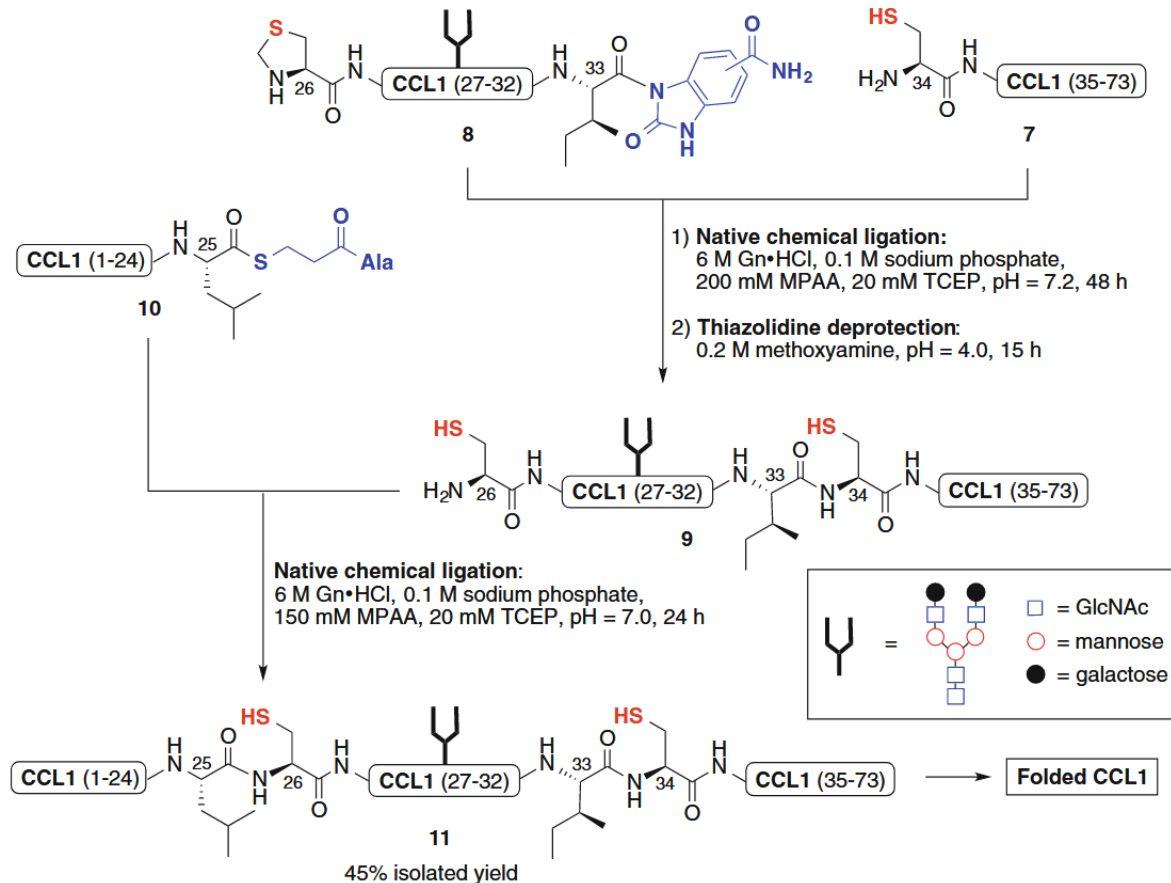
- ❑ Racemization and side reactions
- ❑ Long( > 50 AA) sequences

# Introduction

## Limitations on chain length



## Convergent synthesis strategy



**Fmoc-SPPS**

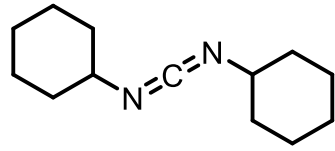
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**Segments Ligation  
Condensation?**

# Introduction

## Condensation

### Carbodiimides



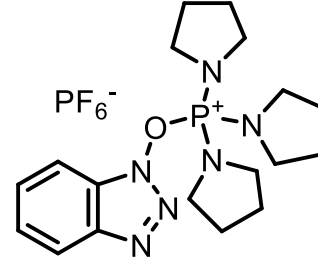
DCC

### Additives



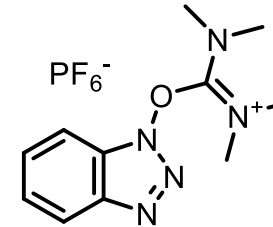
HOBT

### Phosphonium salts



PyBOP

### Uronium/Aminium salts



HBTU

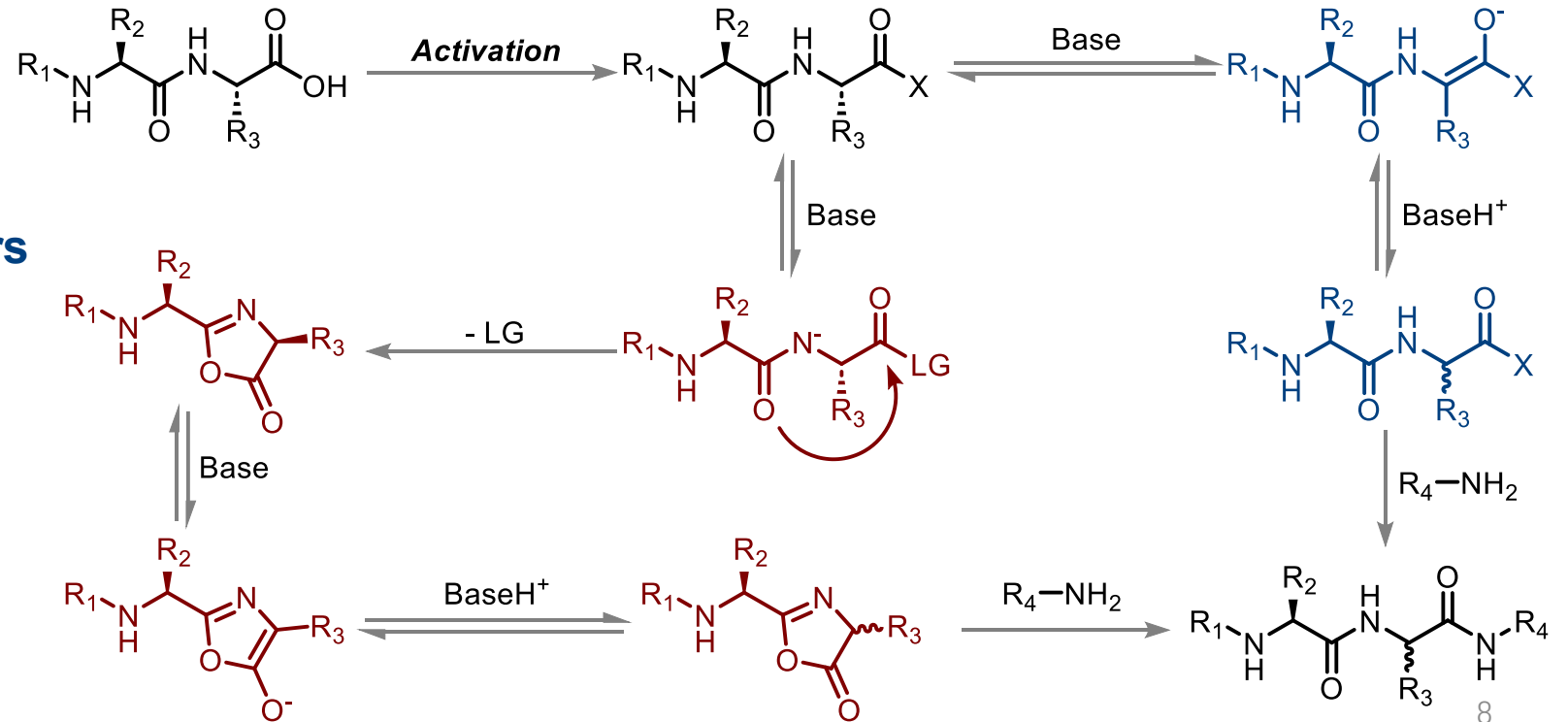
## Racemization

### ■ Oxazolone mechanism

### ■ Enolization of Activated Esters

### ■ Hard to purify

### ■ Unexpected bio-activities



# Introduction

## **Condensation reaction**

Maximum (Full) protection

Harsh, organic solvent

Racemization risk

**Any carboxyl and amino group**

Global deprotection

**Good solubility in Organic solvent**

**Segment synthesis**

## **Native Chemical Ligation**

**Protection-free/Minimal protection**

**Mild, aqueous buffer (near-neutral pH)**

**Almost No Racemization**

C-thioester + N-Cysteine(limited)

**No deprotection**

Hydrophobic segments: poor solubility

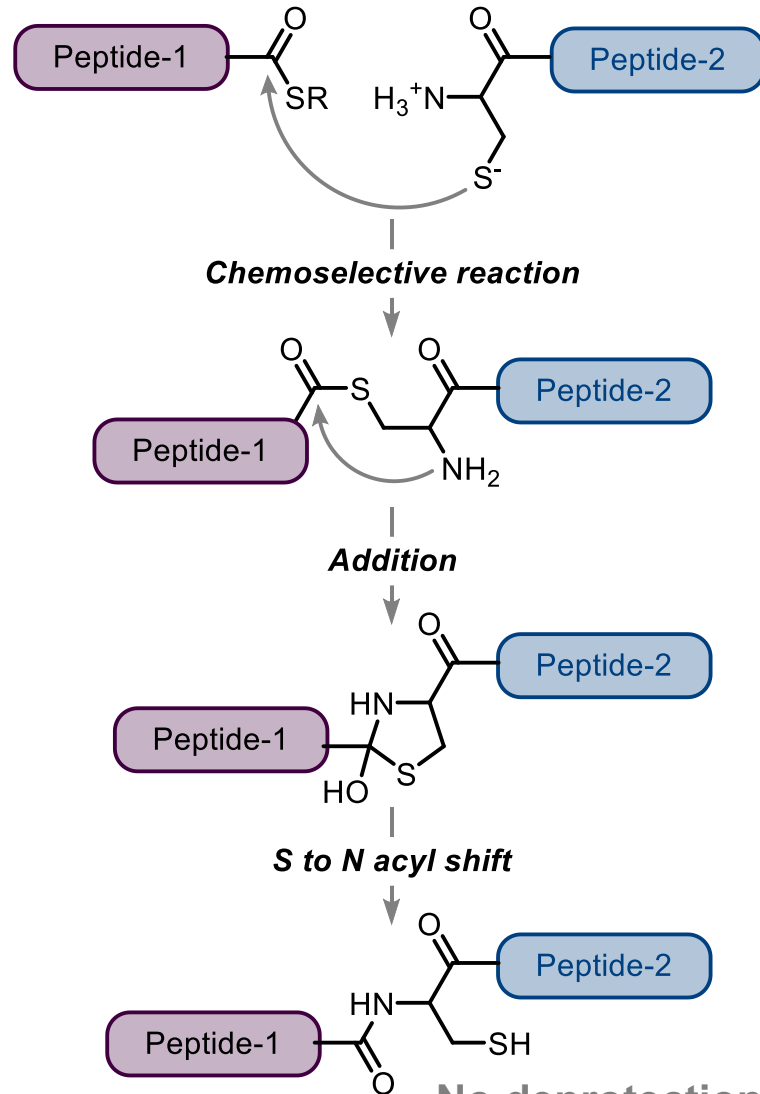
**Segment ligation**

# Outline

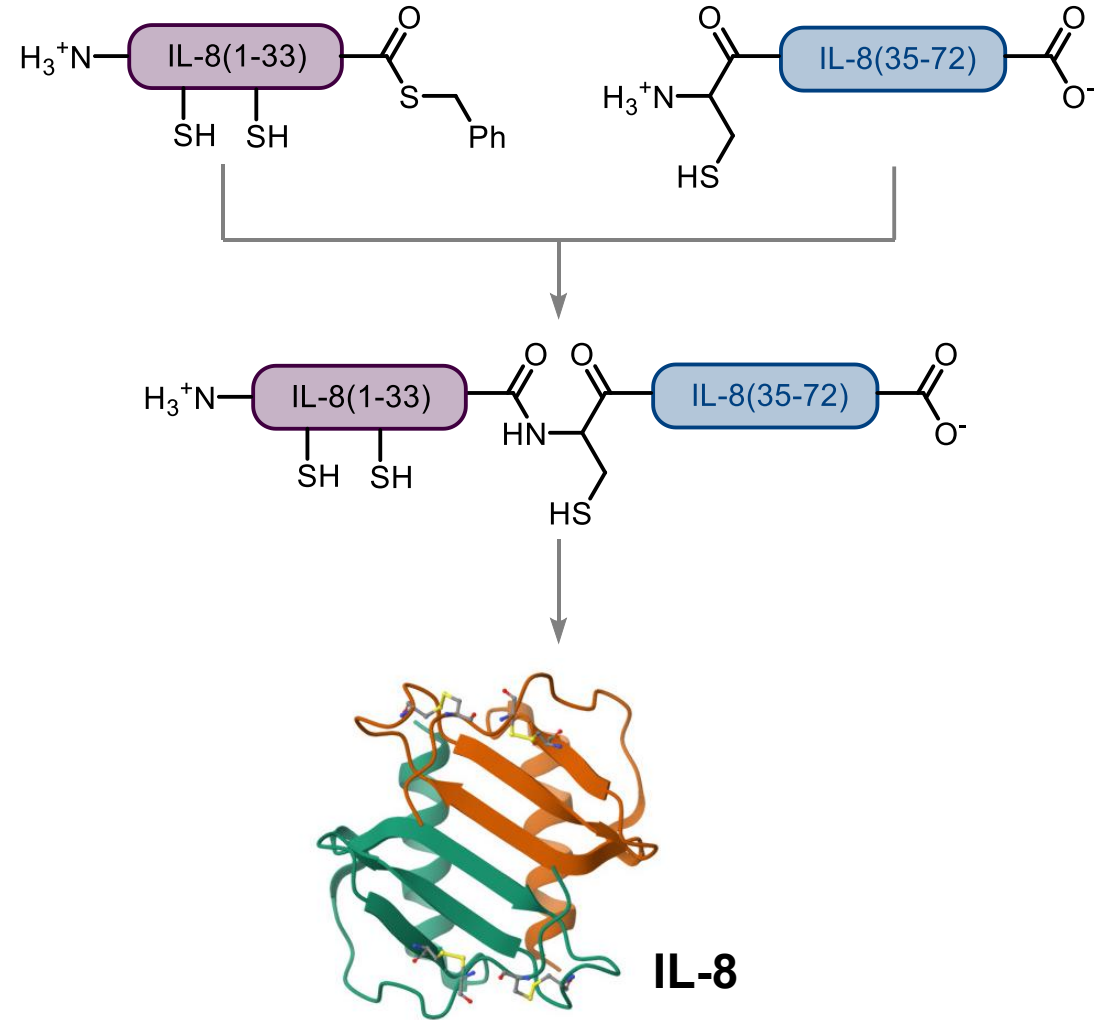
- Introduction
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  - Mechanism
  - Precursor of Thioester
  - Alternative of Cys
    - Auxiliary-based Methods
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# Native Chemical Ligation (NCL)

Reaction between two **unprotected** peptide segments in **aqueous** buffer at **low concentrations**



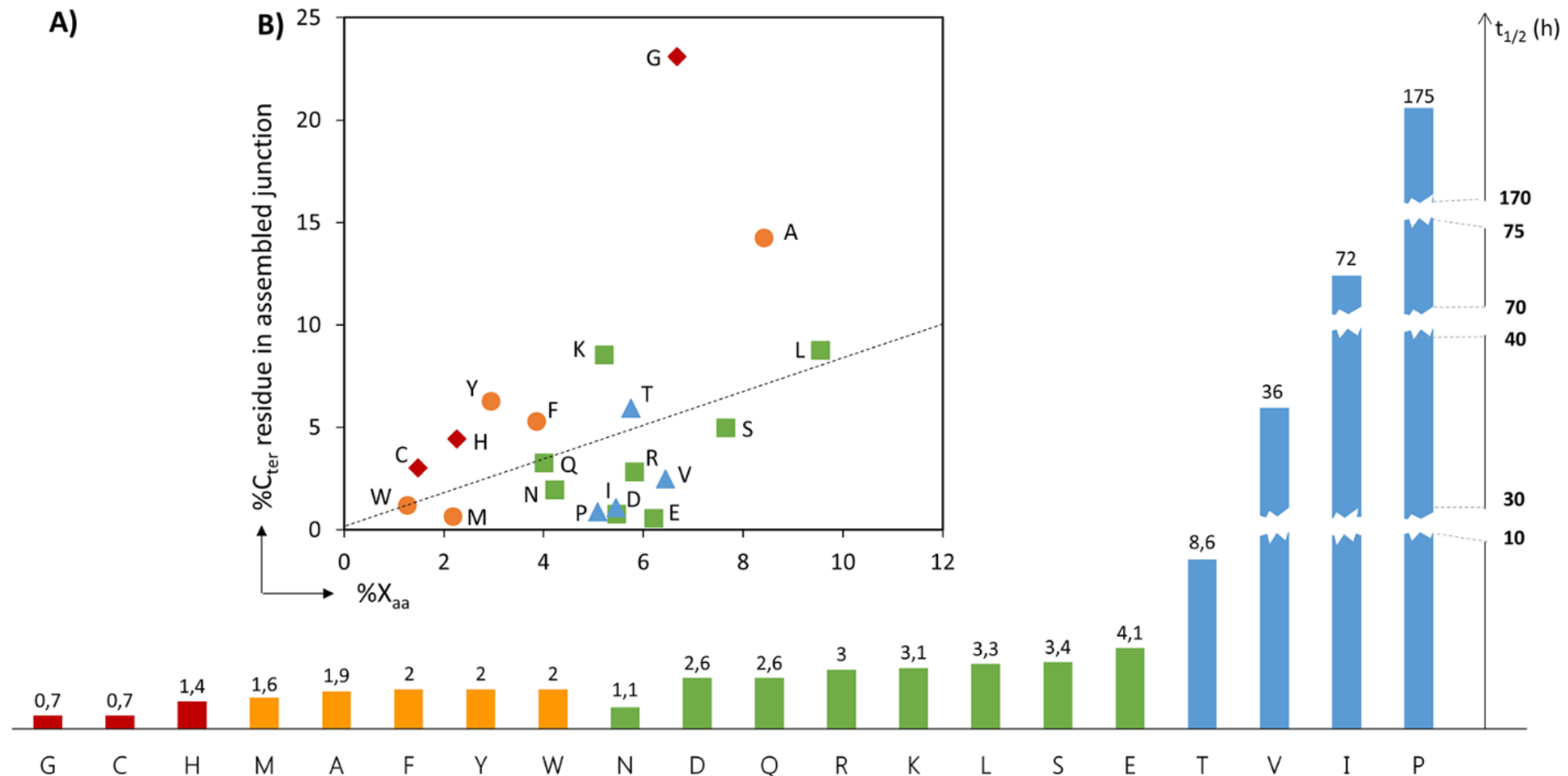
**No deprotection is needed**



**IL-8**

# Native Chemical Ligation(NCL)

## C-terminal residues scope



Residues with slow ligation rate:

