Literature Seminar

東京大学
THE UNIVERSITY OF TOKYO

Peptide and Protein Ligation

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2018.7.21

Contents

東京大学
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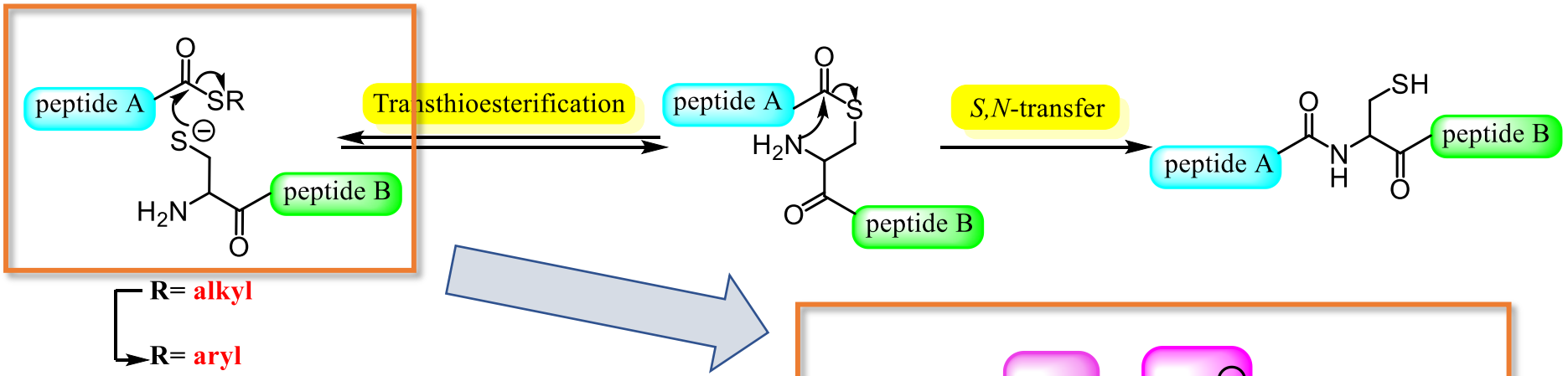
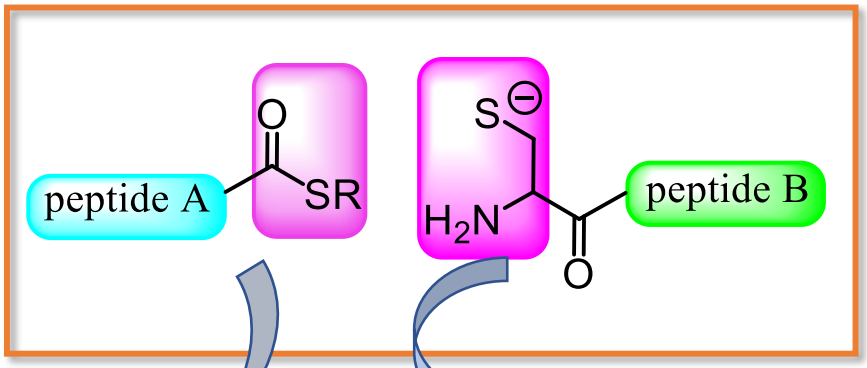
- Introduction
- Extension of **N**ative **C**hemical **L**igation(**NCL**)
- Enzyme-Mediated Ligation
- Summary

Native Chemical Ligation(NCL)



- NCL is an efficient ligation method for native backbone protein ligation.
- NCL is of great use in the cysteine ligation site.



2. *C*-terminal

- Not only limited to thioester
- Modification of this part can also promote the ligation site of *N*-terminal.

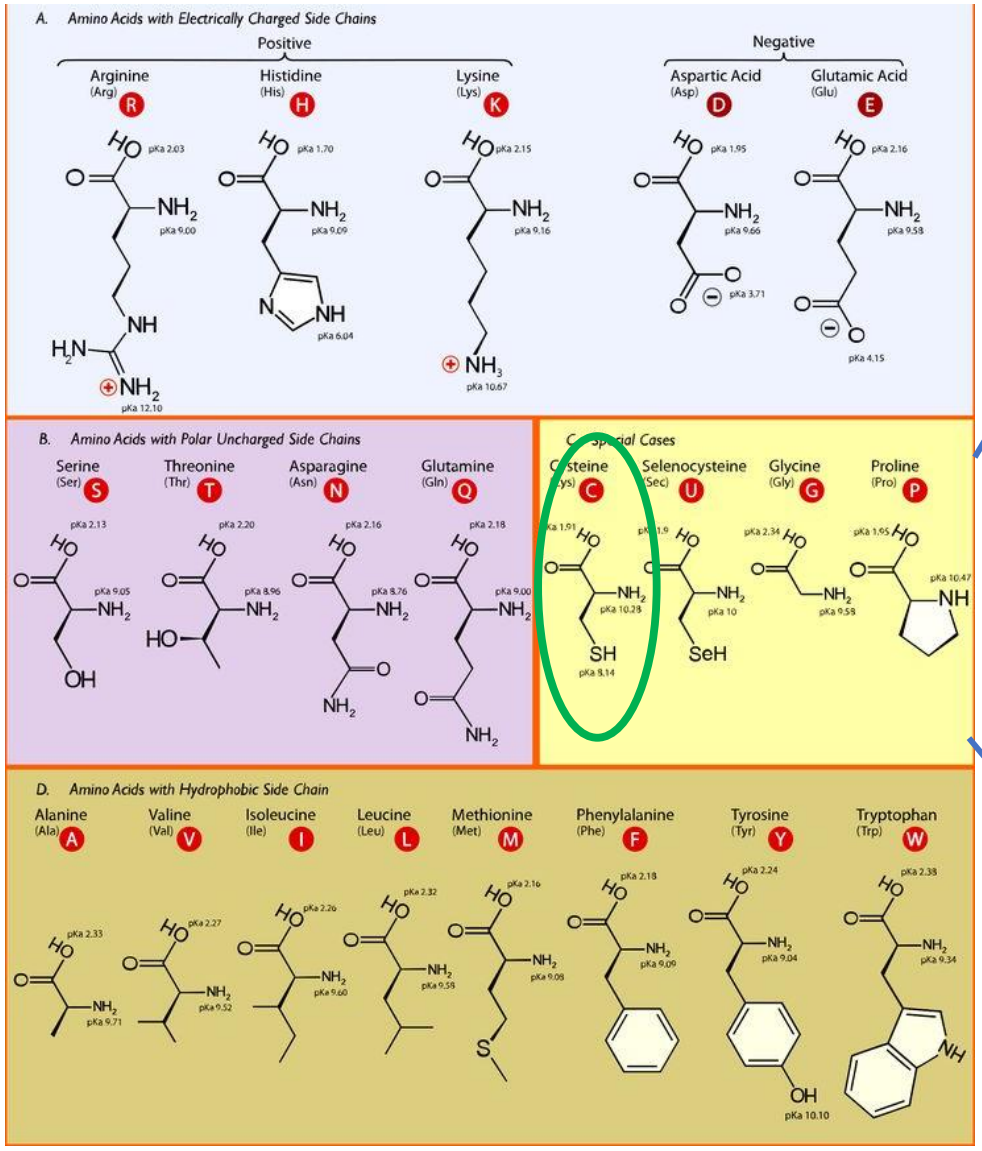
1. *N*-terminal mercaptan

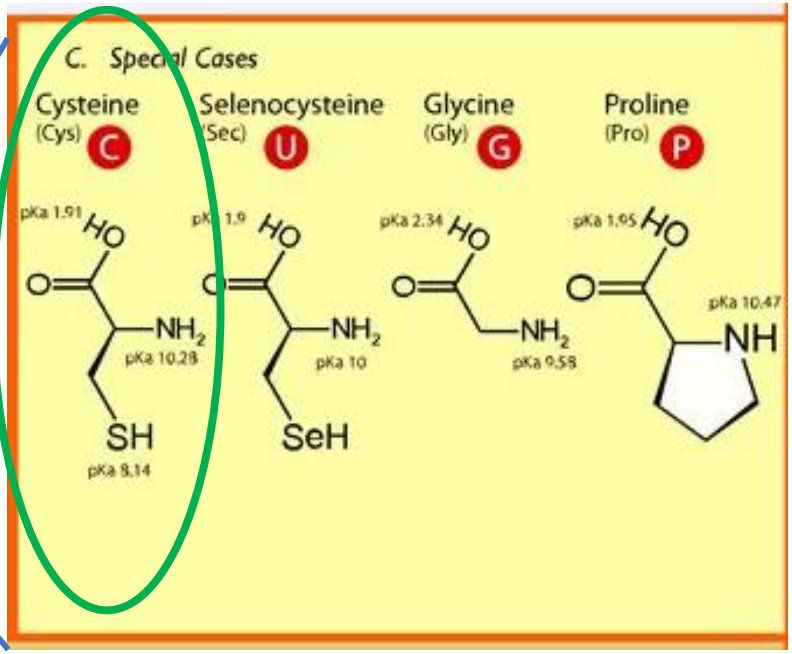
- Only suitable for cysteine.
- Modification for expansion of scope of ligation site.