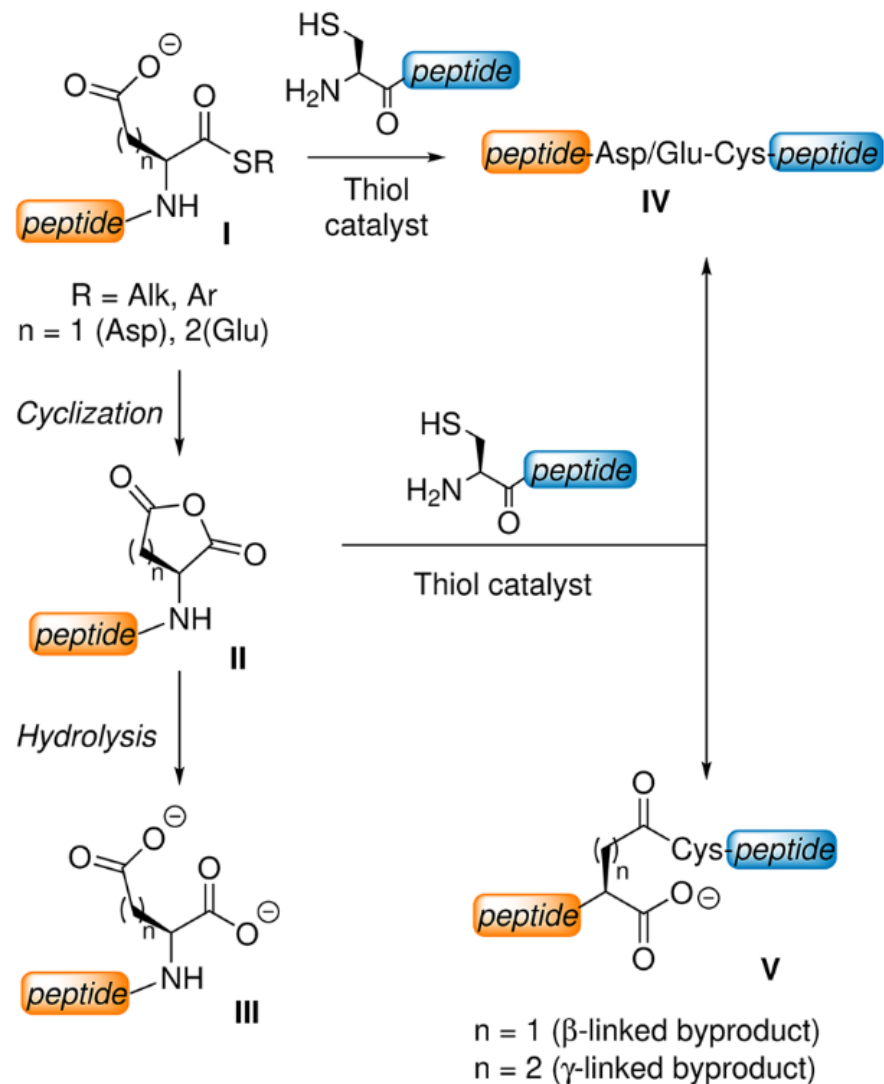
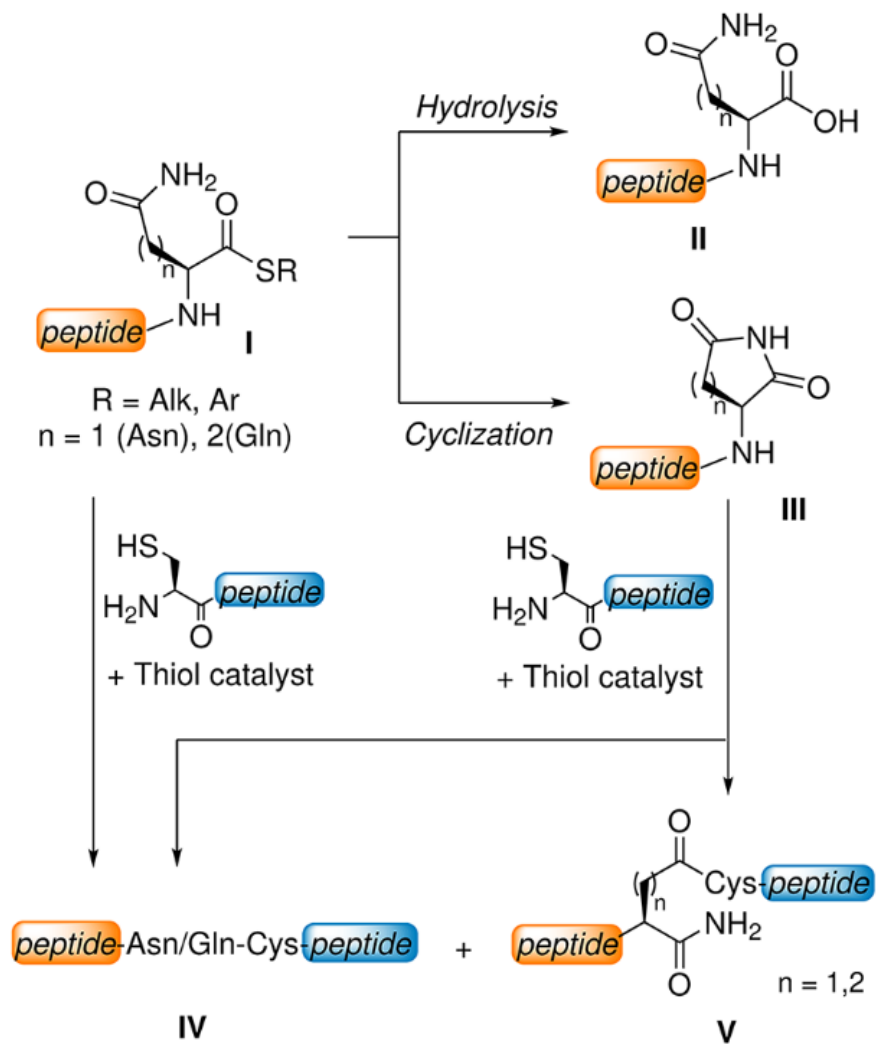
□ Thr, Val, Ile: Sterically hindered

□ Pro: n to π\* electron donation

# Native Chemical Ligation (NCL)

## C-terminal residues scope

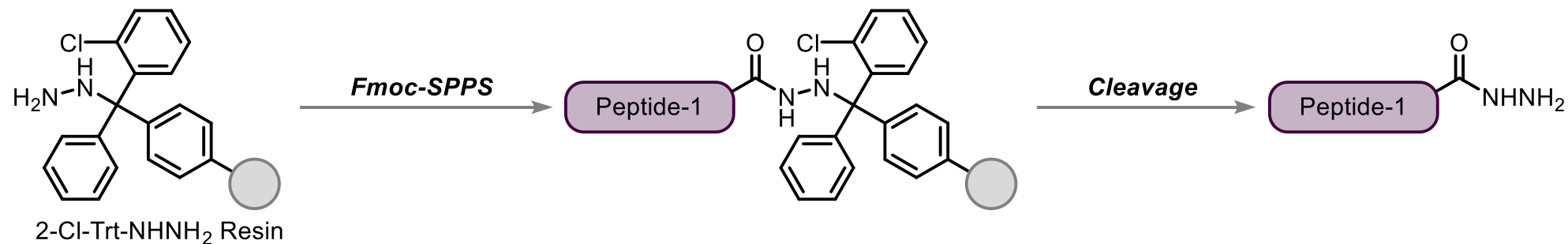
Residues with side reaction: Glu, Gln, Asp, Asn



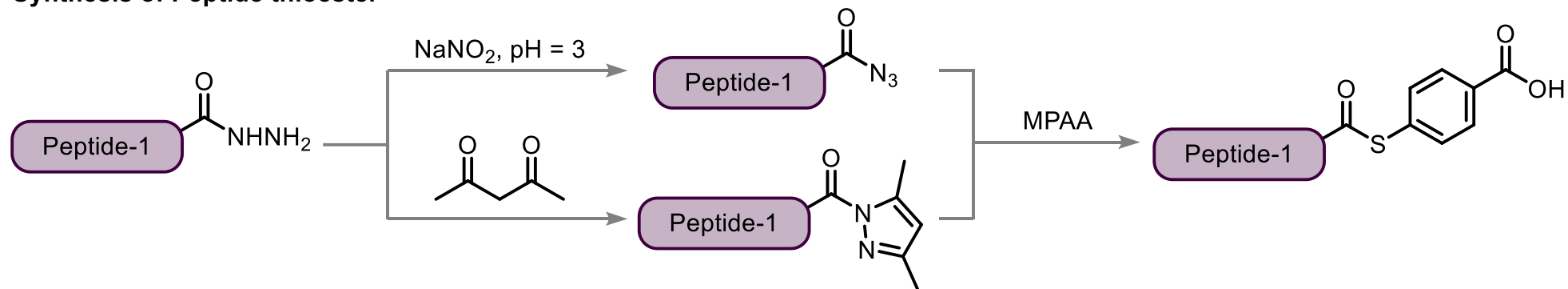
# NCL: Precursor of Thioester

## Peptide hydrazide

### Synthesis of Peptide Hydrazide



### Synthesis of Peptide thioester



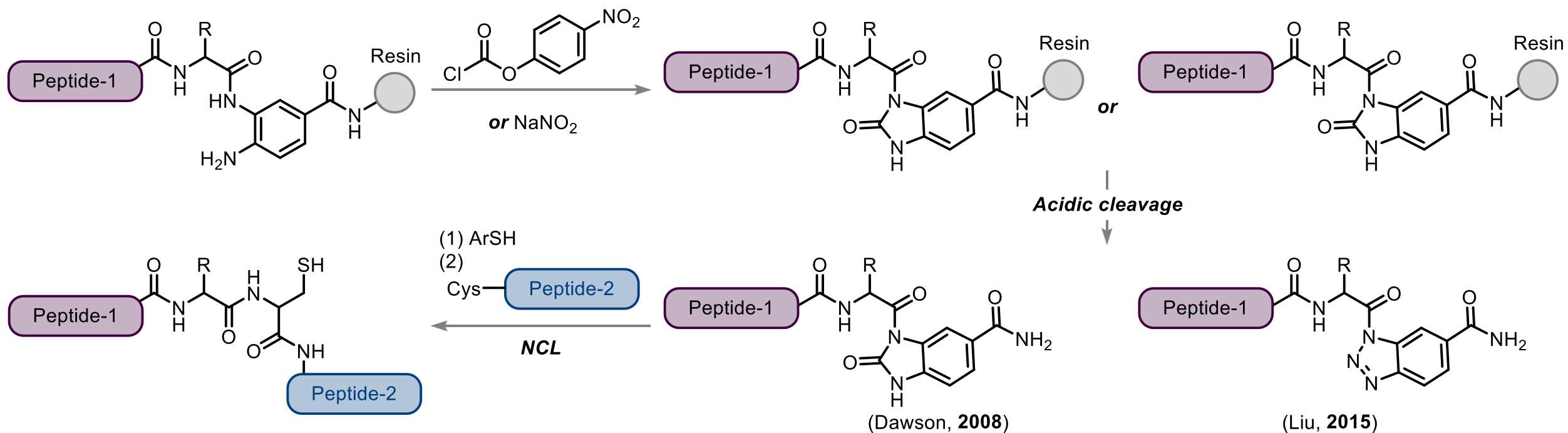
Zheng, J.-S., Liu, L., et al., *Nat Protoc.* **2013**, 8(12), 2483–2495.

Flood, D. T., Dawson, P. E., et al., *Angew. Chem. Int. Ed.* **2018**, 57 (36), 11634–11639.

Fang, G.-M., Liu, L., et al., *Angew. Chem. Int. Ed.* **2011**, 50 (33), 7645–7649

# NCL: Precursor of Thioester

## Peptide N-acyl-benzimidazolines (Nbz) and peptide benzotriazole

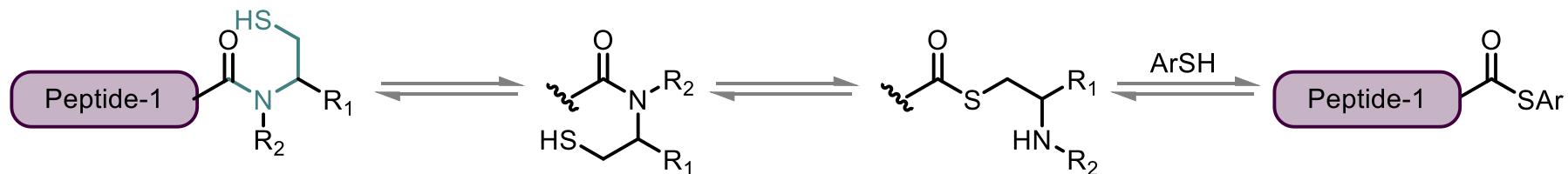


Wang J., Fang, G., Liu, L. *Angew. Chem. Int. Ed.* **2015**, 54(7), 2194–2198.

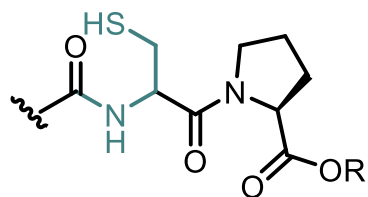
Blanco-Canosa J. B., Dawson P. E., *Angew. Chem. Int. Ed.* **2008**, 47(36), 6851–6855.

# NCL: Precursor of Thioester

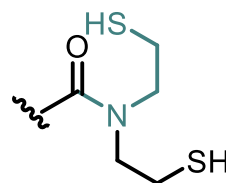
## Peptide crypto-thioesters (N-S Acyl shift)



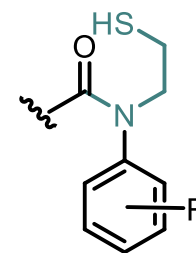
(a) CPE, Aimoto **2007**



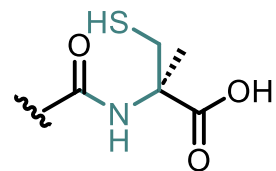
(b) SEA, Melnyk **2010**, Liu **2011**



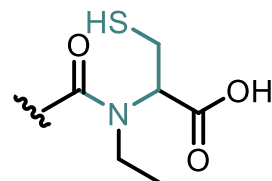
(c) SEAildes, Otaka **2013**



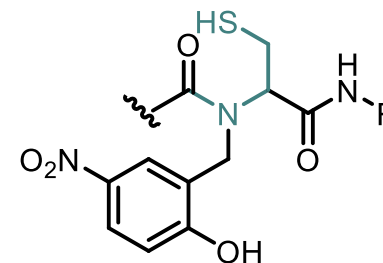
(d)  $\alpha$ -Me-Cys-OH, Offer **2014**



(e) N-Et-Cys-OH, Hojo **2015**



(f) N-Hub-Cys, Aucagne **2016**



# NCL: Alternative of Cys

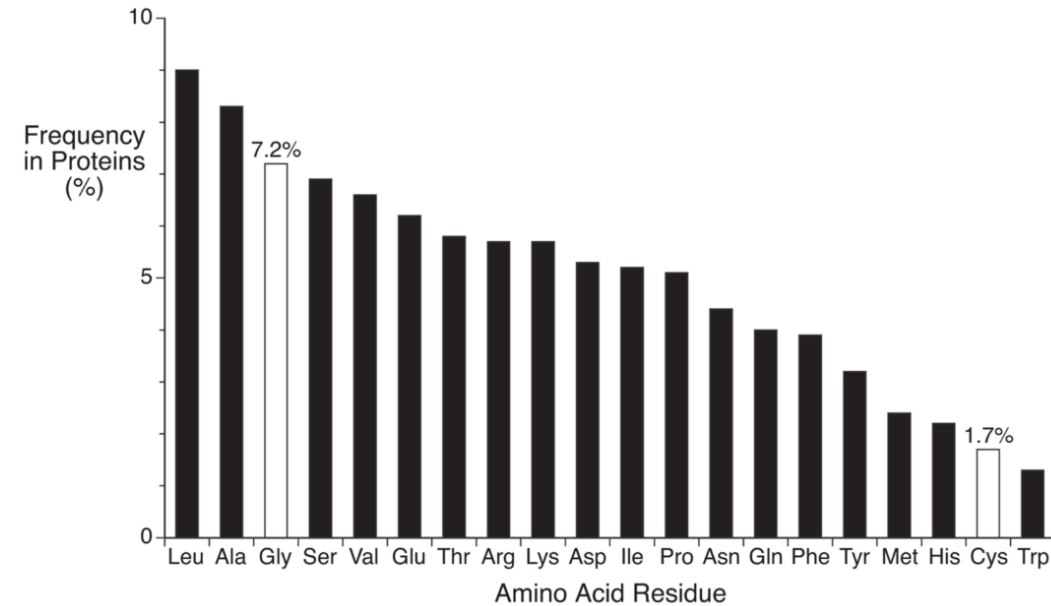
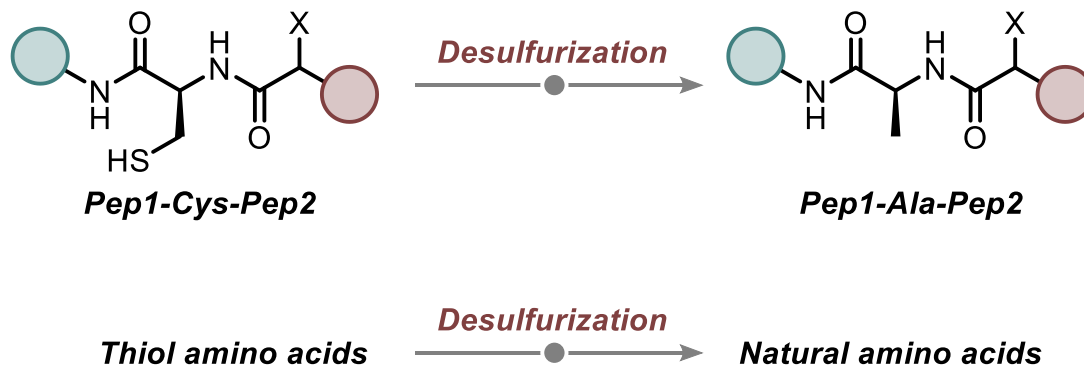
- **Cys: *Low-abundance***(~59AA per Cys)
- In many proteins there are no Cys or the Cys site isn't in a position suitable for the NCL.
- **How to get rid of the limitation of Cys?**