Brik. A *et al. Nature Chem.* **2016**, *8*, 407-418

Bingham. J. P *et al. Molecules* **2014**, *19*, 14461-14483

21 Amino Acid (Ligation Site)





- Cysteine as the ligation site is achieved.

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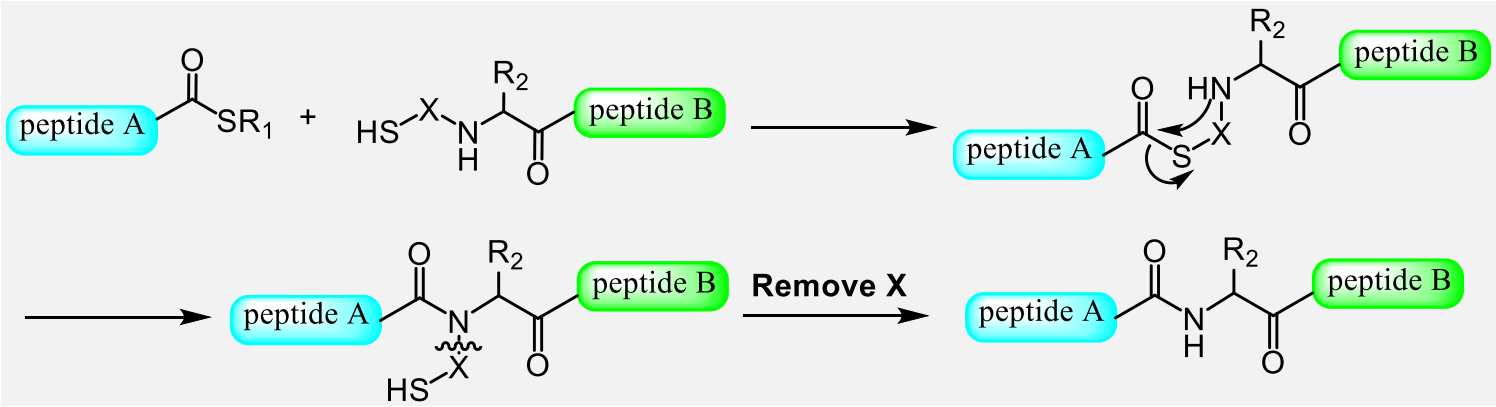
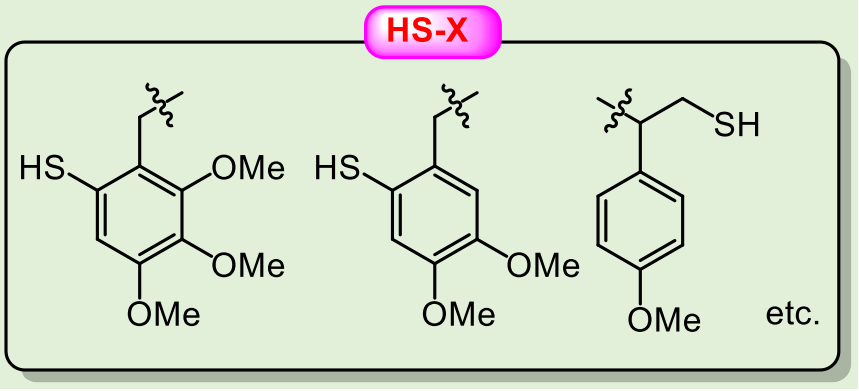
  - ✓ **S**erine/**T**hreonine **L**igation(**STL**)
  - ✓ α-**K**eto**a**cid and a **H**ydroxyl**a**mine (**KAHA** Ligation)
  - ✓ Histidine Ligation
- Enzyme-Mediated Ligation
- Summary

Auxiliary Mediated Ligation

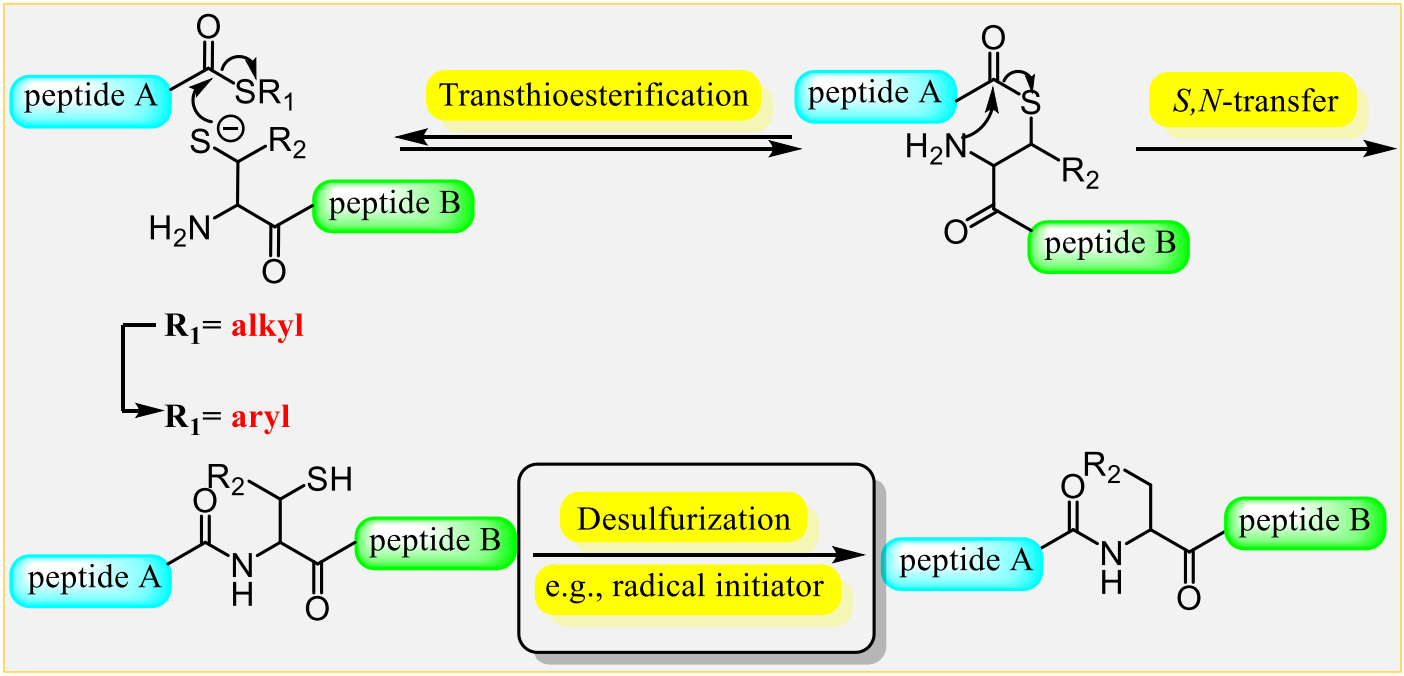


- *C*-terminal usually uses thioester.
- *N*-terminal has the auxiliary electron-rich group.
- Auxiliary group should be removed, such as TFA, HF.
- Requirement is the presence of glycine at one of the ligation sites, due to the bulkiness of the auxiliary group.