## □ Auxiliary-Based Methods

### □ N-terminal auxiliaries

### □ Side-chain auxiliaries

## □ Ligation-desulfurization Strategy



## □ New Cys-free ligation methods

### □ Serine/Threonine Ligation(STL)

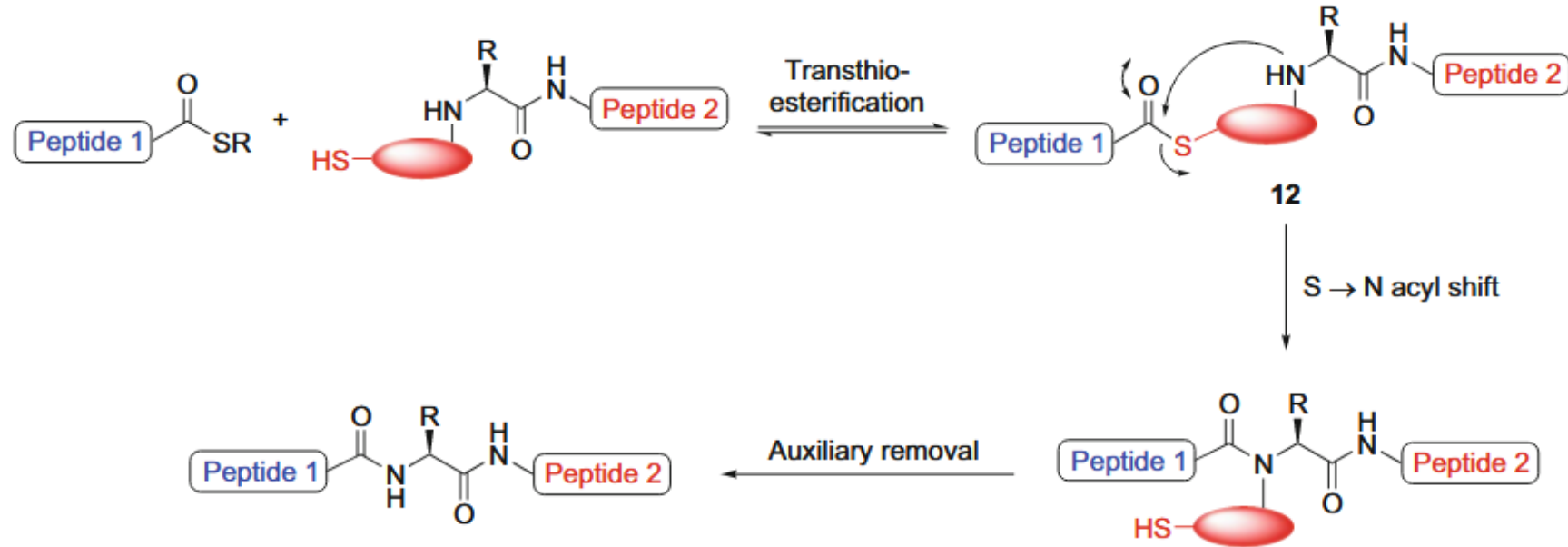
### □ Cysteine/Penicillamine Ligation(CPL)

### □ Diselenide-Selenoester Ligation(DSL)

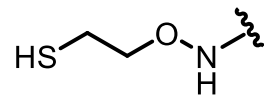
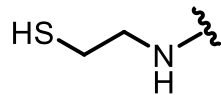
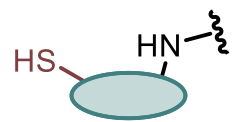
### □ *etc...*

# Auxiliary Based Methods

## N-terminal auxiliaries

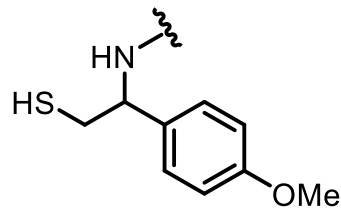
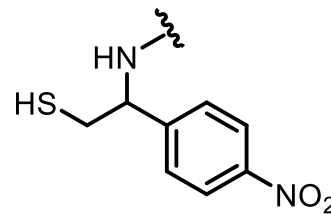
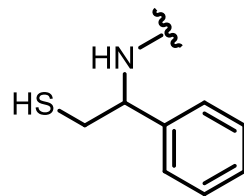


*Ethanethiol-type*

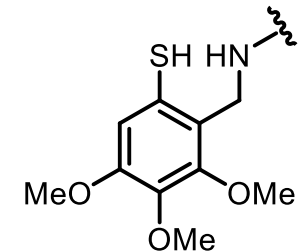
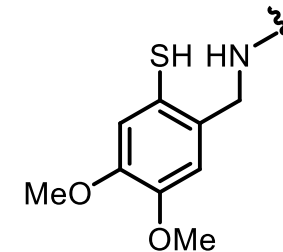
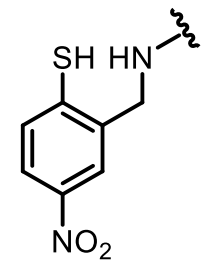
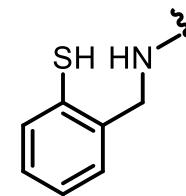


(Kent, 1996)

*1-Phenylethanethiol-type*

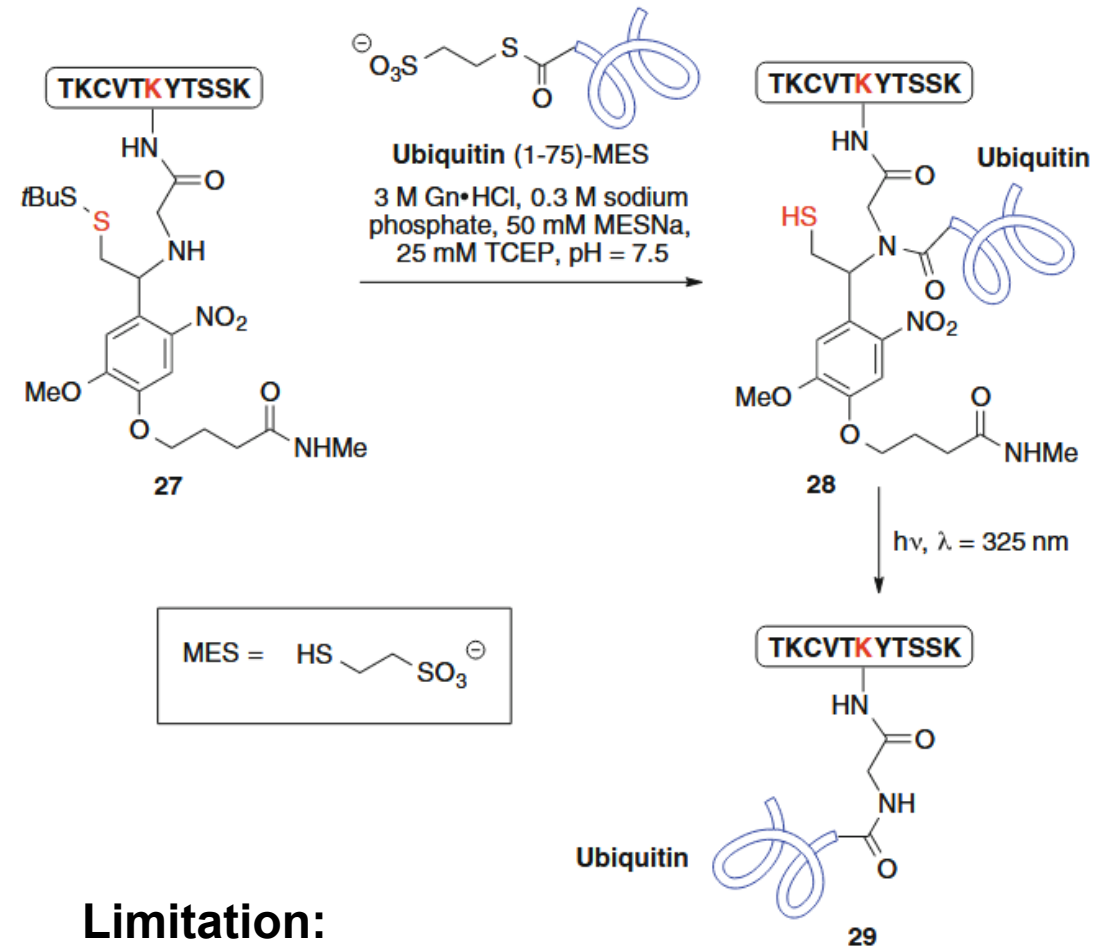
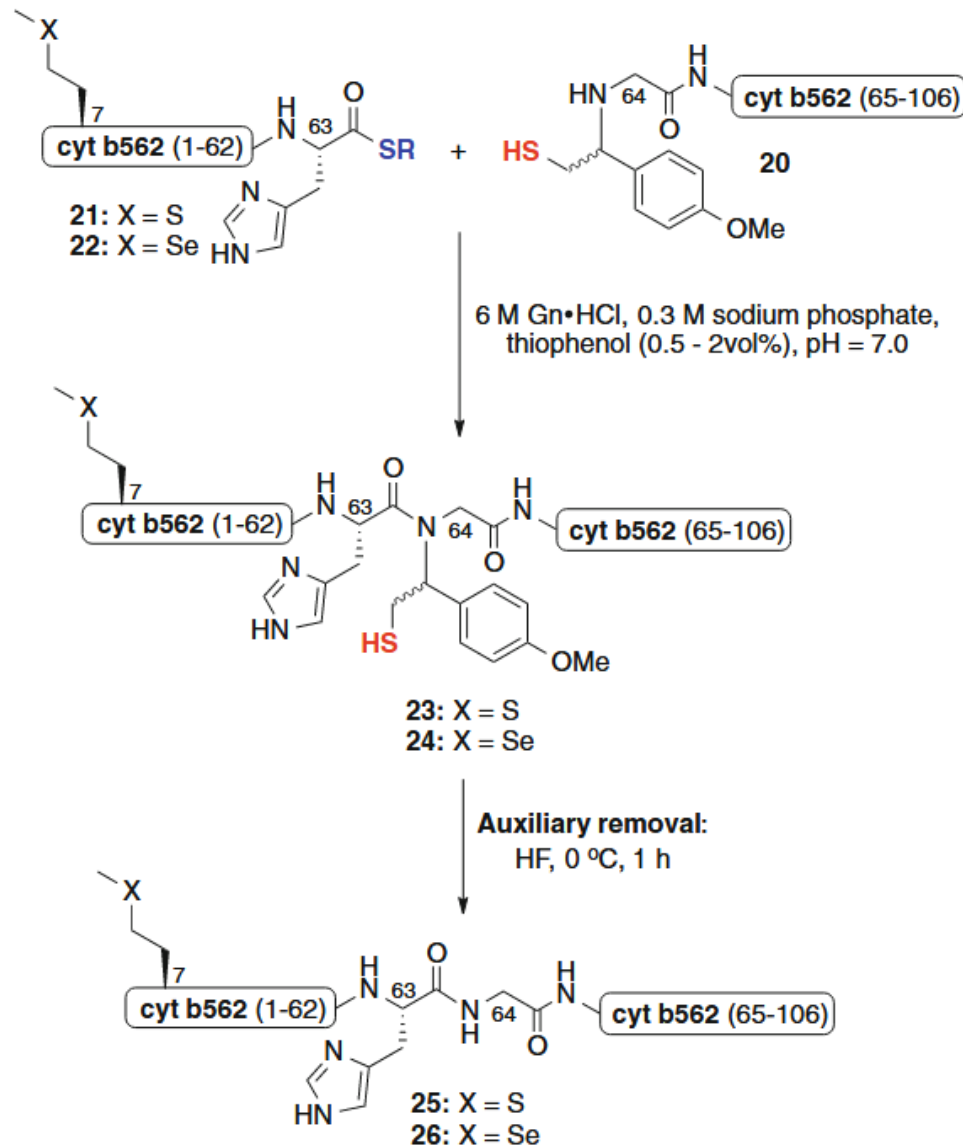


*2-mercaptobenzyl-type*



# Auxiliary Based Methods

## N-terminal auxiliaries

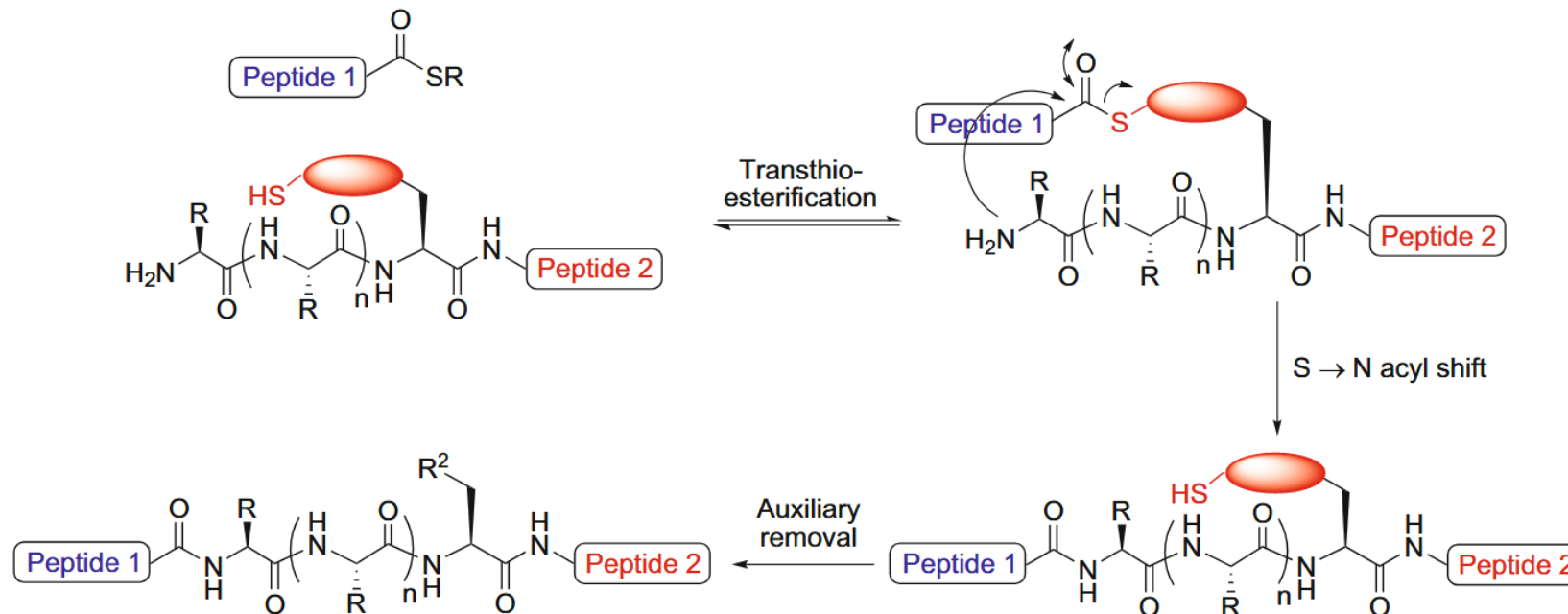


### Limitation:

- ❑ lower ligation rate
- ❑ Harsh conditions for the auxiliaries removal
- ❑ poor sequence tolerance(limit to Gly)

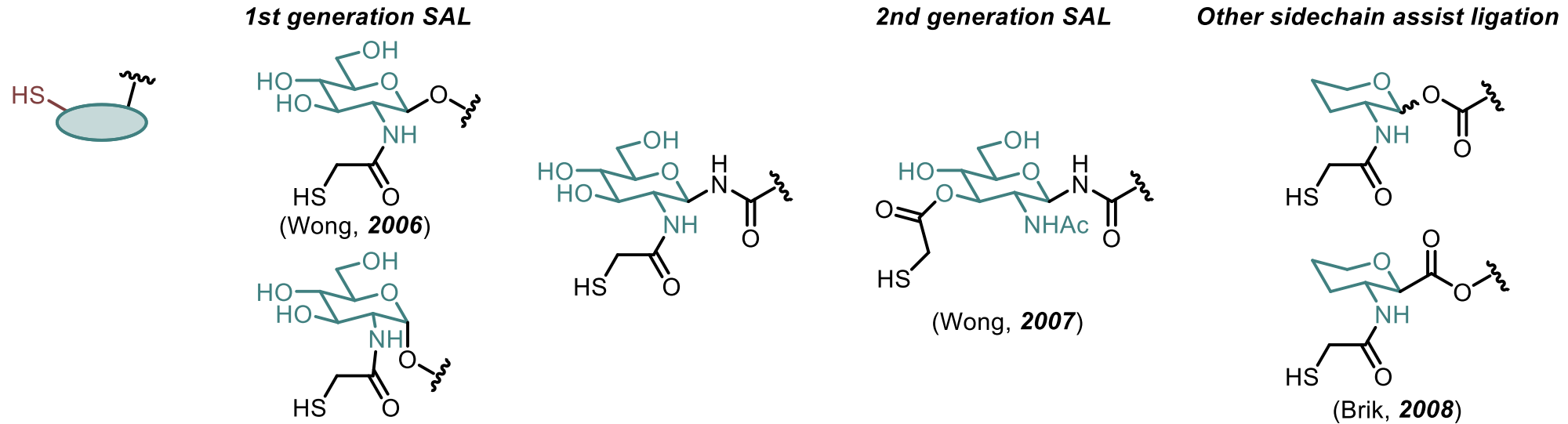
# Auxiliary Based Methods

## Side-chain auxiliaries (Sugar assist ligation, SAL; etc.)



# Auxiliary Based Methods

## Side-chain auxiliaries (Sugar assist ligation, SAL; etc.)



### ■ Generation 1

- Reductive desulfurization
- Limit: incompatible with cysteine

### ■ Generation 2

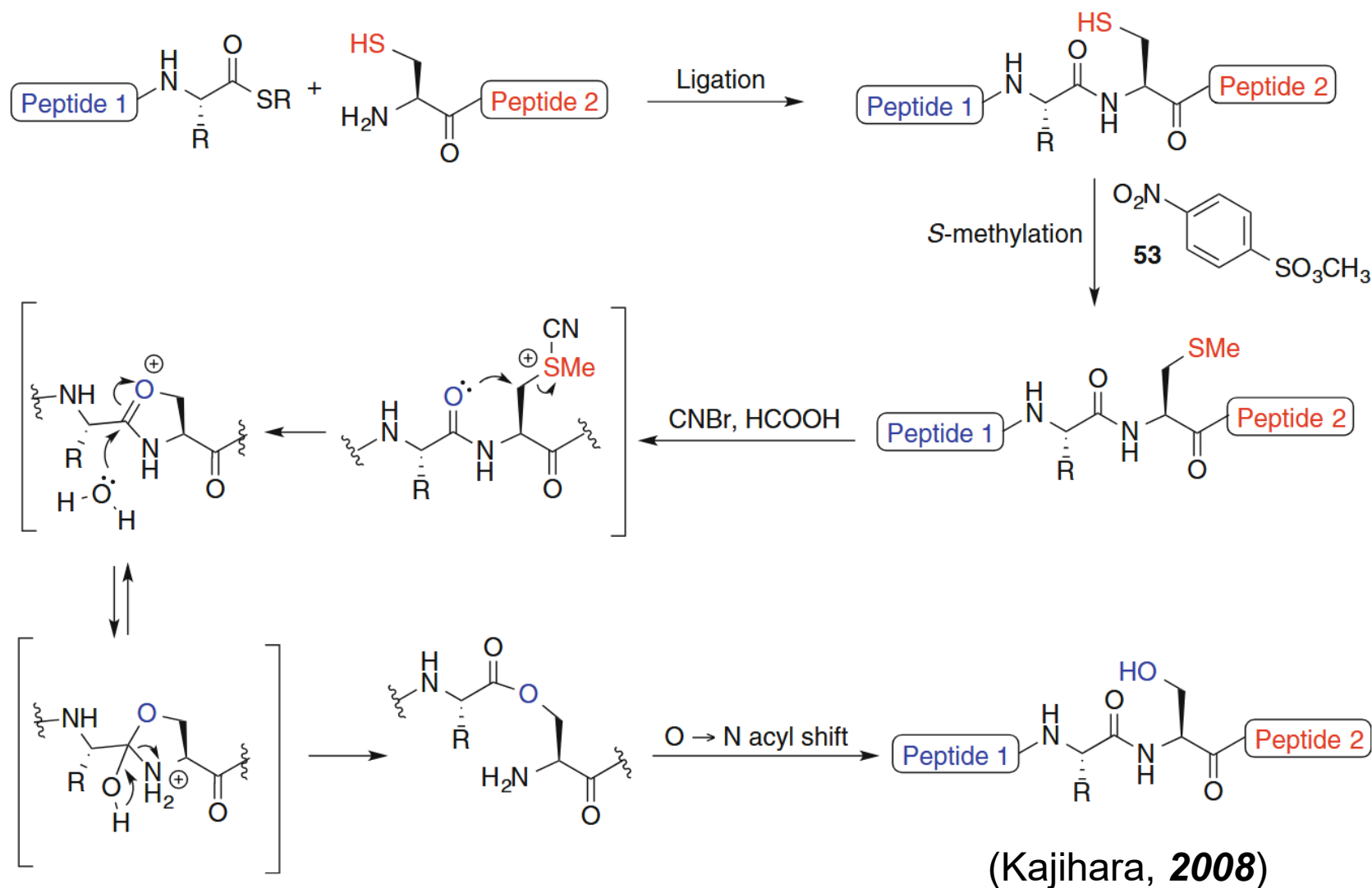
- Removed via hydrazinolysis

### ■ Cyclohexyl auxiliary

- Link to the side-chain of Asp, Glu or Ser
- Rapidly cleaved in situ by the addition of NaOH

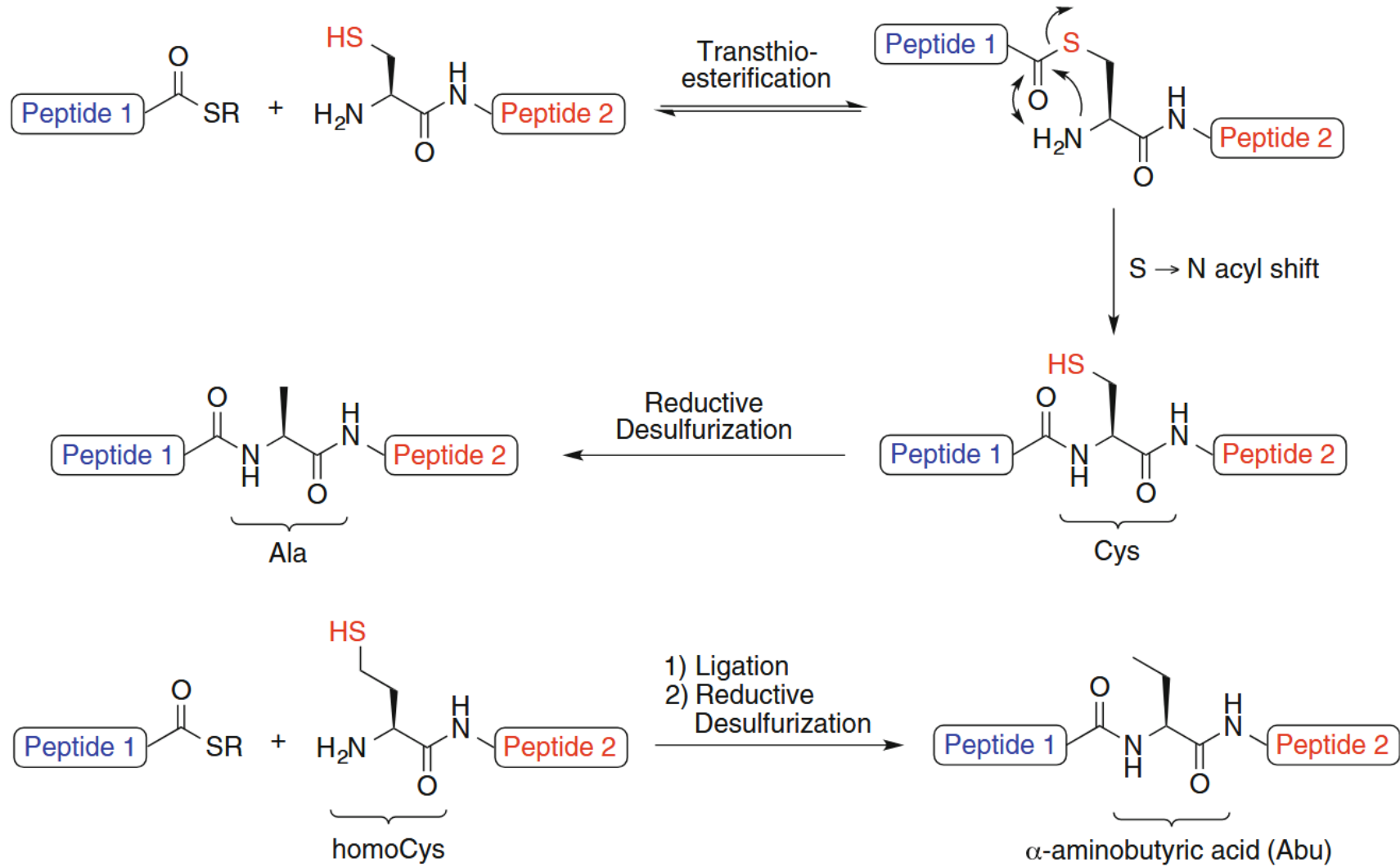
# Ligation-desulfurization Chemistry

## Cys to Ser



# Ligation-desulfurization Chemistry

## Cys to Ala:



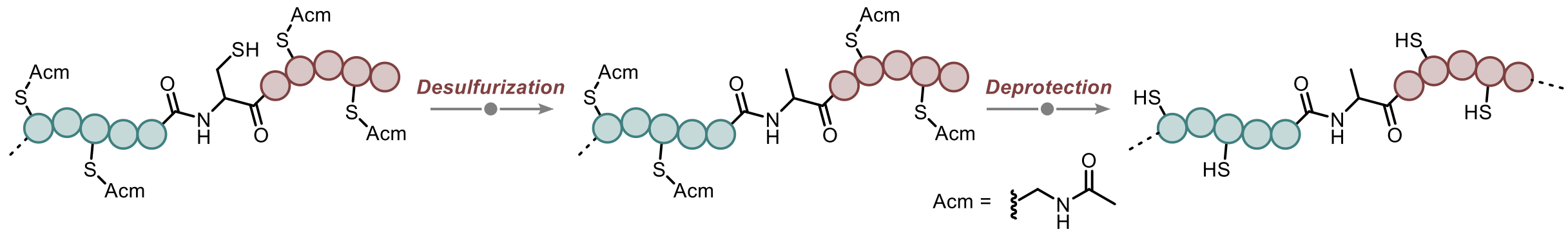
(Dawson, 2001)

# Ligation-desulfurization Based on Ala

## Metal-based Desulfurization

entry	metal reagent	reaction medium	yield (%)	advantage or disadvantage
1	Pd/Al <sub>2</sub> O <sub>3</sub> (10%)	0.1 M phosphate, 6 M guanidine, pH 7.5	90	disulfide formation
2	Pd/Al <sub>2</sub> O <sub>3</sub> (10%)	0.1 M acetate, 6 M guanidine, pH 4.5	>99	desalting required
3	Pd/Al <sub>2</sub> O <sub>3</sub> (10%)	20% aqueous AcOH	>99	directly lyophilizable
4	Pd/Carbon (10%)	20% aqueous AcOH	>99	directly lyophilizable
5	Pd/BaSO <sub>4</sub> (10%)	20% aqueous AcOH	>90	directly lyophilizable
6	PdO	20% aqueous AcOH	<30	reaction incomplete
7	Raney nickel	20% aqueous AcOH	>99	directly lyophilizable

- **Pd/Al<sub>2</sub>O<sub>3</sub>: high-yielding global desulfurization of Cys residues while minimizing the over-reduction of Tyr, Phe, Trp.**



- **Raney Ni: Compatible with Met and Cys(Acm)**

Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*(4), 526–533.

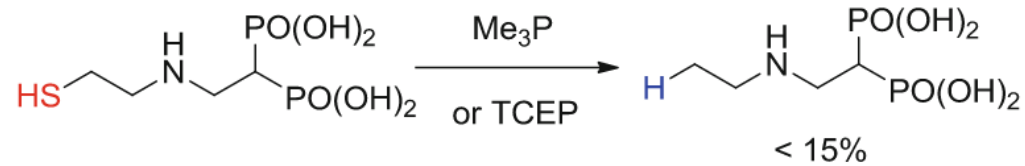
Pentelute, B. L.; Kent, S. B. H., *Org. Lett.* **2007**, *9*(4), 687–690.

*Protein Ligation and Total Synthesis I*, Liu, L., Springer International Publishing: Cham, **2015**; Vol. 362.

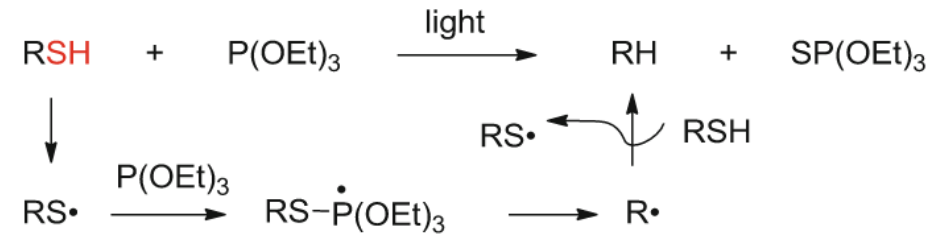
# Ligation-desulfurization Based on Ala

## Metal-free Desulfurization

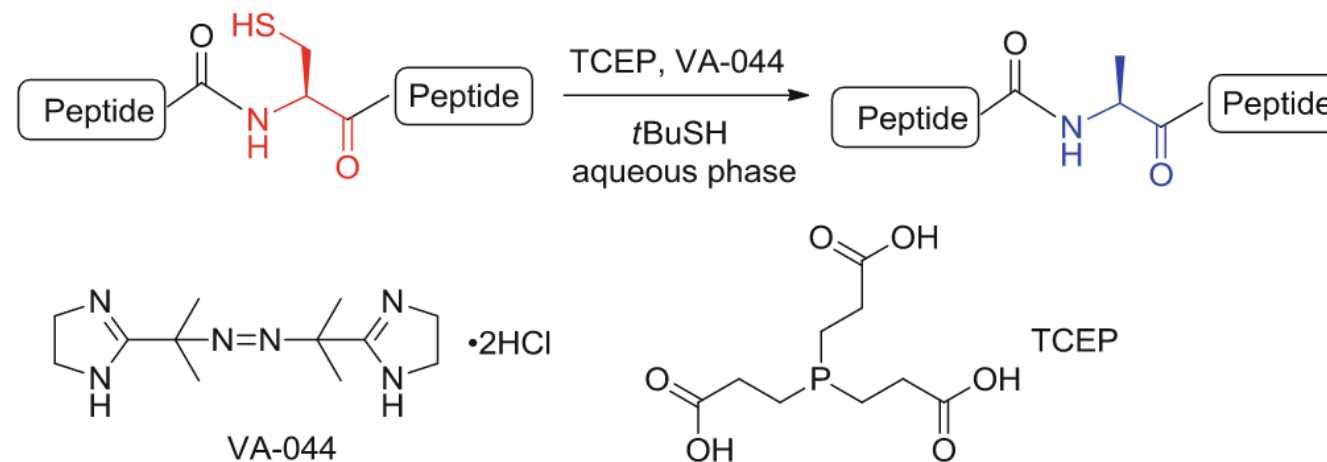
1. Unexpected desulfurization by Alferiev *et al.* (2005)



2. Radical promoted desulfurization by Hoffmann and Walling *et al.* (1956, 1957, 1960)

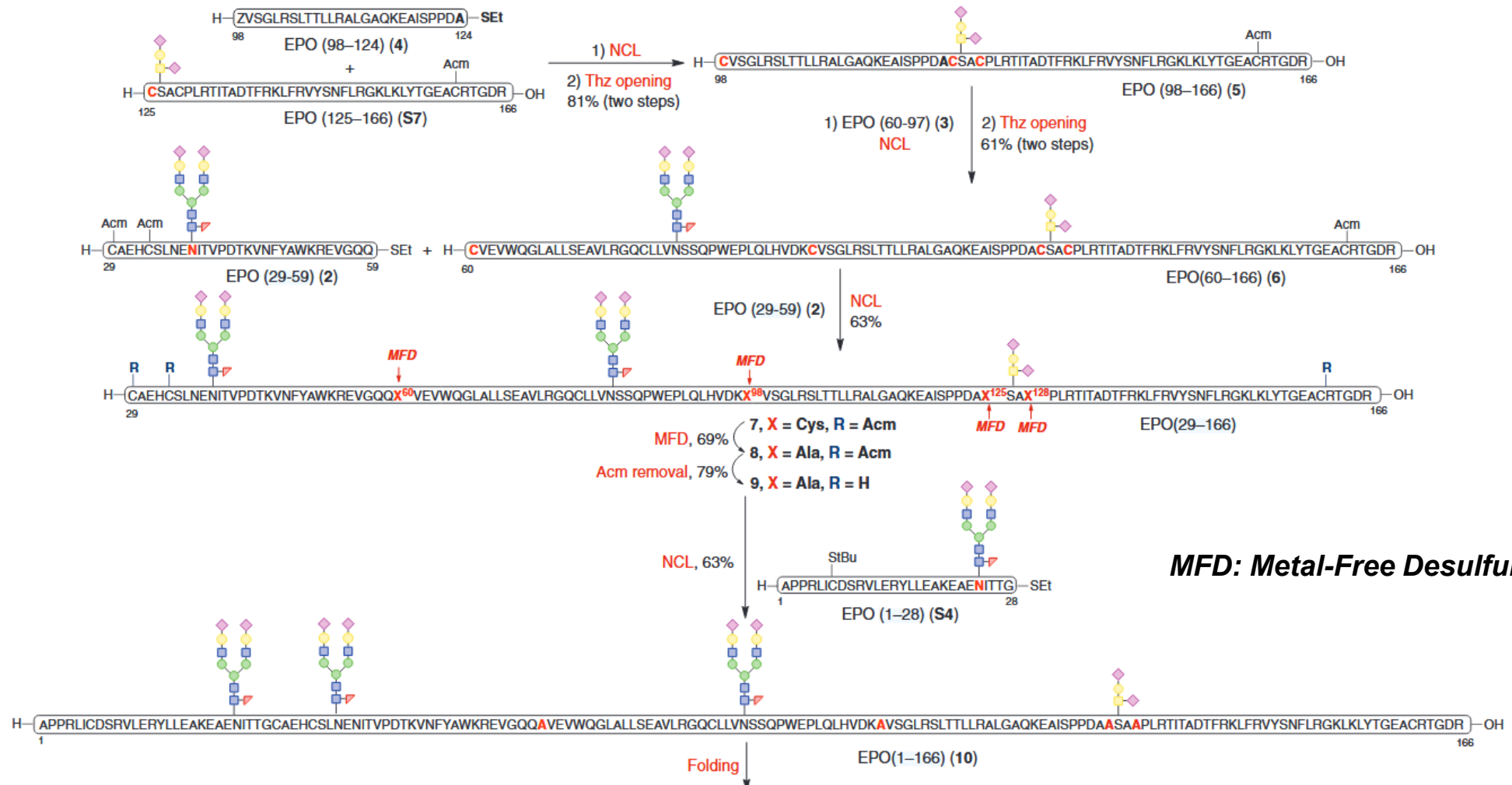


3. Metal-free desulfurization on cysteine by Wan and Danishefsky (2007)

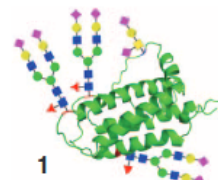


# Ligation-desulfurization Based on Ala

## Metal-free Desulfurization



**MFD: Metal-Free Desulfurization**



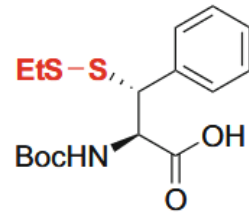
Wang, P., Danishefsky, S. J., et al., *Science* **2013**, 342(6164), 1357–1360.