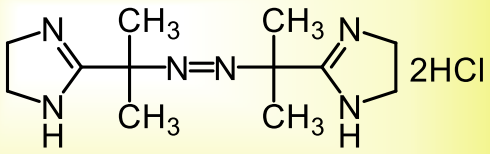
Dawson. P. E *et al. J. Am. Chem. Soc*. **2002**, *124*, 4642-4646
Cardona. V. M. F *et al, J. Peptide Res*. **2003**, *61*, 152-157
Bingham. J. P *et al. Molecules* **2014**, *19*, 14461-14483

NCL-Desulfurization



Usual Desulfurization Methods

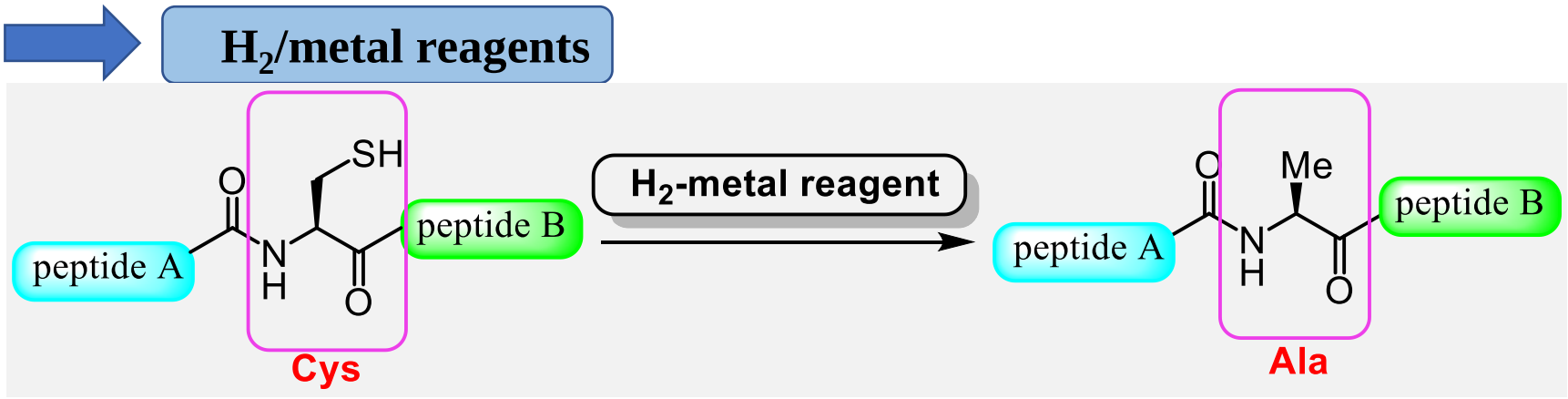
- H_2/metal reagents, such as Pd/Al_2O_3, Pd/C, Raney nickel etc.
- Metal-free reductant, VA-044.



VA-044

Dawson. P. E *et al. Isr. J. Chem.* **2011**, *51*, 862 – 867

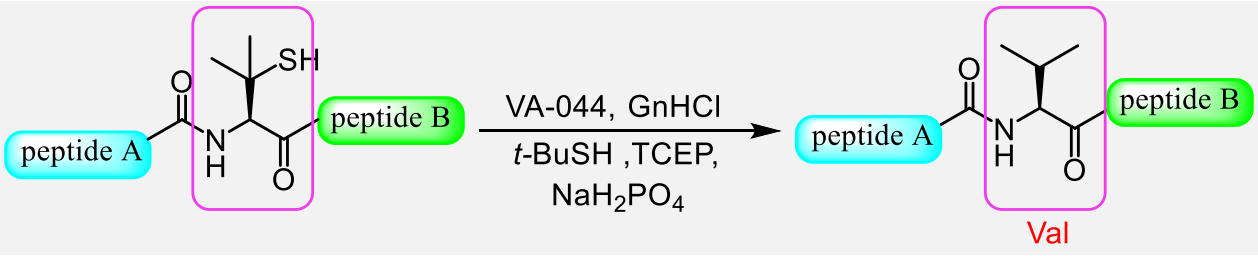
NCL-Desulfurization



- H_2-mental reagent includes Pd/Al_2O_3, $Pd/BaSO_4$, Pd/C and Raney nickel, which are efficient for desulfurization for cysteine.
- Cysteine on side chain should be protected by acetamidomethyl(Acm) group.
- Undesired side reaction such as hydrogenation of tryptophan and demethylthiolation of methionine might occur.
- Low yield of product due to adsorption on large metal surface.

Dawson. P. E *et al. J. Am. Chem. Soc*. **2001**, *123*, 526-533
Danishefsky. S. J *et al. Angew. Chem. Int. Ed*. **2007**, *46*, 9248-9252
Bingham. J. P *et al. Molecules* **2014**, *19*, 14461-14483

NCL-Desulfurization

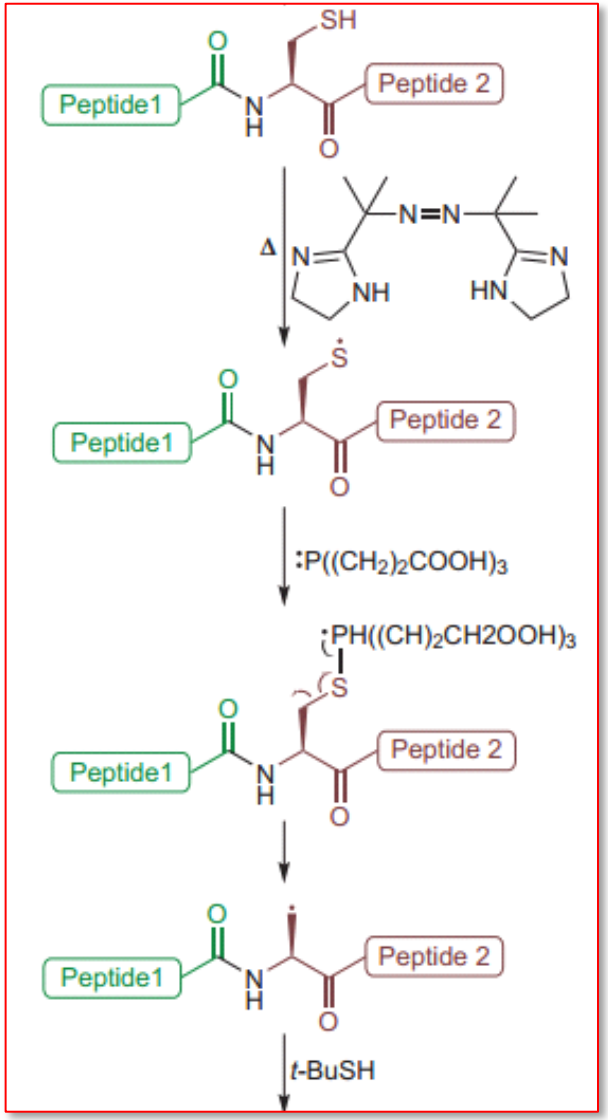


Advantages compared to H_2/metal

- Tryptophan and Methionine in side chain is stable.
- VA-044 can afford high yield, but sometimes is not effective enough in some desulfurization reaction.

Limitation:

- Cysteine on side chain should also be protected by acetamidomethyl(Acm) group.
- Not efficient for some desulfurization.