◆ sialic acid ■ glucosamine  
● galactose ▲ fucose  
● mannose ■ galactosamine

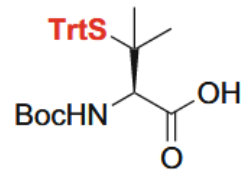


# Ligation-desulfurization of Other AAs

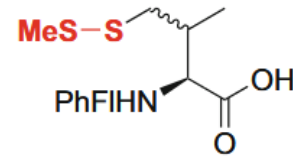
## Thiol amino acid



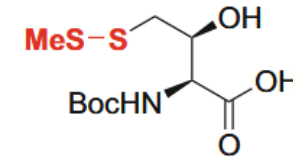
Phe  
84



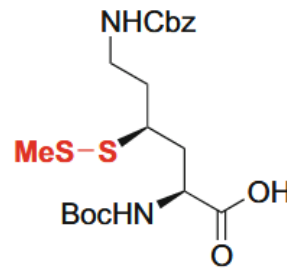
Val  
85



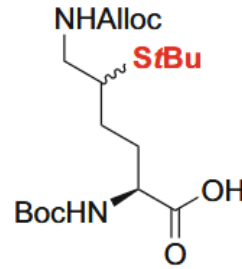
Val  
86



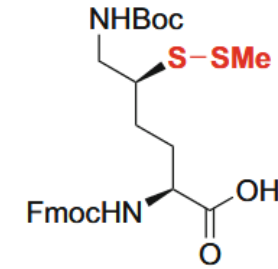
Thr  
87



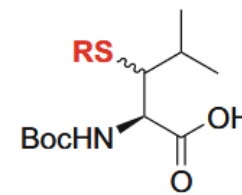
Lys  
88



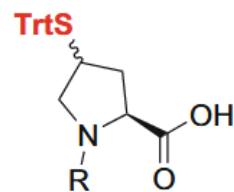
Lys  
89



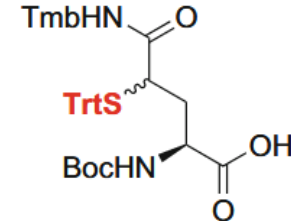
Lys  
90



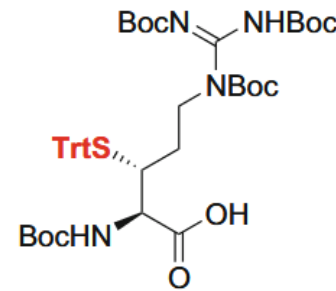
Leu  
91: R = SMe  
92: R = PMB



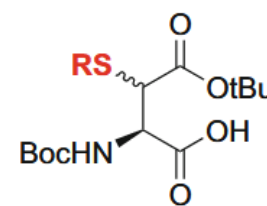
Pro  
93: R = Fmoc  
94: R = Boc



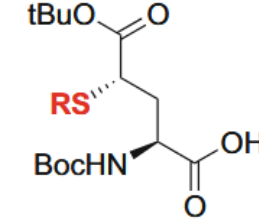
Gln  
95



Arg  
96



Asp  
97: R = Tmb  
98: R = Trt

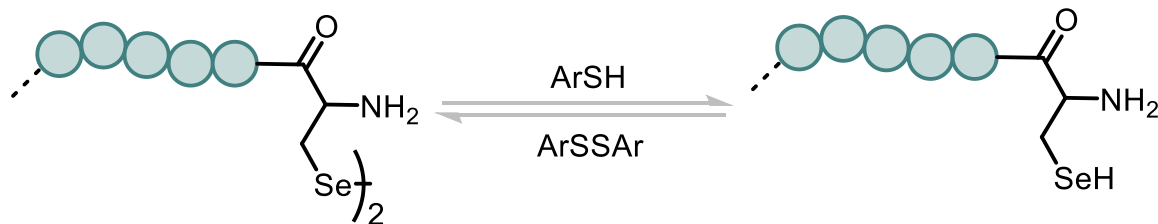


Glu  
99: R = Dmb  
100: R = SMe

# Diselenide-Selenoester Ligation(DSL)

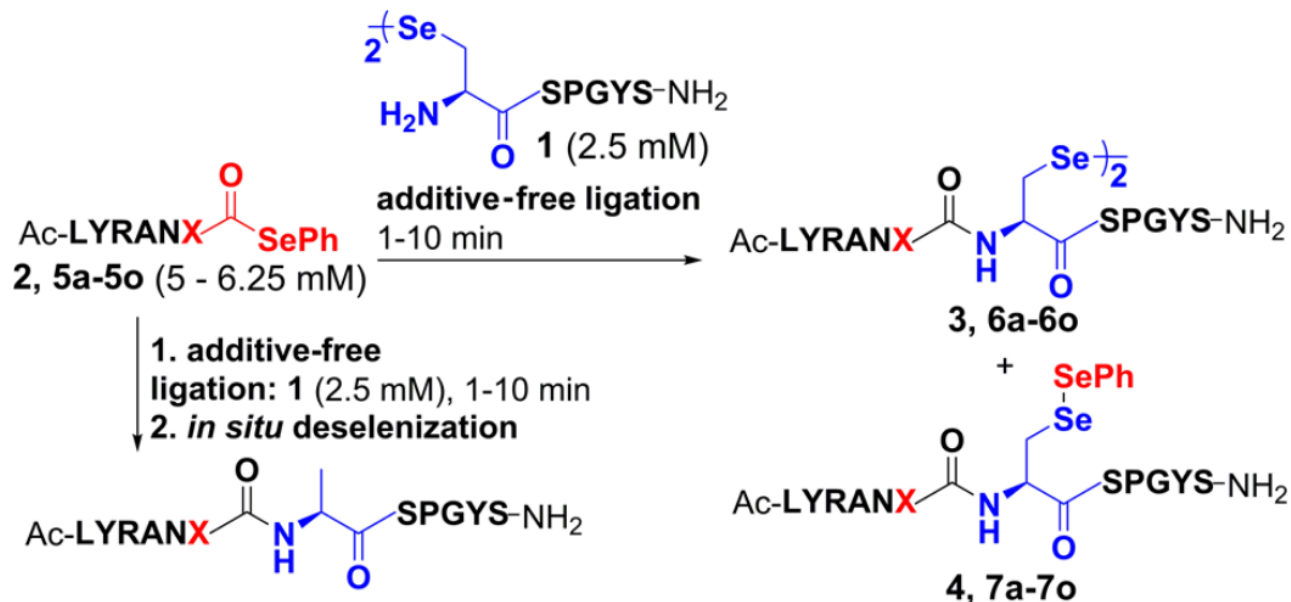
## Desulfurization strategy:

- Low ligation rates at sterically hindered C-terminal thioesters.
- Incompatible with Cys etc. elsewhere in the sequence.



□ Higher nucleophilicity

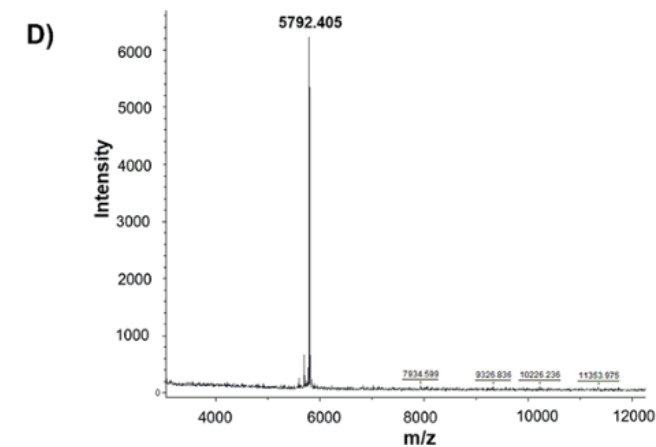
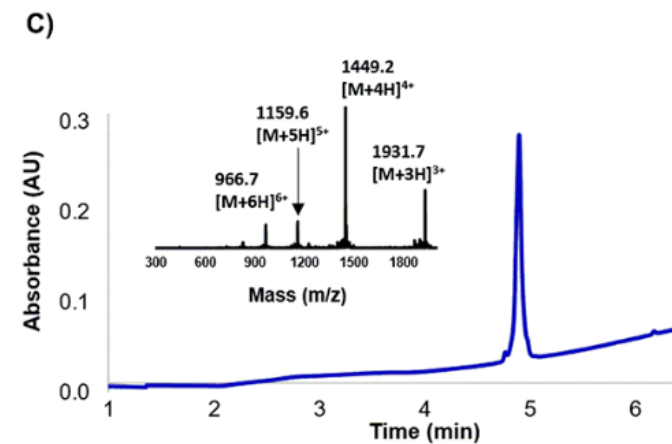
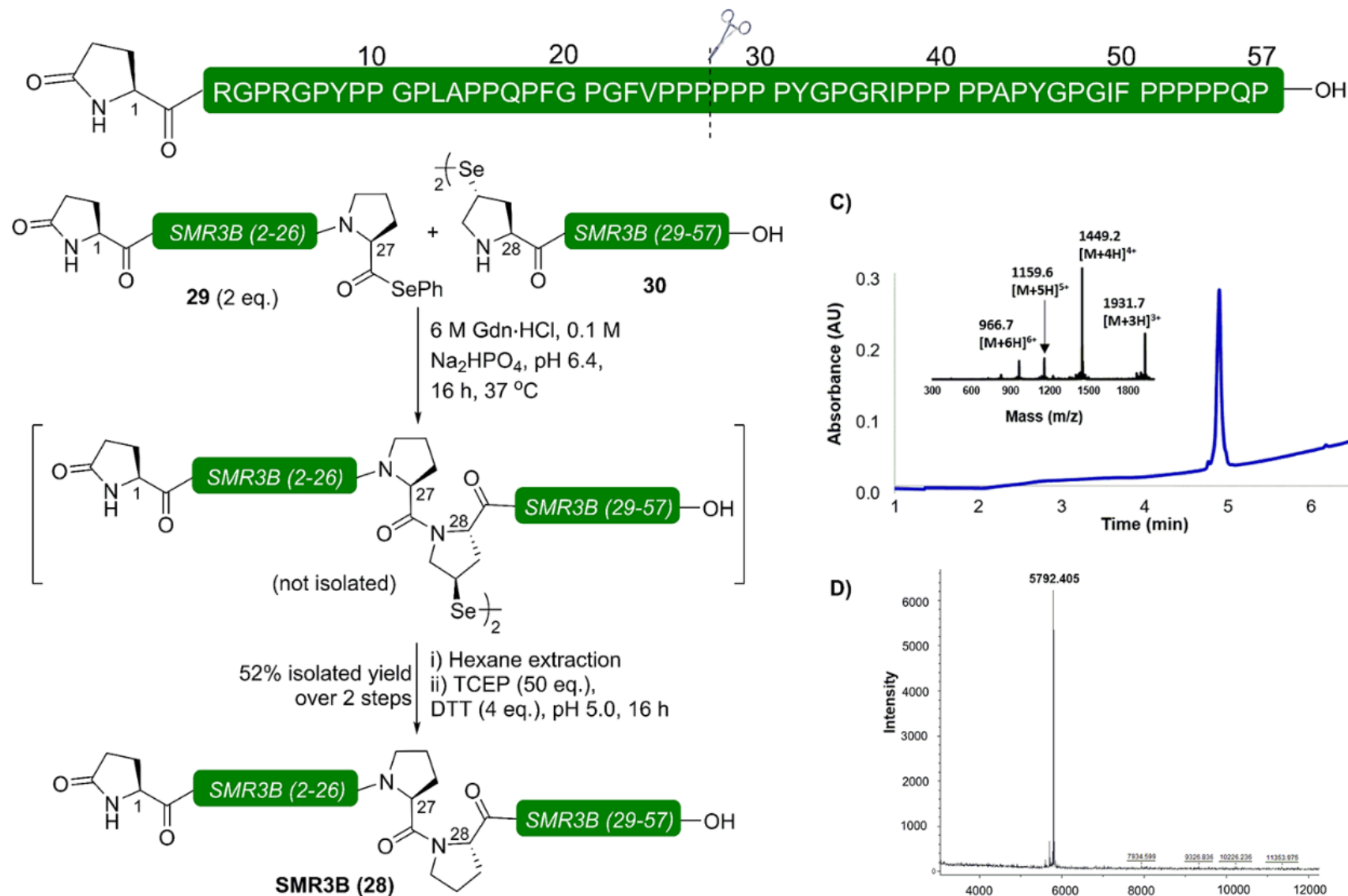
□ Lower steady-state concentration



3 + 4: <b>X = A</b> (72%) <sup>[b]</sup>	6h + 7h: <b>X = R</b> (74%) <sup>[b]</sup>
6a + 7a: <b>X = S</b> (73%) <sup>[b]</sup>	6i + 7i: <b>X = N</b> (88%) <sup>[b]</sup>
6b + 7b: <b>X = T</b> (84%) <sup>[b]</sup>	6j + 7j: <b>X = Q</b> (80%) <sup>[b]</sup>
6c + 7c: <b>X = L</b> (87%) <sup>[b]</sup>	6k + 7k: <b>X = Y</b> (93%) <sup>[b]</sup>
6d + 7d: <b>X = F</b> (83%) <sup>[b]</sup>	6l + 7l: <b>X = W</b> (88%) <sup>[b]</sup>
6e + 7e: <b>X = M</b> (72%) <sup>[b]</sup>	6m + 7m: <b>X = I</b> (63%) <sup>[c]</sup>
6f + 7f: <b>X = K</b> (79%) <sup>[b]</sup>	6n + 7n: <b>X = V</b> (67%) <sup>[c]</sup>
6g + 7g: <b>X = G</b> (96%) <sup>[b]</sup>	6o + 7o: <b>X = P</b> (NR)



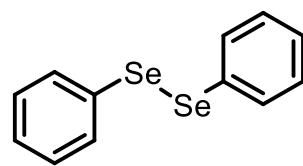
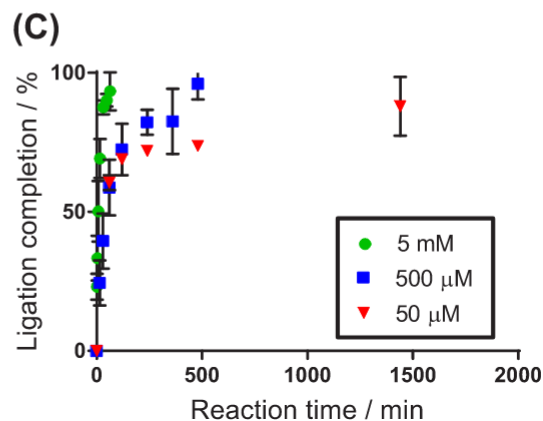
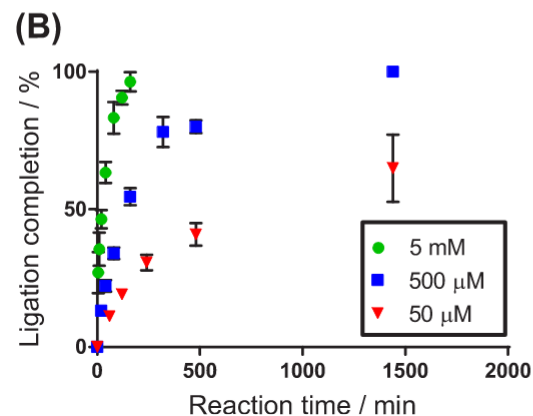
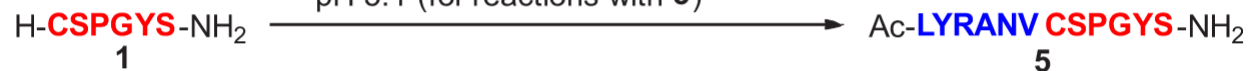
# Diselenide-Selenoester Ligation(DSL)



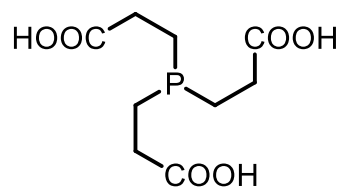
# Diselenide-Selenoester Ligation(DSL)

## reductive-DSL

2 eq. Ac-LYRANV -XR  
 (2: XR = SCH<sub>2</sub>CF<sub>3</sub>, 3: XR = SePh)  
 6 M Gdn•HCl, 0.2 M BIS-TRIS,  
 50 mM TCEP, 37 °C  
 pH 6.2 (for reactions with 2)  
 pH 5.1 (for reactions with 3)

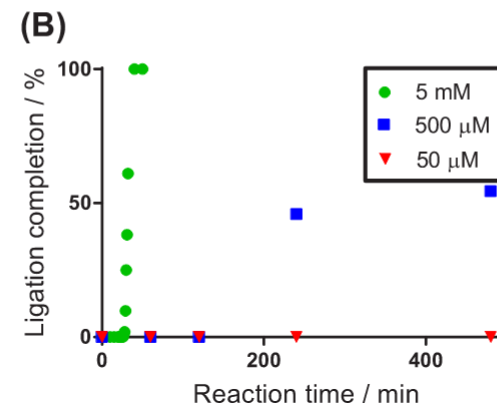
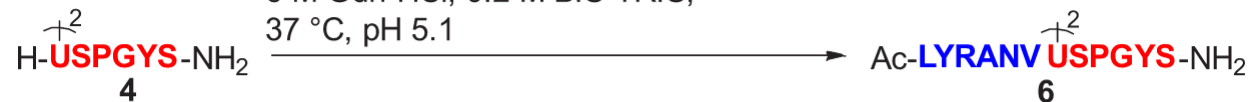


DPDS

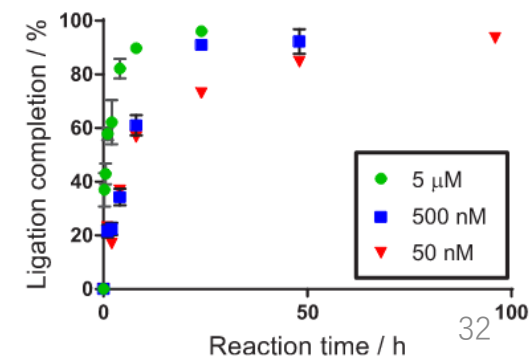
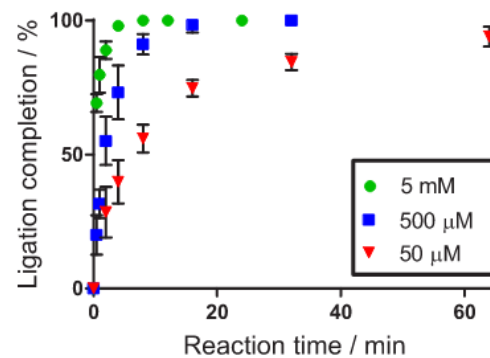
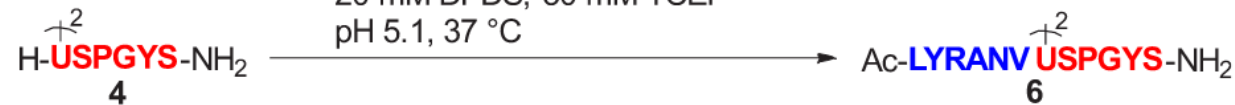


TCEP

2 eq. Ac-LYRANV -SePh **3** (relative to monomeric **4**)  
 6 M Gdn•HCl, 0.2 M BIS-TRIS,  
 37 °C, pH 5.1



2 eq. Ac-LYRANV -SePh (**3**)  
 6 M Gdn•HCl, 0.2 M BIS-TRIS,  
 20 mM DPDS, 30 mM TCEP  
 pH 5.1, 37 °C

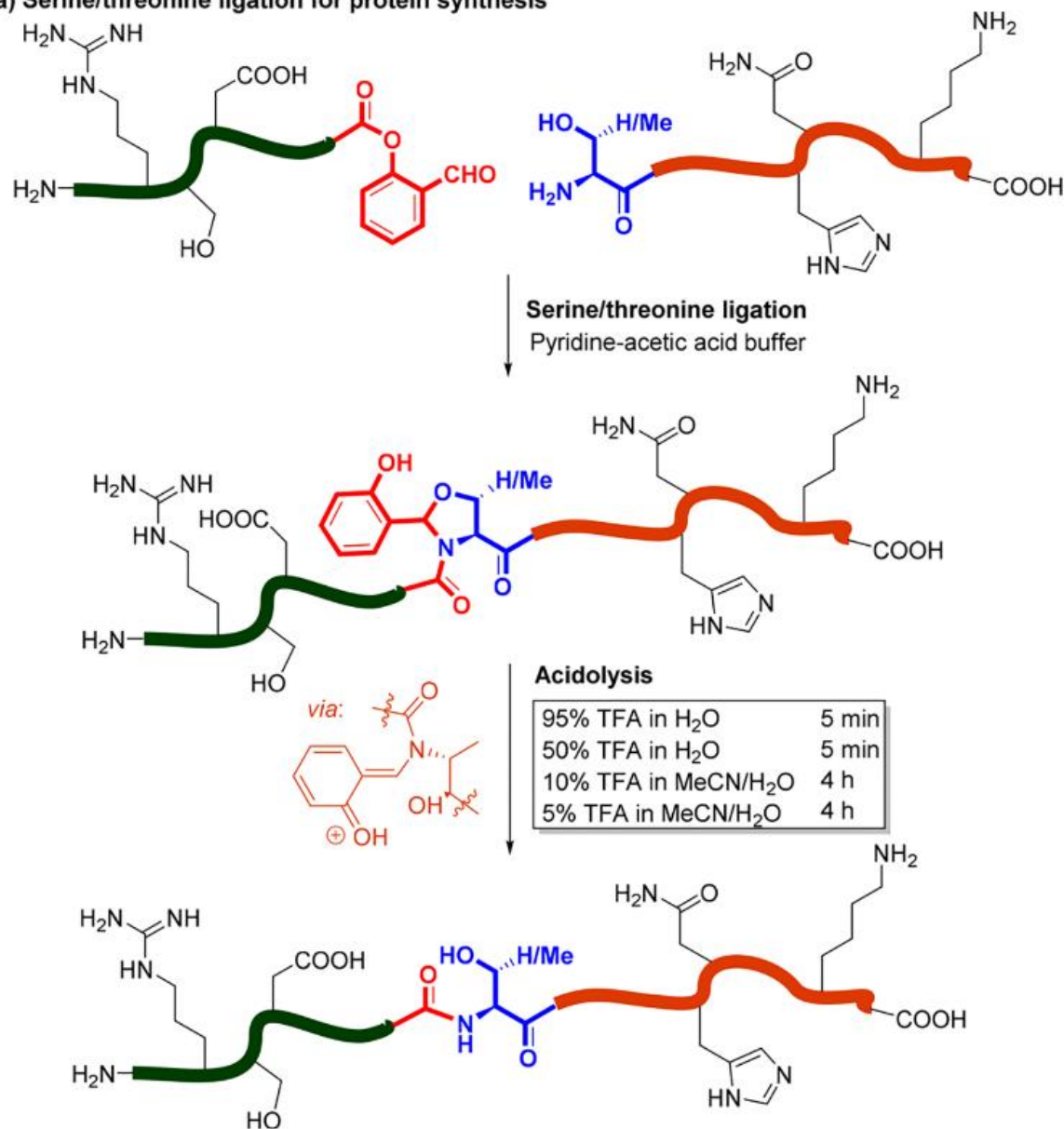


# Outline

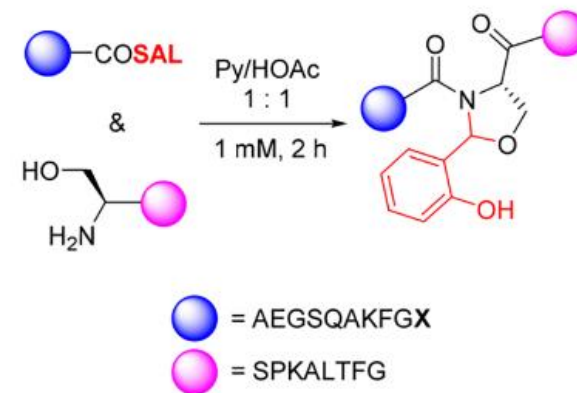
- Introduction
- Native Chemical Ligation(NCL)
  - Mechanism
  - Precursor of Thioester
  - Alternative of Cys
    - Auxiliary-based Methods
    - Ligation-desulfurization
    - Diselenide-Selenoester Ligation(DSL)
- **Serine/Threonine Ligation(STL) & Cysteine/Penicillamine Ligation(CPL)**
- $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation
- Staudinger Ligation
- Summary and Outlook
- Acknowledgement

# Serine/Threonine Ligation(STL)

a) Serine/threonine ligation for protein synthesis



b) Effect of C-terminal residues on STL

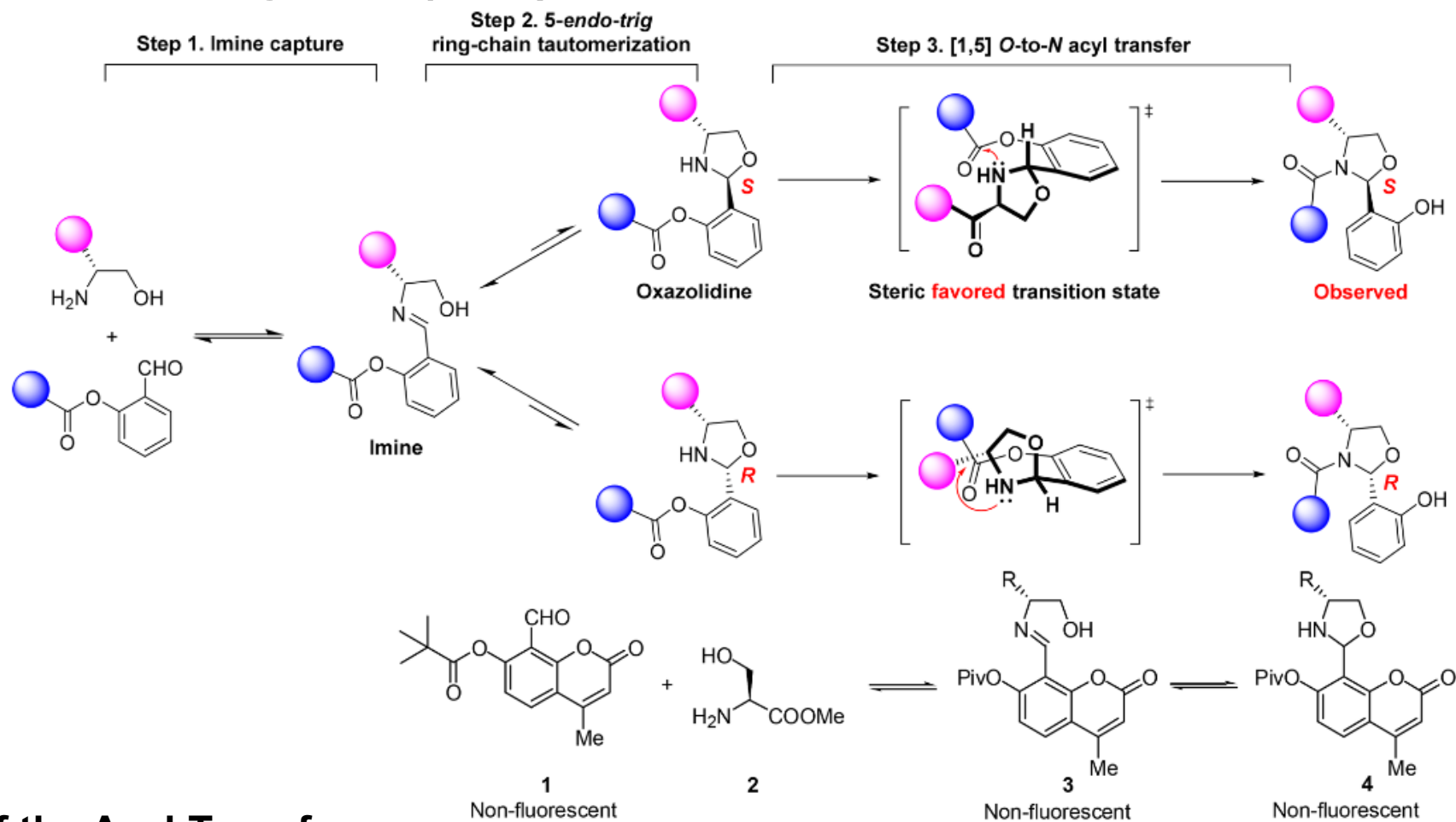


C-terminal residues (X) Conversion (%) at 2 h

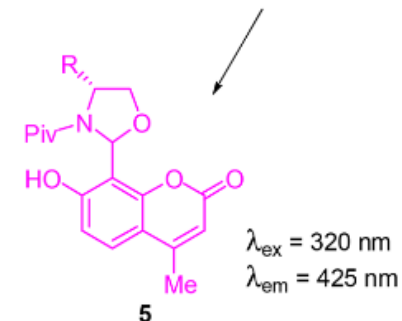
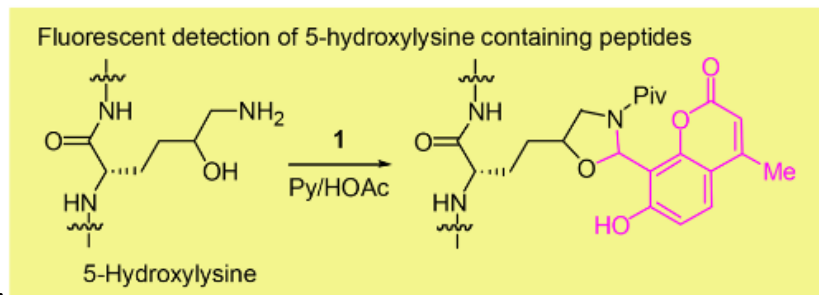
	Ala	87.1
	Gly	85.9
F	Ser	84.8
A	Gln	78.7
S	Thr	71.3
T	Phe	67.7
	Cys(SStBu)	65.2
M	Val	45.5
E	Ile	41.8
DI	Met	38.5
U	Asn	38.5
M	Tyr	33.7
	Leu	33.4
S	His	28.6
L	Trp	24.8
O	Arg	20.5
W	Pro	7.9
	Lys	Decompose
	Asp	N.D.
	Glu	N.D.

# Serine/Threonine Ligation (STL)

## Mechanism



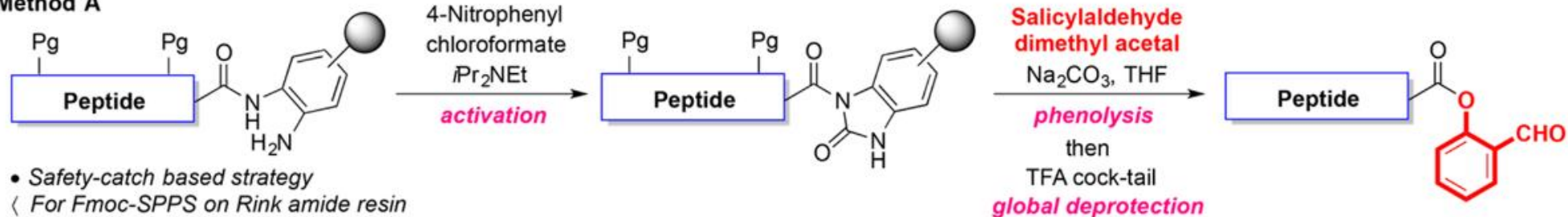
## Monitoring of the Acyl Transfer:



# Serine/Threonine Ligation(STL)

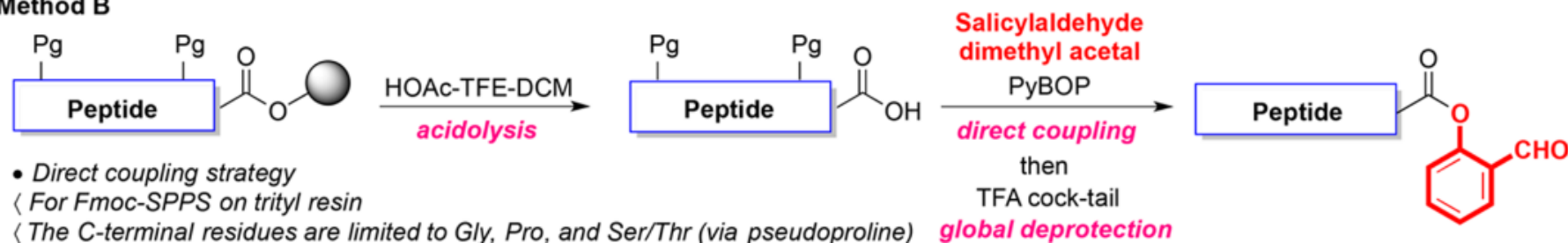
Liu, H., Li, X., *Acc. Chem. Res.* **2018**, *51*(7), 1643–1655.

## Method A



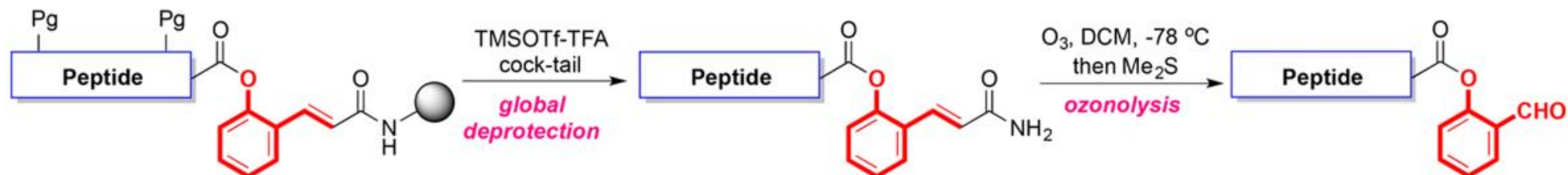
- Safety-catch based strategy
- ⟨ For Fmoc-SPPS on Rink amide resin
- ⟨ No limitation for C-terminal residue

## Method B



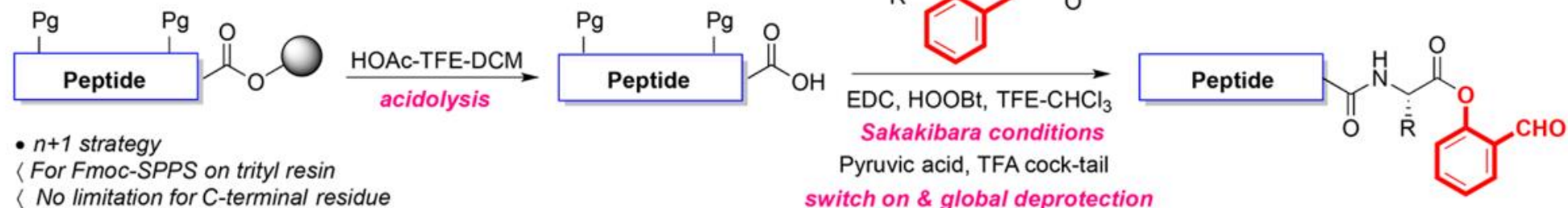
- Direct coupling strategy
- ⟨ For Fmoc-SPPS on trityl resin
- ⟨ The C-terminal residues are limited to Gly, Pro, and Ser/Thr (via pseudoproline)

## Method C



- Ozonolysis based strategy
- ⟨ For Boc-SPPS on AM resin
- ⟨ Limited to peptides without Trp/Cys/Met in the sequences.