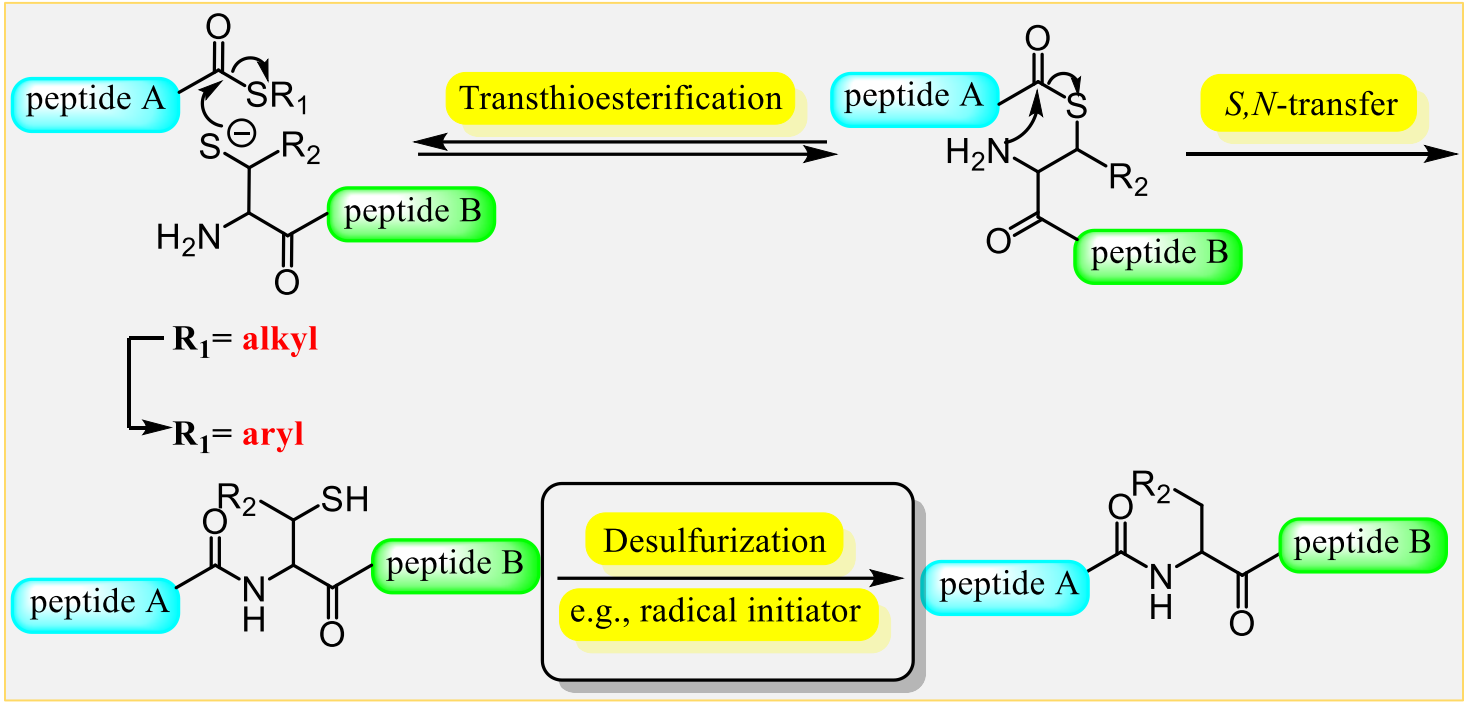


Seitz. O C *et al. Angew. Chem. Int. Ed*. **2008**, *47*, 6807 –6810

Brik. A *et al. Nature Chem.* **2016**, *8*, 407-418

Malins. L. R *et al. Chem. Sci.* **2014**, *5*, 260–266

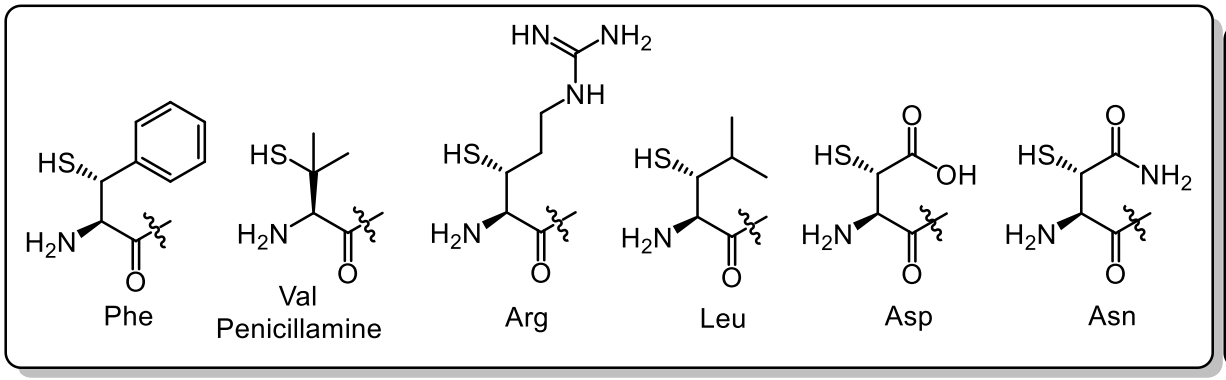
NCL-Desulfurization

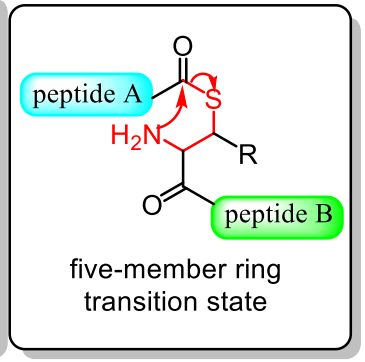


- Development of desulfurization expands the scope of ligation site.
- *C*-terminal is thioester.
- *N*-terminal is not only limited to Cystine, compared to NCL.
- Cysteine on side chain should be protected by acetamidomethyl(Acm) group.

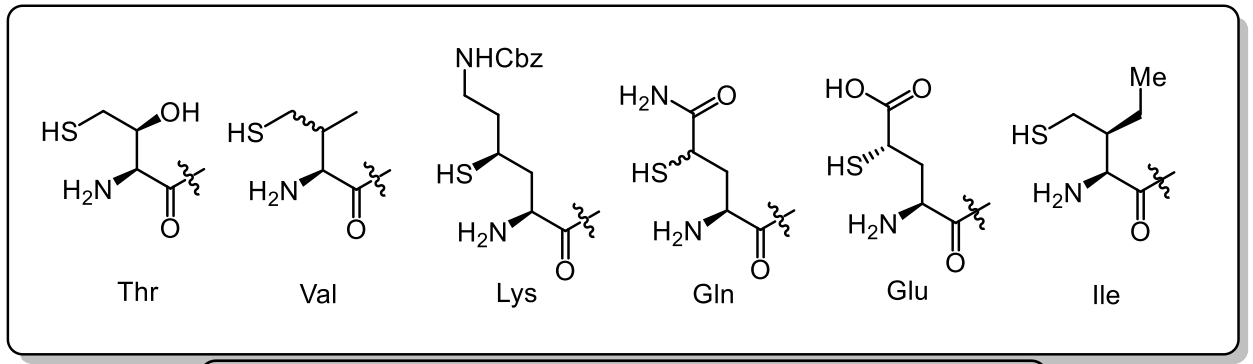
Brik. A *et al. Nature Chem.* **2016**, *8*, 407-418

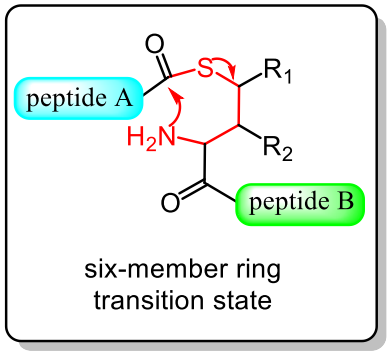
NCL-Desulfurization



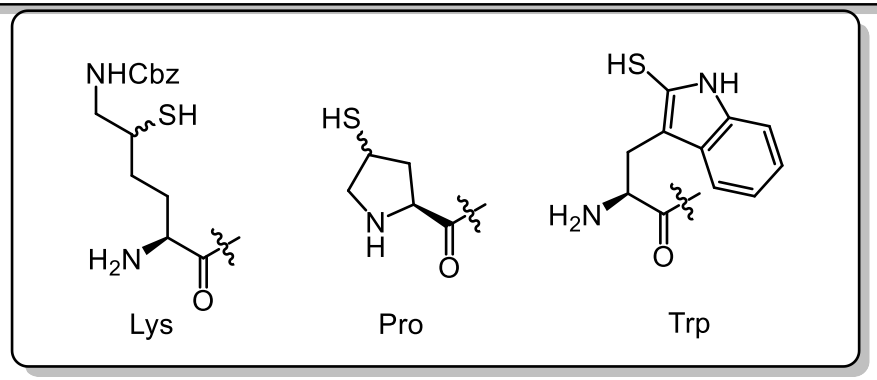


β-thiol amino acid





γ-thiol amino acid



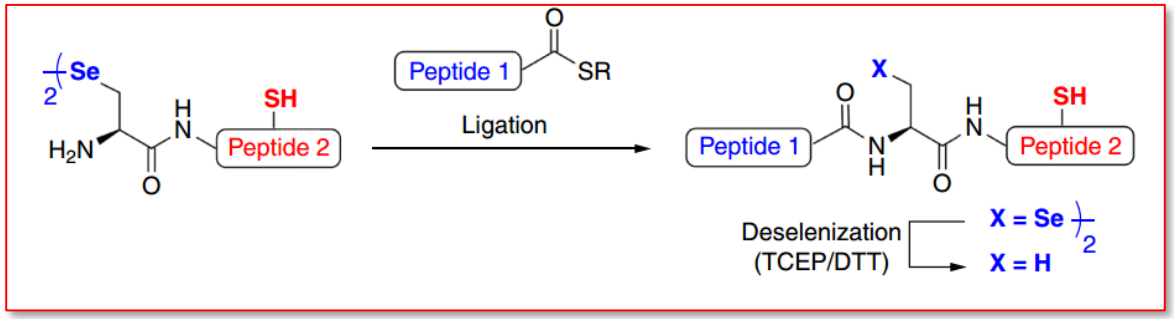
- Chemical ligation, followed by desulfurization expand the scope of ligation site to these amino acids, shown above.
- During desulfurization, Cys needs to be protected.

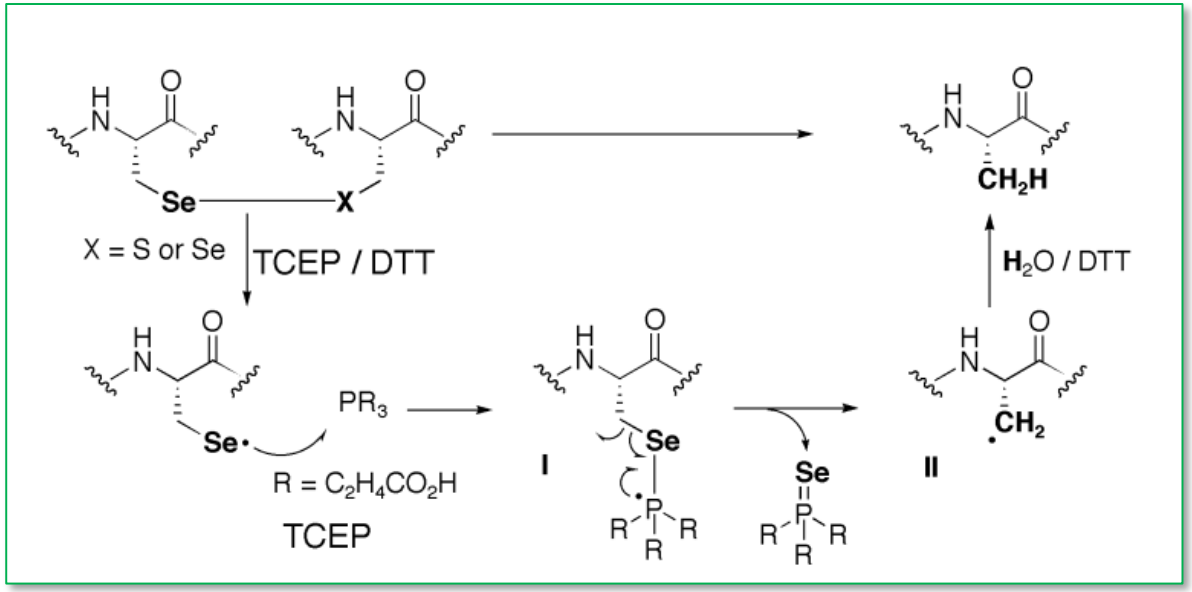
Payne. R. J *et al. Curr. Opin. Chem. Biol.* **2014**, *22*, 70-78

Payne. R. J *et al. Org. Lett*. **2015**, *17,* 4902−4905

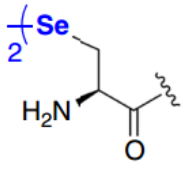
Chuanfa. L *et al. Org. Lett.* **2016**, *18,* 2696−2699

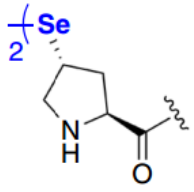
NCL-Desulfurization(Deselenization)

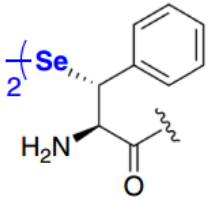




- Weak C-Se bond makes the deselenizaion occur without radical initiator.
- Selective deselenization can be achieved with the retention of unprotected cysteine.
- Proline selenoesters have superior leaving group ability compared to the corresponding thioester analogs.


Sec


Pro

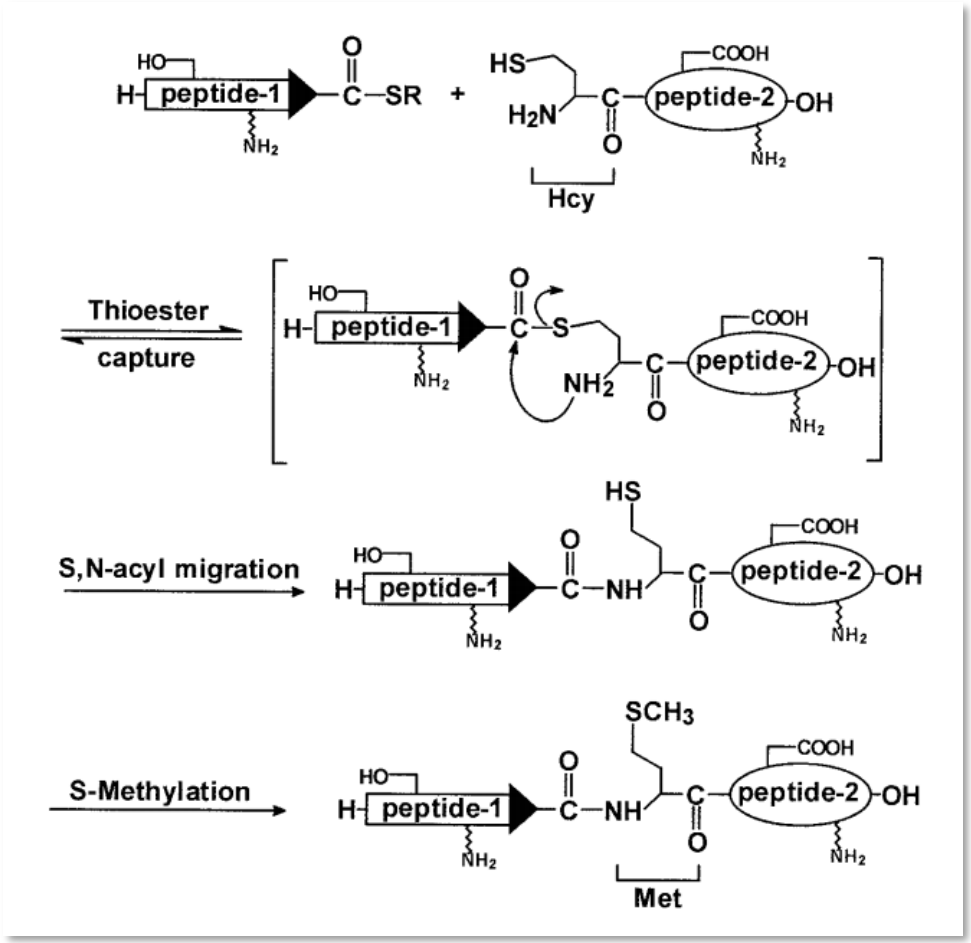

Phe

Dawson. P. E *et al. Angew. Chem. Int. Ed.* **2010**, *49*, 7049 –7053

Payne. R. J *et al. Curr. Opin. Chem. Biol.* **2014**, *22*, 70-78

NCL-Desulfurization

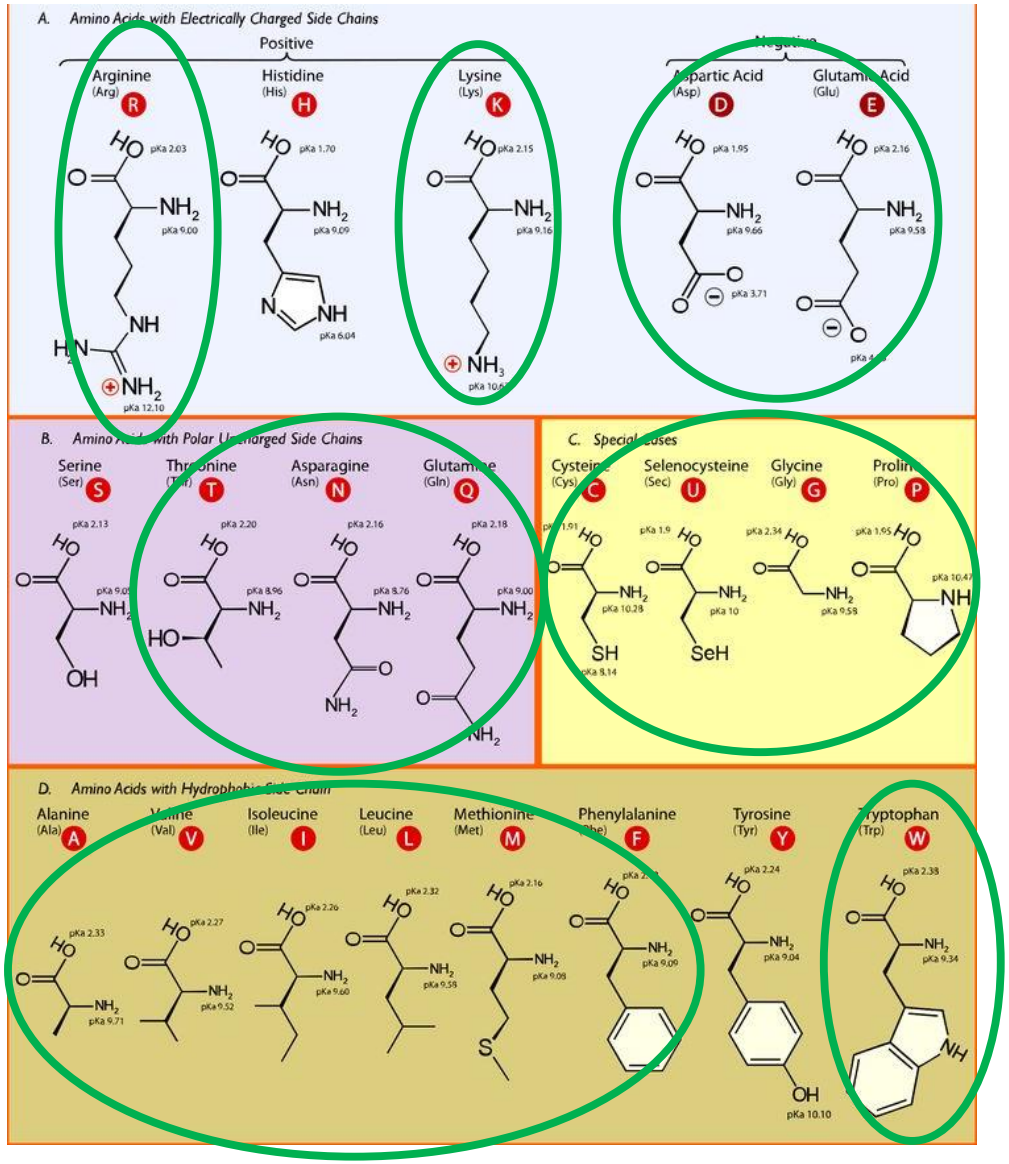
Methionine Ligation using Homocysteine



- With *C*-terminal thioester and homocysteine(Hcy), followed by methylation, we can get methionine on the ligation site.
- Homocysteine can be obtained from methylation.
- Methyl *p*-nitrobenzenesulfonate as the methyl donor in the *S*-methylation step.