## Method D



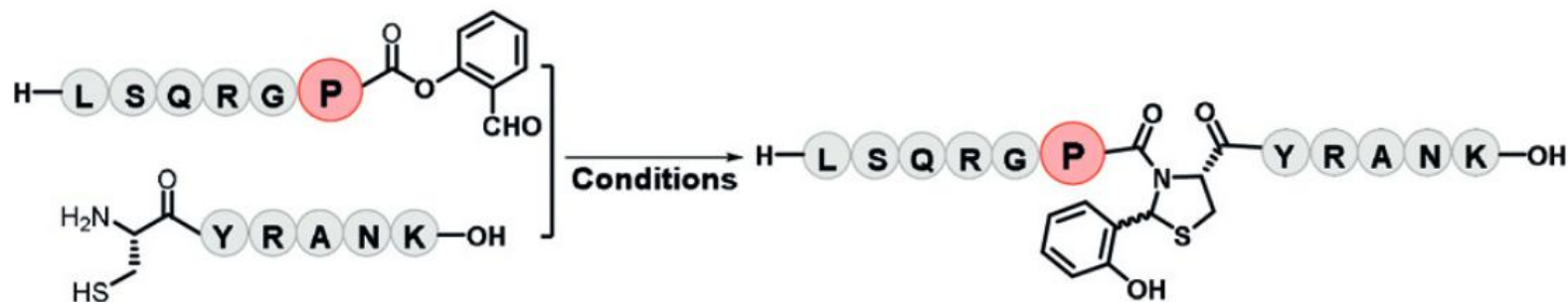
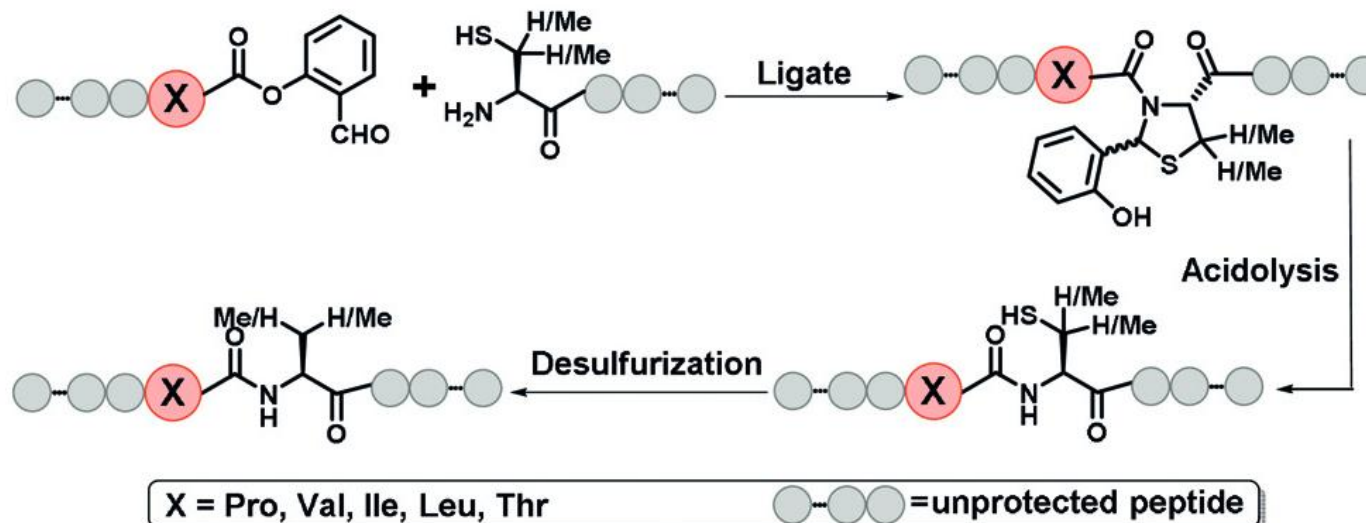
- n+1 strategy
- ⟨ For Fmoc-SPPS on trityl resin
- ⟨ No limitation for C-terminal residue



# Cysteine/Penicillamine Ligation (CPL)

## ■ C-terminal Pro/Val/Ile: Low reactivity in NCL/STL

## CPL: combination of NCL & STL

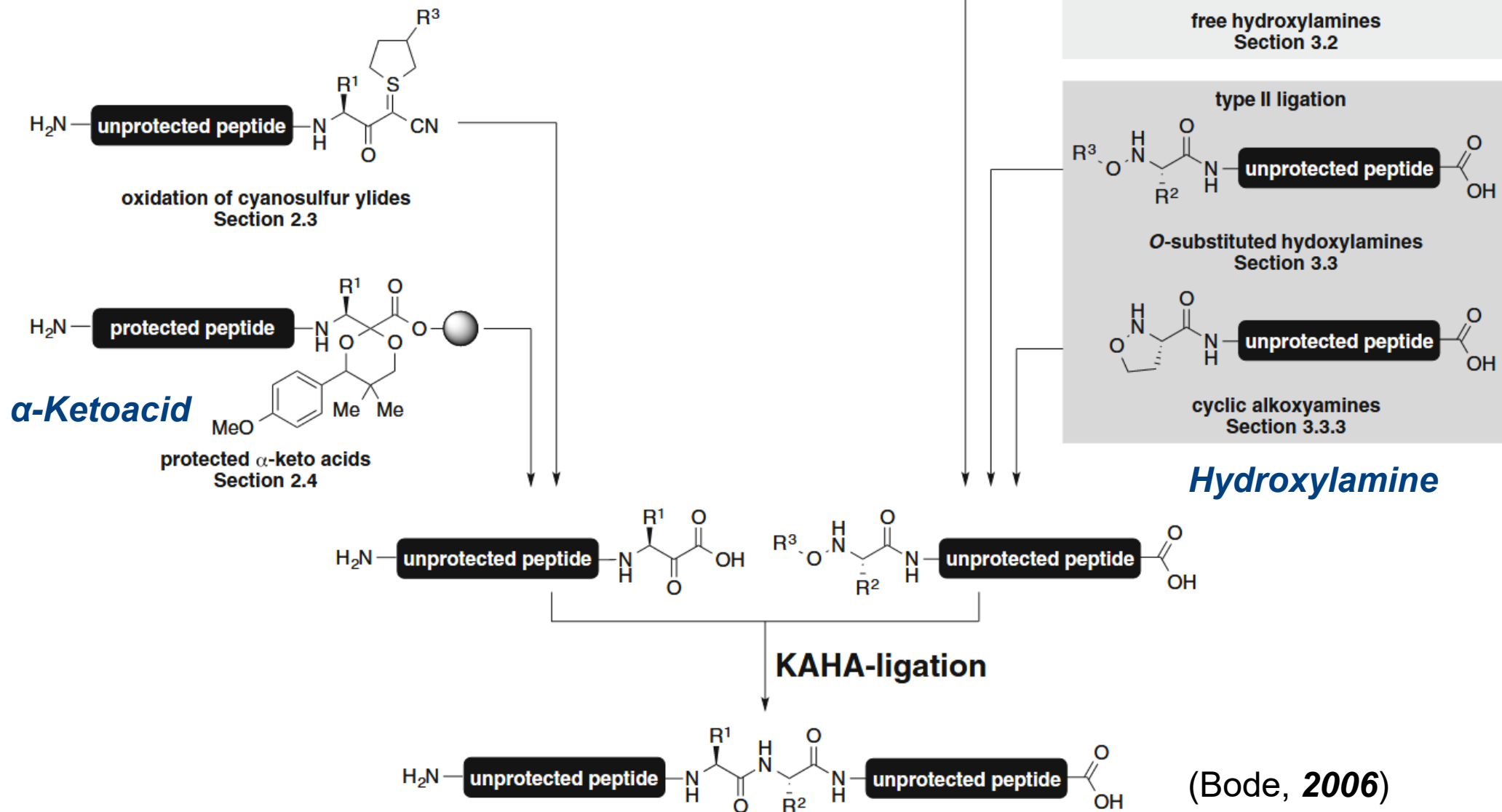


Entry	Reaction conditions <sup>[a]</sup>	<i>t</i> [h]	Conversion [%] <sup>[b]</sup>
1	Phosphate solution containing 6 m Gn·HCl, pH 4	24	90
2	Pyridine/Acetic acid = 1/1, (mol/mol)	2	> 95

# Outline

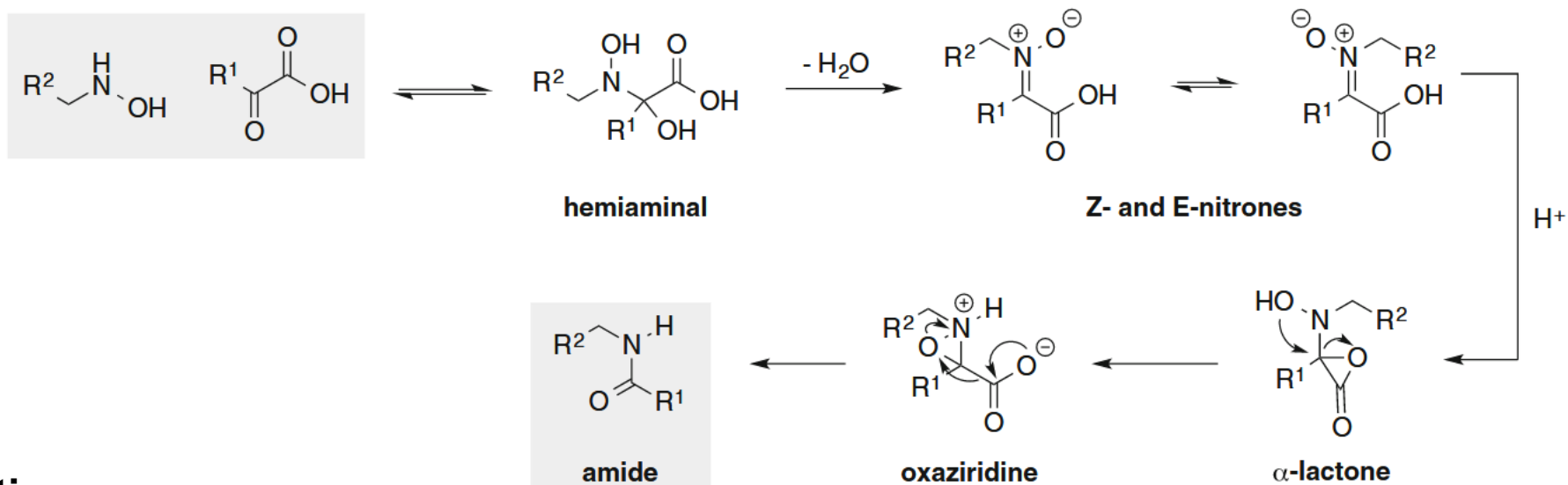
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# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

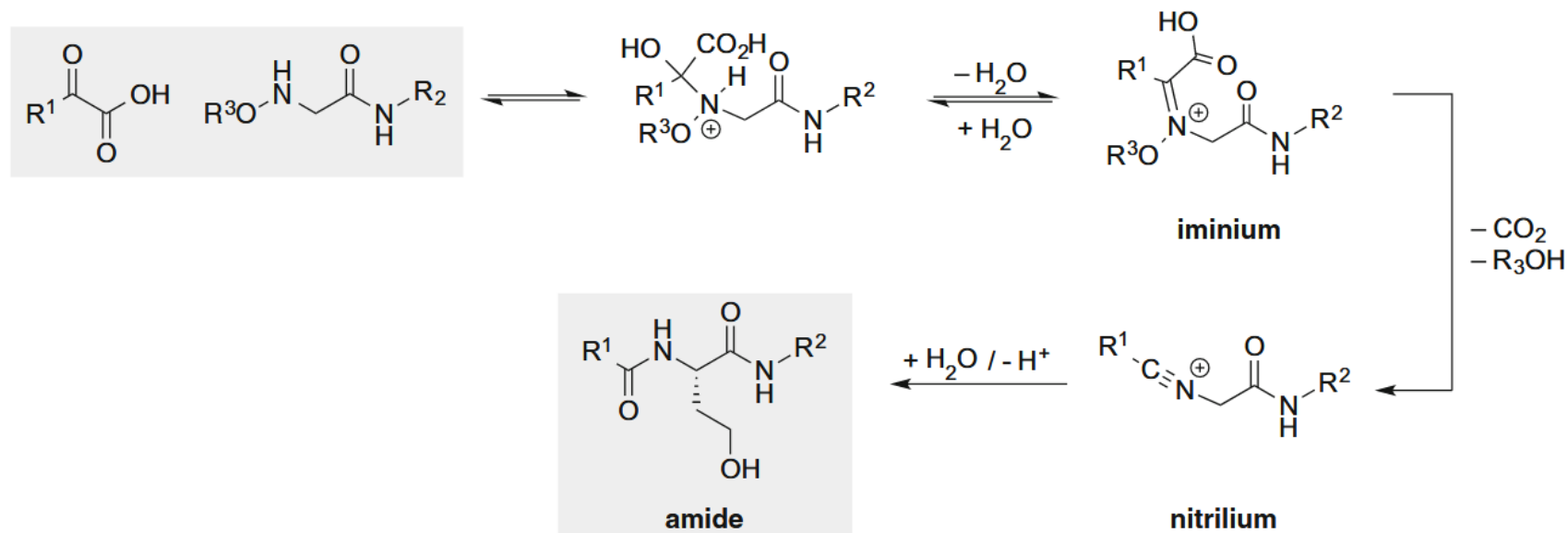


# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

## Type I Ligation:

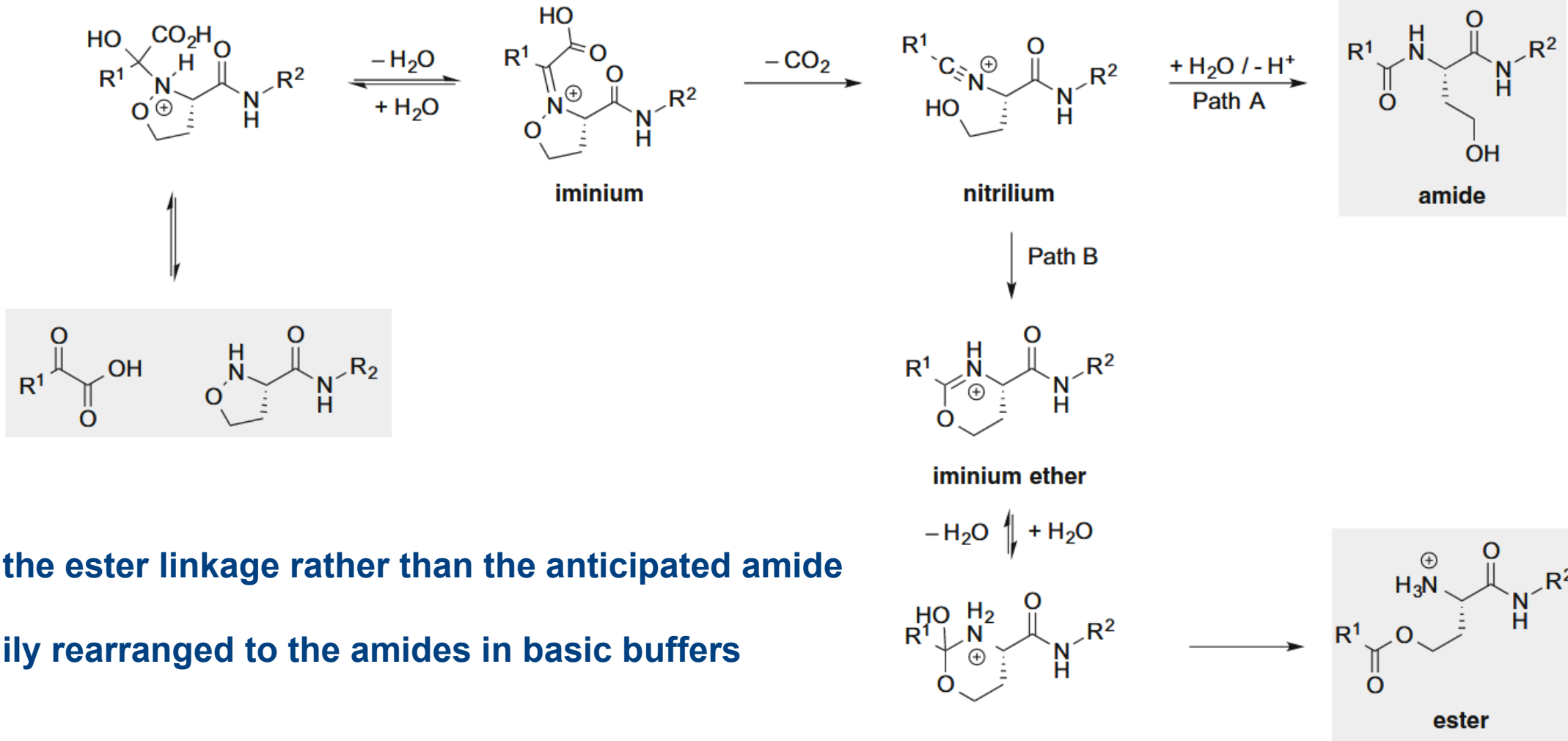


## Type II Ligation:



# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

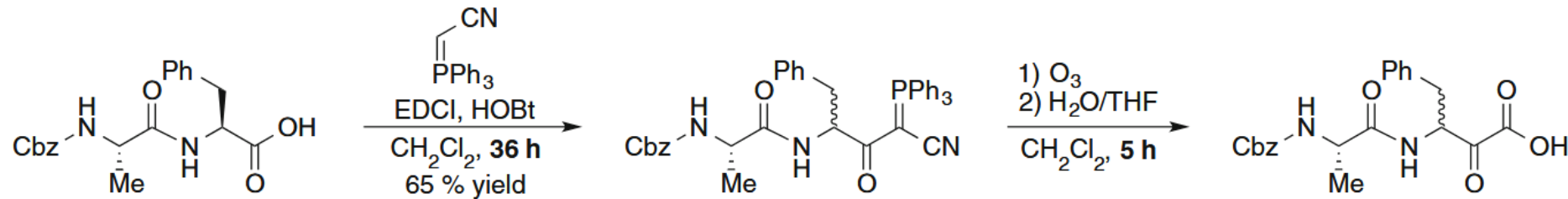
Ligation with 5-oxaproline:



# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

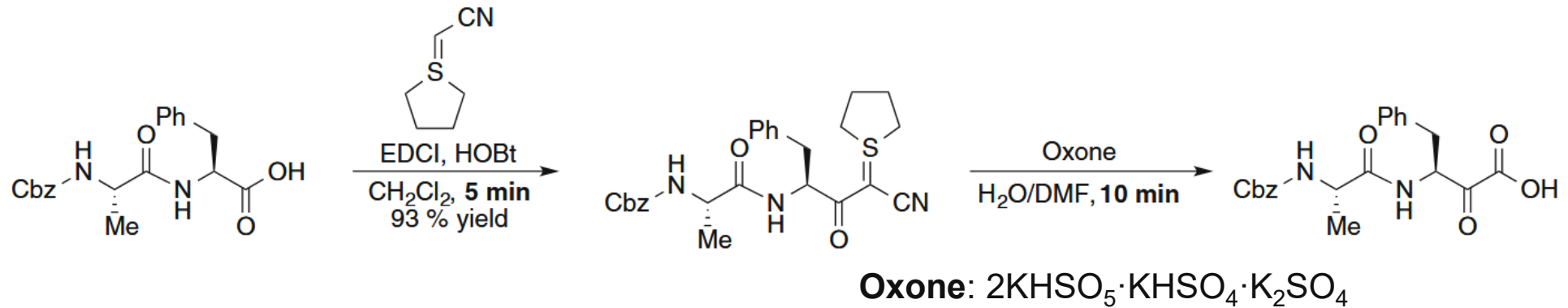
## Preparation of Ketoacid

□ *via* phosphorus ylide(Wasserman 1992):



□ long reaction times, resulting in epimerization

□ *via* sulfur ylide(Bernatowicz 1983):