Tam. J. P *et al. Biopolymers,* **1998,** *46*, 319–327

21 Amino Acid (Ligation Site Achieved)



Short Summary

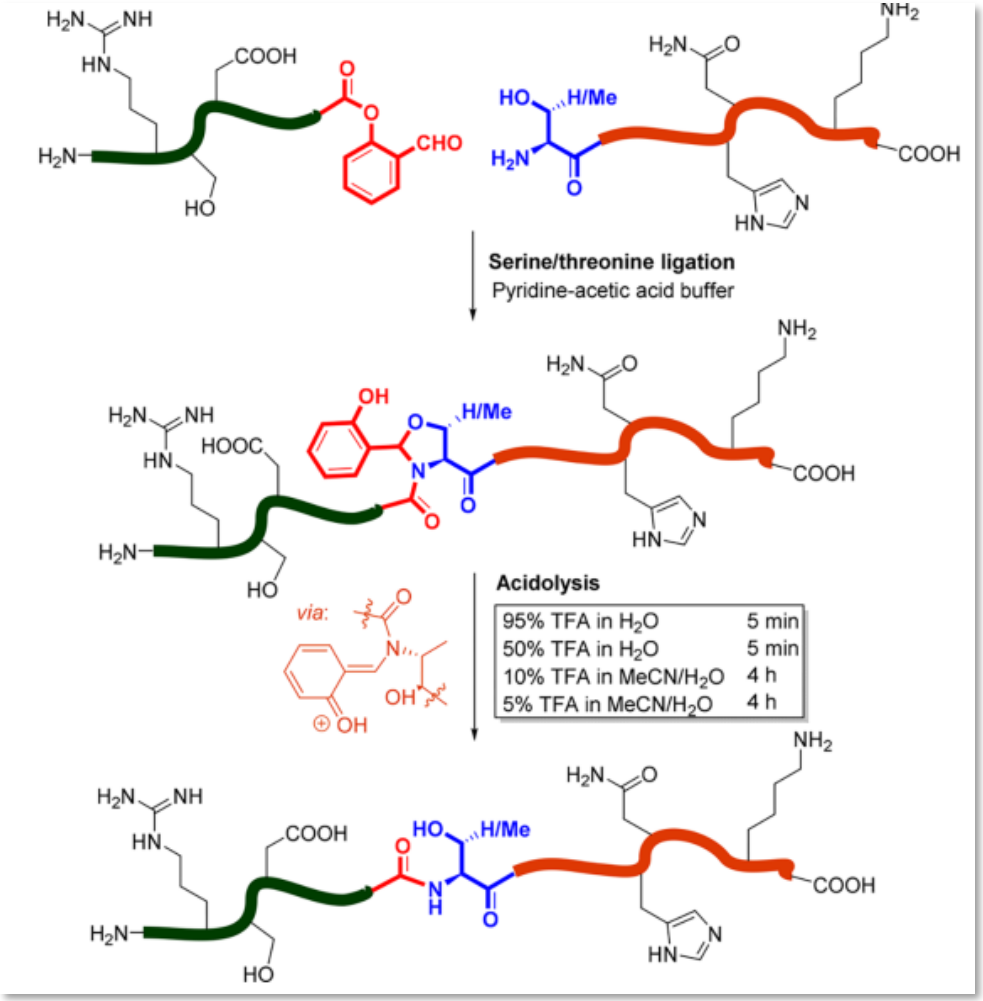
- By the method of NCL, NCL-desulfurization, and auxiliary mediated ligation, most of the ligation site are achieved.
- Up to now, *C*-terminal is thioester, and all the modifications are based on *N*-terminal.
- Ligation site of **serine, tyrosine, histidine** haven't been achieved.

Next Section

- Some special method to expand the ligation site.

Serine Ligation Site

Serine/Threonine Ligation(STL)

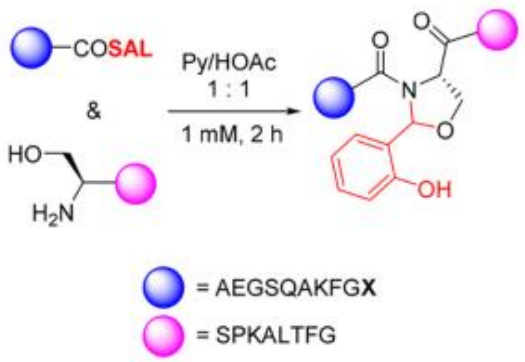


- STL originates from a chemoselective reaction between an unprotected peptide with a C-terminal salicylaldehyde (SAL) ester and another unprotected peptide with an N-terminal serine or threonine residue.
- After acidolysis, the product has the nature peptide ligation site.
- Mild reaction condition and short reaction time.

Xuechen. L *et al. Org. Lett*., **2010**, *12*, 1724-1727

Acc. Chem. Res. **2018**, DOI: 10.1021/acs.accounts.8b00151

Serine Ligation Site



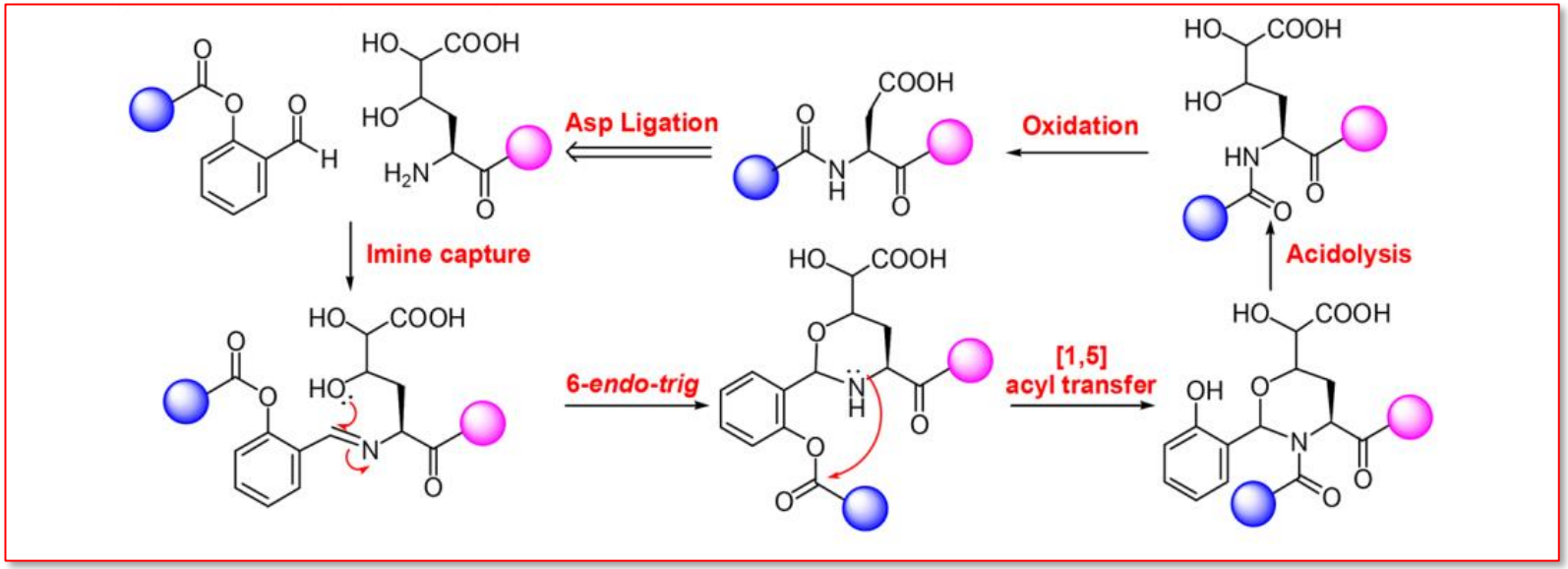
	C-terminal residues (**X**)	Conversion (%) at 2 h
FAST	Ala	87.1
	Gly	85.9
	Ser	84.8
	Gln	78.7
	Thr	71.3
	Phe	67.7
	Cys(SS*t*Bu)	65.2
MEDIUM	Val	45.5
	Ile	41.8
	Met	38.5
	Asn	38.5
	Tyr	33.7
	Leu	33.4
SLOW	His	28.6
	Trp	24.8
	Arg	20.5
	Pro	7.9
	Lys	Decompose
	Asp	N.D.
	Glu	

- The hindered β-branched amino do not retard the formation of product significantly.
- Side chain is tolerant to some nucleophilic amino acid such as lysine, which might react with the aldehyde group, but the reaction is reversible and unable to generate a stable product.
- The *C*-terminal Asn sometime poses a problem in NCL, while under the STL condition, the ligation proceeded smoothly without significant side products.
- The peptide SAL esters with *C*-terminal Lys/Asp/Glu could not be prepared as stable compounds, because the first amino acid contains nucleophilic side-chain functionalities.