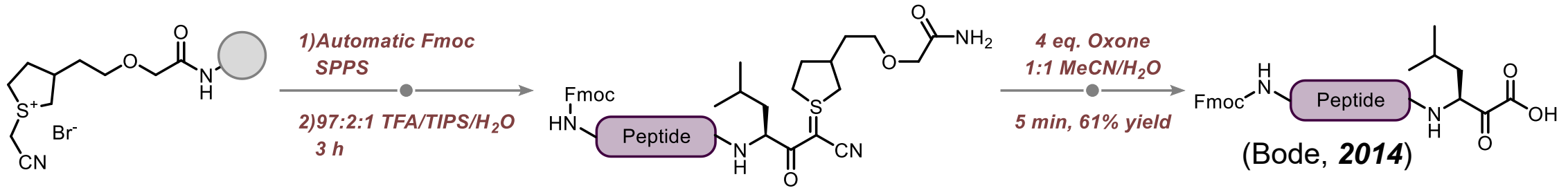


Tolerates all unprotected AA except for Cys & Met, Try sometimes problematic

# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

## Preparation of Ketoacid

### □ Oxidative cleavage after SPPS:

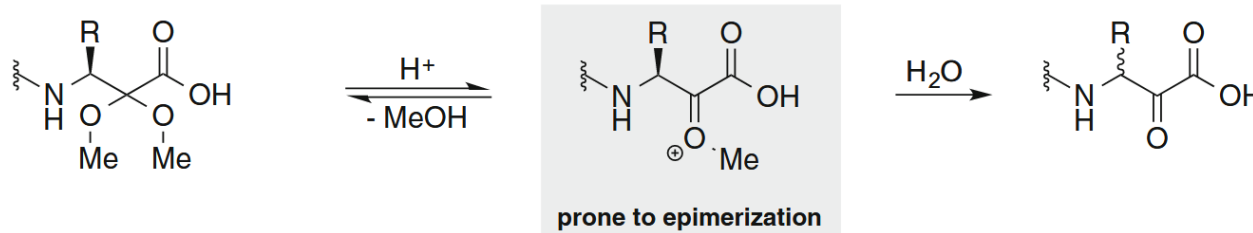


➤ incompatible with Cys, Met and Try

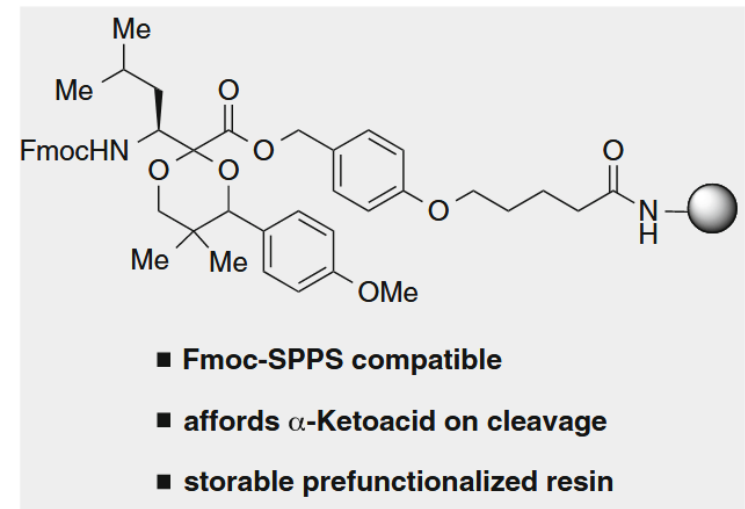
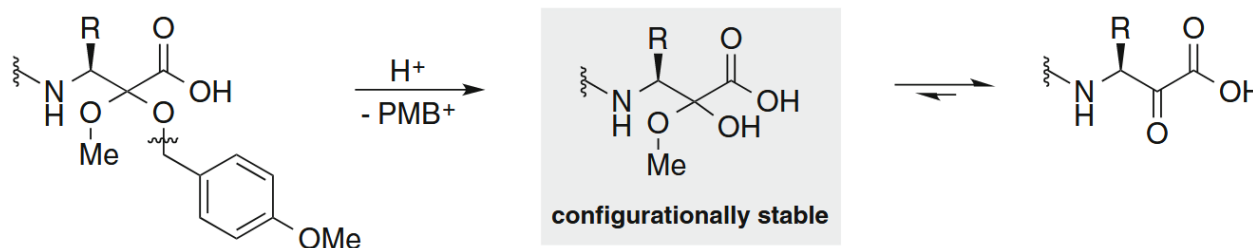
➤ additional purification at the late stage of the synthesis.

### □ Protecting groups:

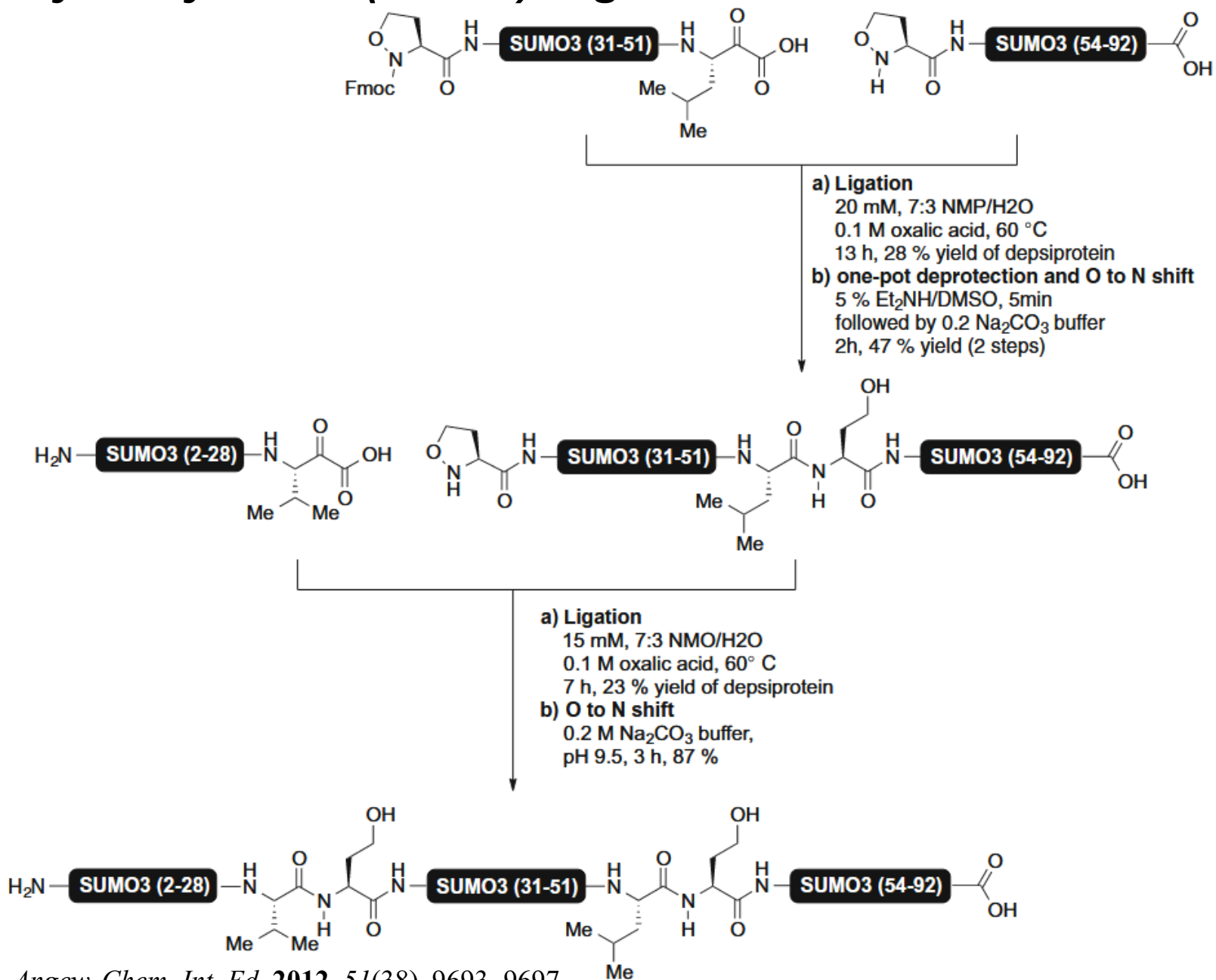
Deprotection of traditional acetal protecting groups



Proposed acid-labile protecting group for enantiopure  $\alpha$ -ketoacids



# $\alpha$ -Ketoacid Hydroxylamine(KAHA) Ligation

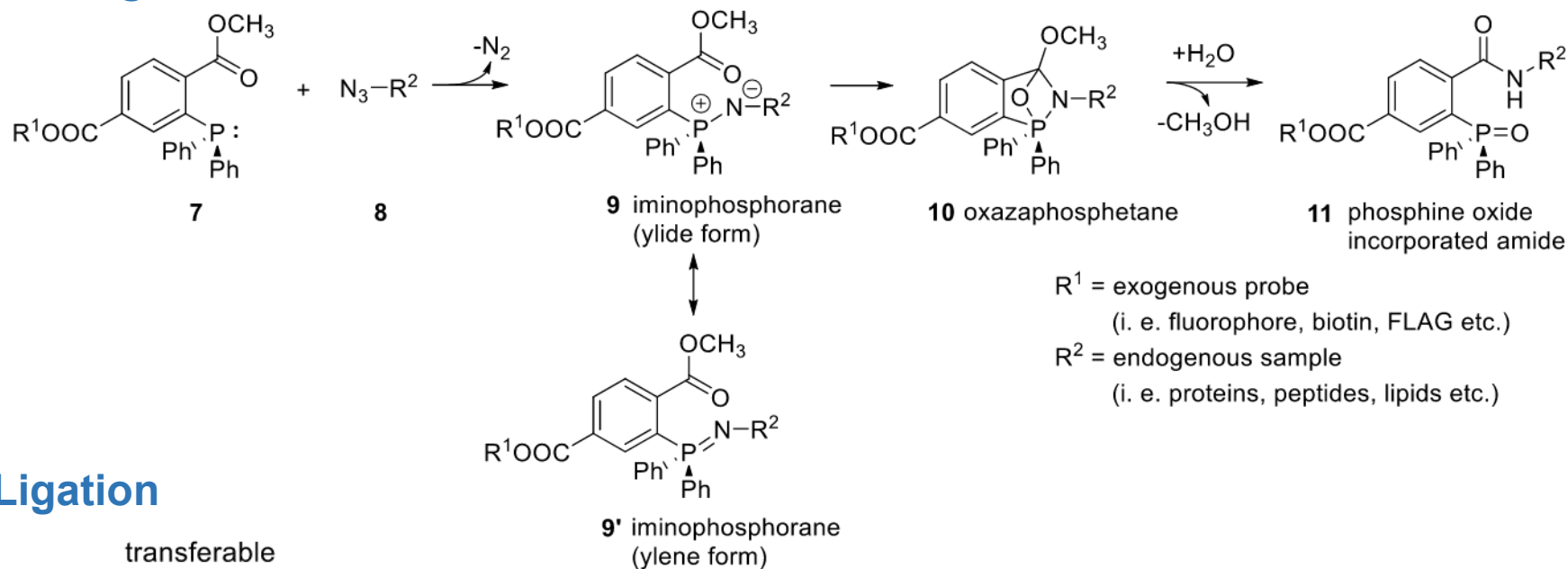


# Outline

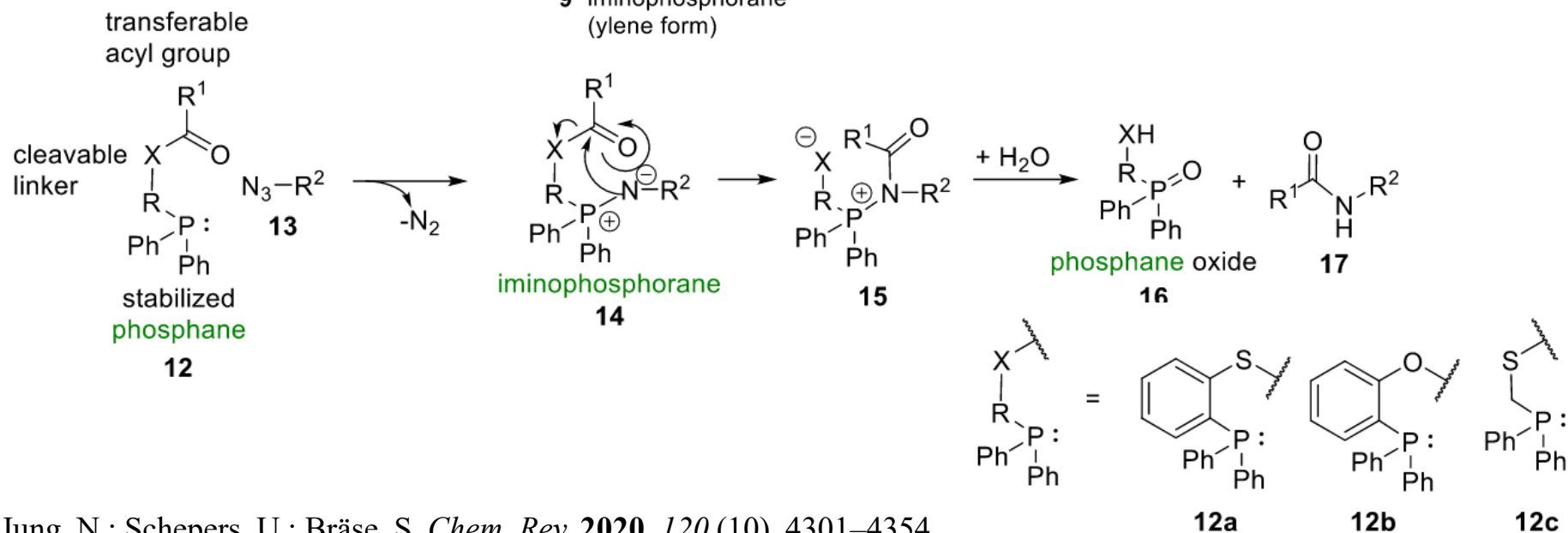
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# Staudinger Ligation

## Non-traceless Ligation



## Traceless Ligation

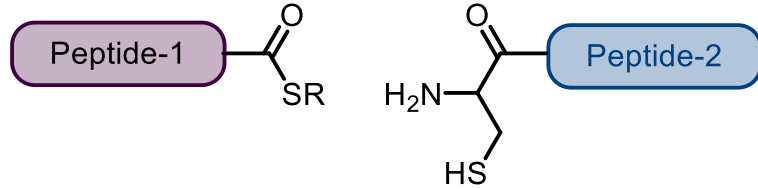


# Outline

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# Summary

## ■ Native Chemical Ligation(NCL)



### □ Thioester precursor

#### □ Hydrazide

### □ Alternative of Cys

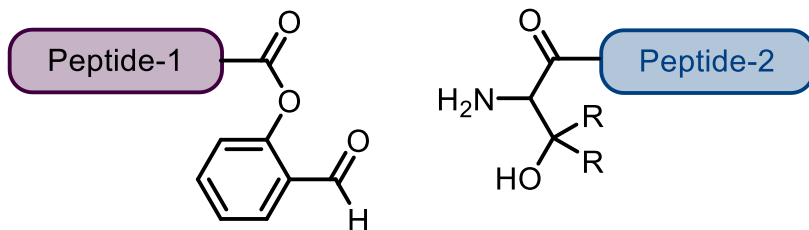
#### □ Auxiliary: N-terminal, Sidechain

#### □ Ligation-desulfurization

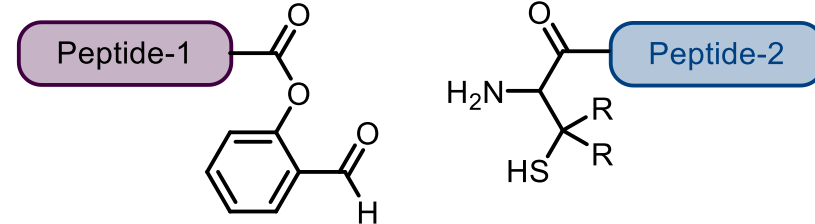
#### □ Ligation-deselenization

#### □ DSL: additive-free, reductive-DSL

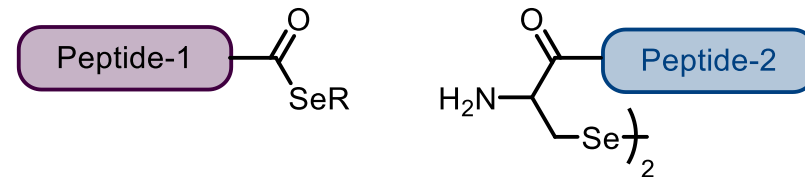
## ■ Serine/Threonine Ligation(STL)



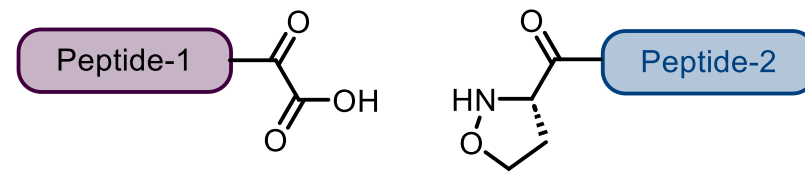
## ■ Cysteine/Penicillamine Ligation(CPL)



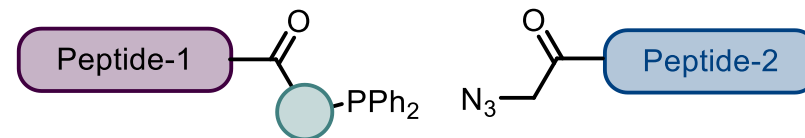
## ■ Diselenide-Selenoester Ligation(DSL)



## ■ α-Ketoacid Hydroxylamine (KAHA) Ligation



## ■ Staudinger Ligation



# ***Outlook***

- **Orthogonal ligation**
- **Synthesis of hydrophobic sequences**
- **Expansion of ligation site scope**

# Acknowledgement

- *Prof. Yan Xu*
- *All members in Xu group*
- *Everyone here*

## Quiz

- Among the chemical ligation methods described above, which one(s) are not based on an acyl transfer process?
- Which C-terminal residues lead to poor efficiency in NCL reactions? Why?
- List several peptide thioester precursors.

# Quiz

- Among the chemical ligation methods described above, which one(s) are not based on an acyl transfer process?
  - KAHA ligation and Staudinger ligation
- Which C-terminal residues lead to poor efficiency in NCL reactions? Why?
  - Val, Ile, Thr: sterically hindered
  - Pro: O-n to C-O- $\pi^*$  electron donation
  - Asn, Asp, Gln, Glu: Cyclization reaction
- List several peptide thioester precursors.
  - Peptide hydrazide
  - Peptide Nbz
  - N-to-S Acyl shift