Xuechen. L *et al. Front. Chem.* **2014**, *2*, 28.
Acc. Chem. Res. **2018**, DOI: 10.1021/acs.accounts.8b00151

Aspartic Acid Ligation Site

Derivates of Serine/Threonine Ligation(STL)



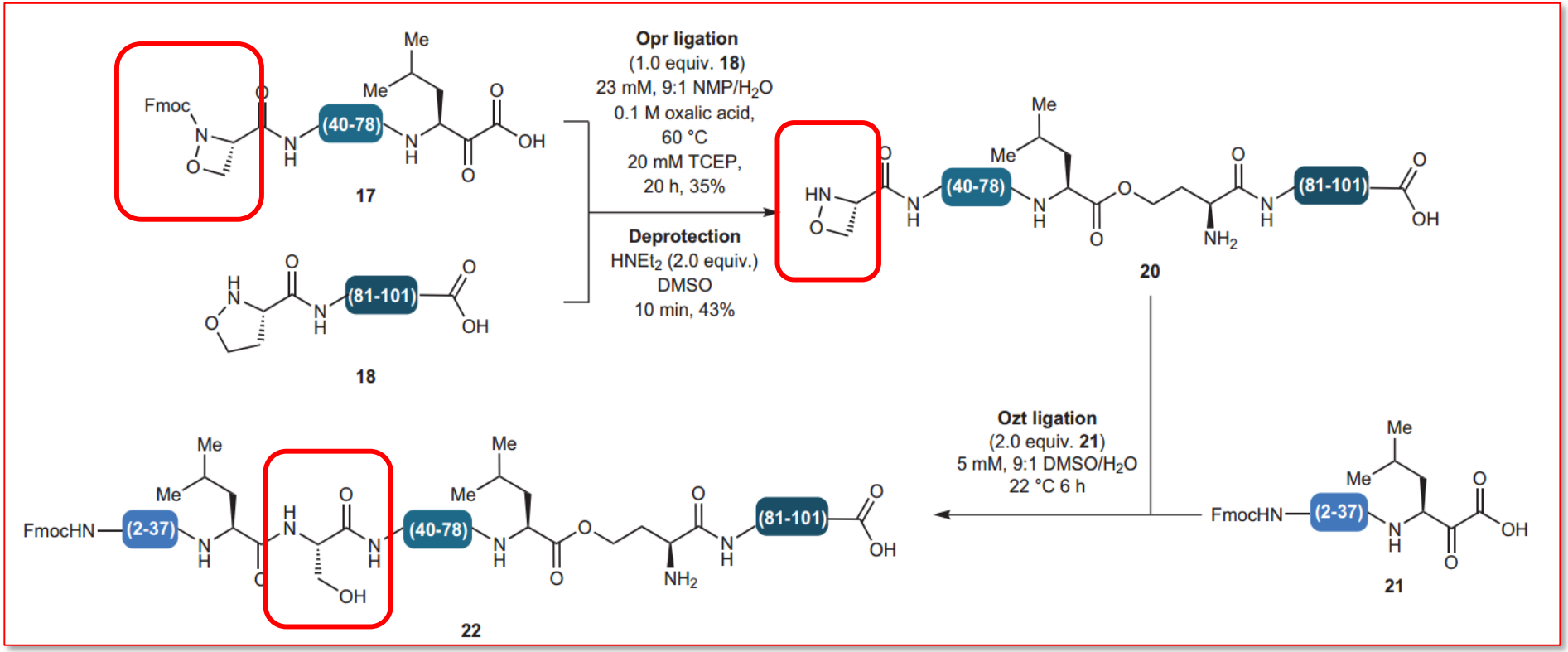
- The amino acid bearing vicinal diol functionality was incorporated into the N-terminus of the peptide.
- The obtained peptide did undergo imine capture with the peptide SAL ester followed by 6-endo-trig tautomerization, [1,5] acyl transfer, and acidolysis to give the peptidic linkage.
- Further selective oxidation of the vicinal diol under mild conditions ($NaIO_4/NaClO_2/H_2O_2$)

Xuechen. L *et al. Chinese. Chem. Lett.* **2018,** *29,* 1119–1122

Acc. Chem. Res. **2018**, DOI: 10.1021/acs.accounts.8b00151

Serine Ligation Site

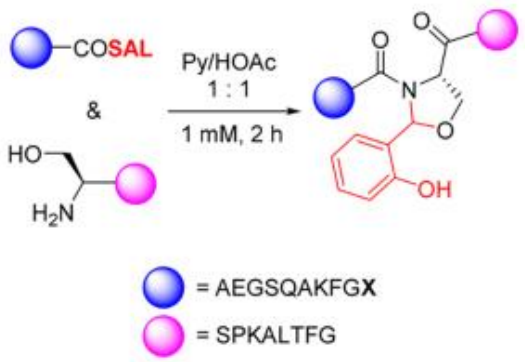
KAHA Ligation



- Fmoc-protected **Ozt**(oxazetidine) is stable, and after removing the Fmoc group, the **ozt** has high reactivity.
- Compared to **Opr**(oxaproline), reaction condition of **Ozt** is of lower concentration and mild temperature.

Bode. J. W *et al. Nature. Chem*. **2015**, *7*, 668-672

Serine Ligation Site



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	Glu	

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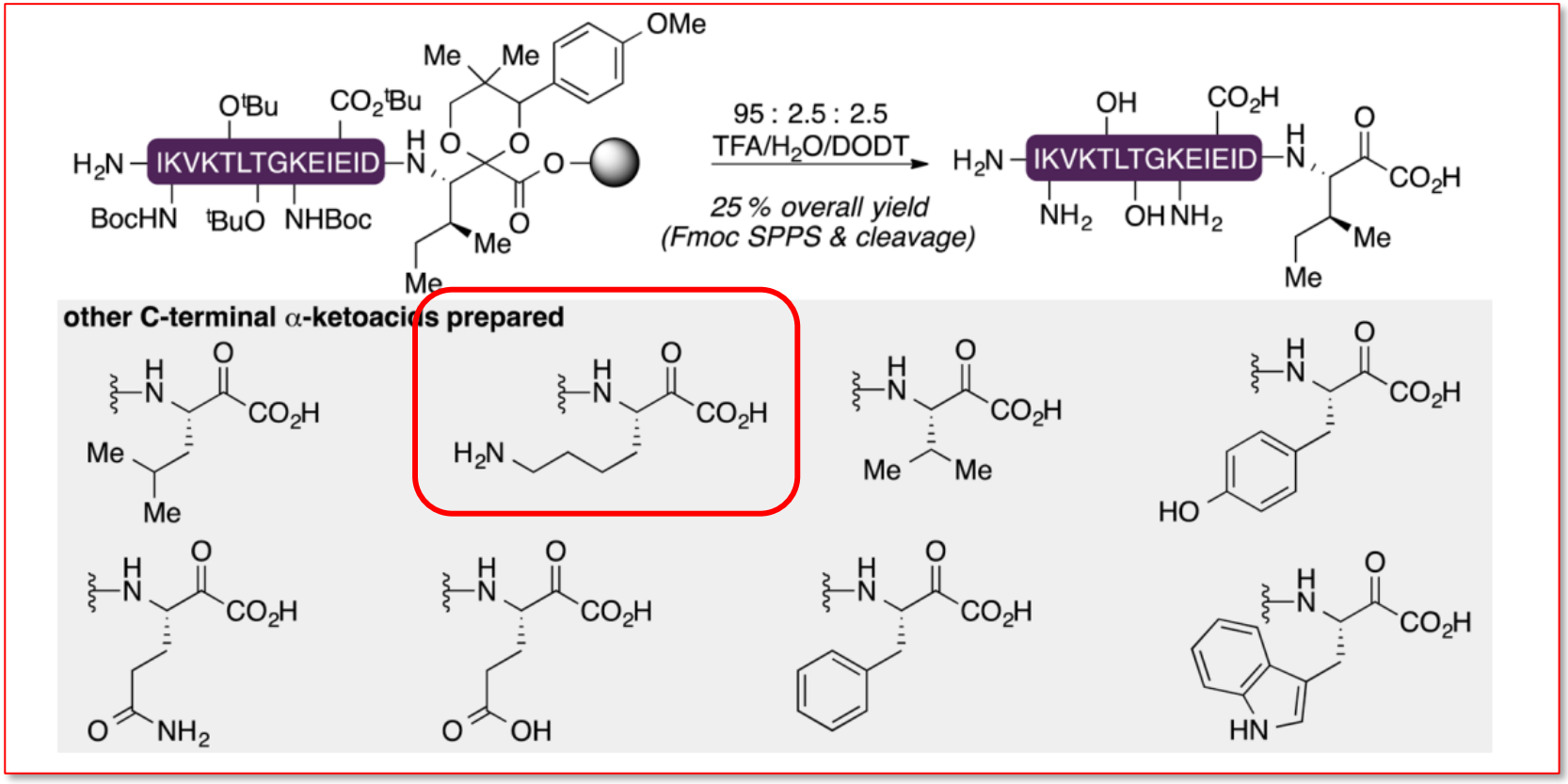
Acc. Chem. Res. **2018**, DOI: 10.1021/acs.accounts.8b00151

Serine Ligation Site

KAHA Ligation

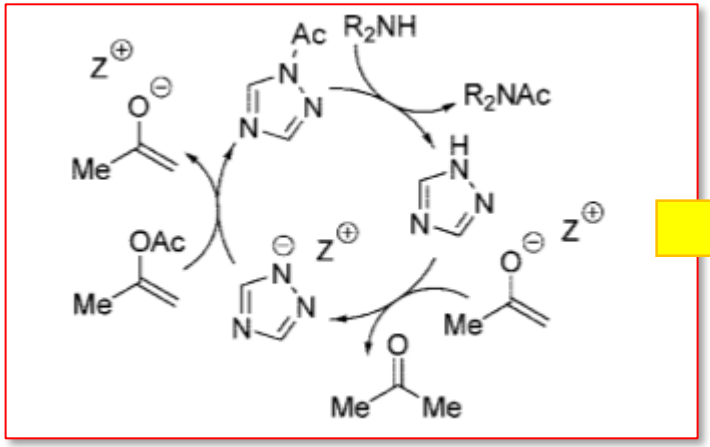


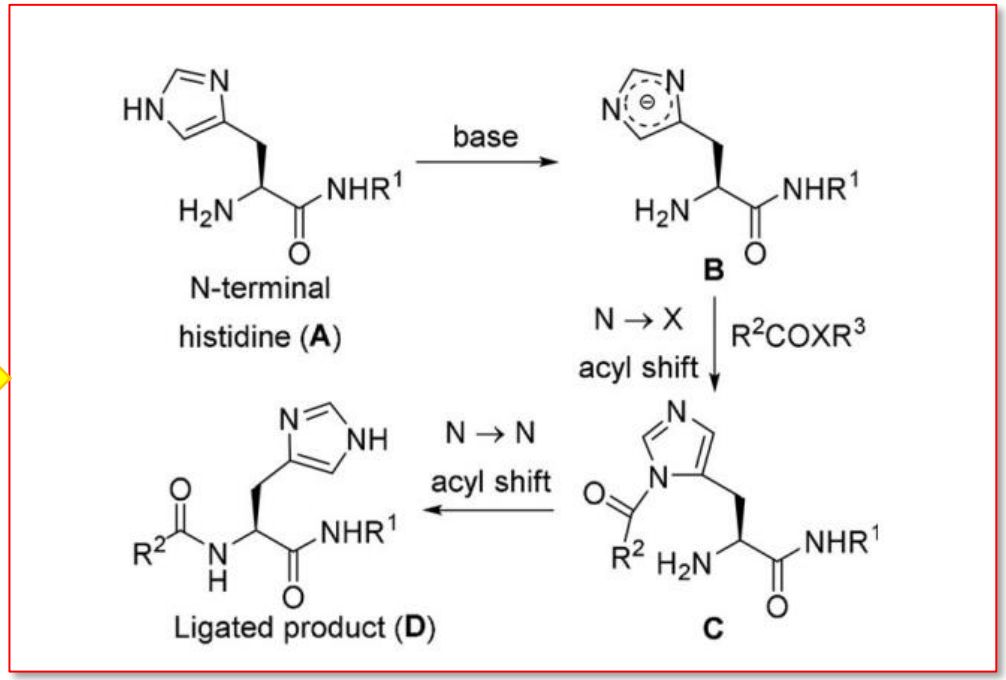
- The ability to synthesis lysine-type α-ketoacid might allow the ligation between lysine and serine.

Bode. J. W *et al. Acc. Chem. Res.* **2017,** *50,* 2104−2115

Histidine Ligation Site

Motivation





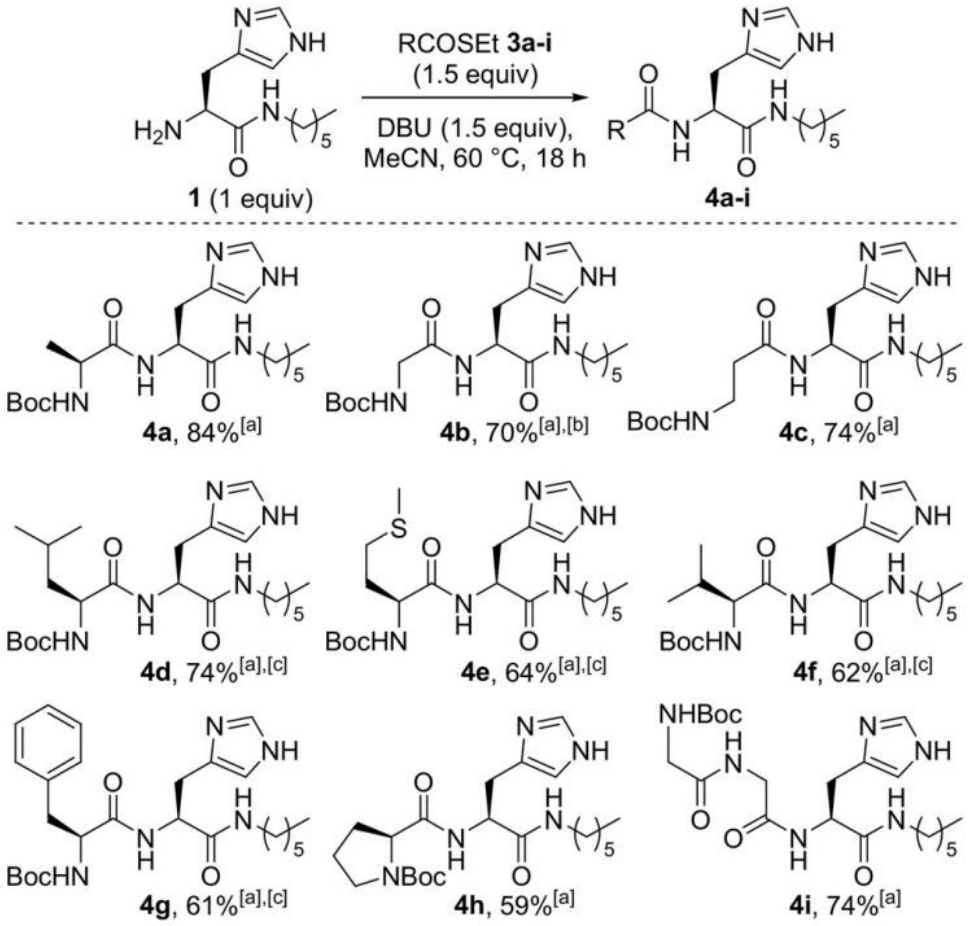
Difficulty of Histidine Ligation

- Imidazole sidechain of histidine, if unprotected, can react with activated carboxylic acid during coupling thus reducing the amount of activated acid for reaction.
- The imidazole moiety of histidine promotes epimerization during coupling and results in mixtures of enantiomeric products.
- Common N-terminal protecting groups like Boc or Fmoc, however, cannot be used since protecting group orthogonality with the N-terminal amine need to be achieved for effective coupling.

Birman. V. B *et al. Org. Lett*., **2009,** *11*, 1499-1502

Yuya. A. L *et al. Chem. Asian J.* **2018**, *13*, 400 – 403

Histidine Ligation Site

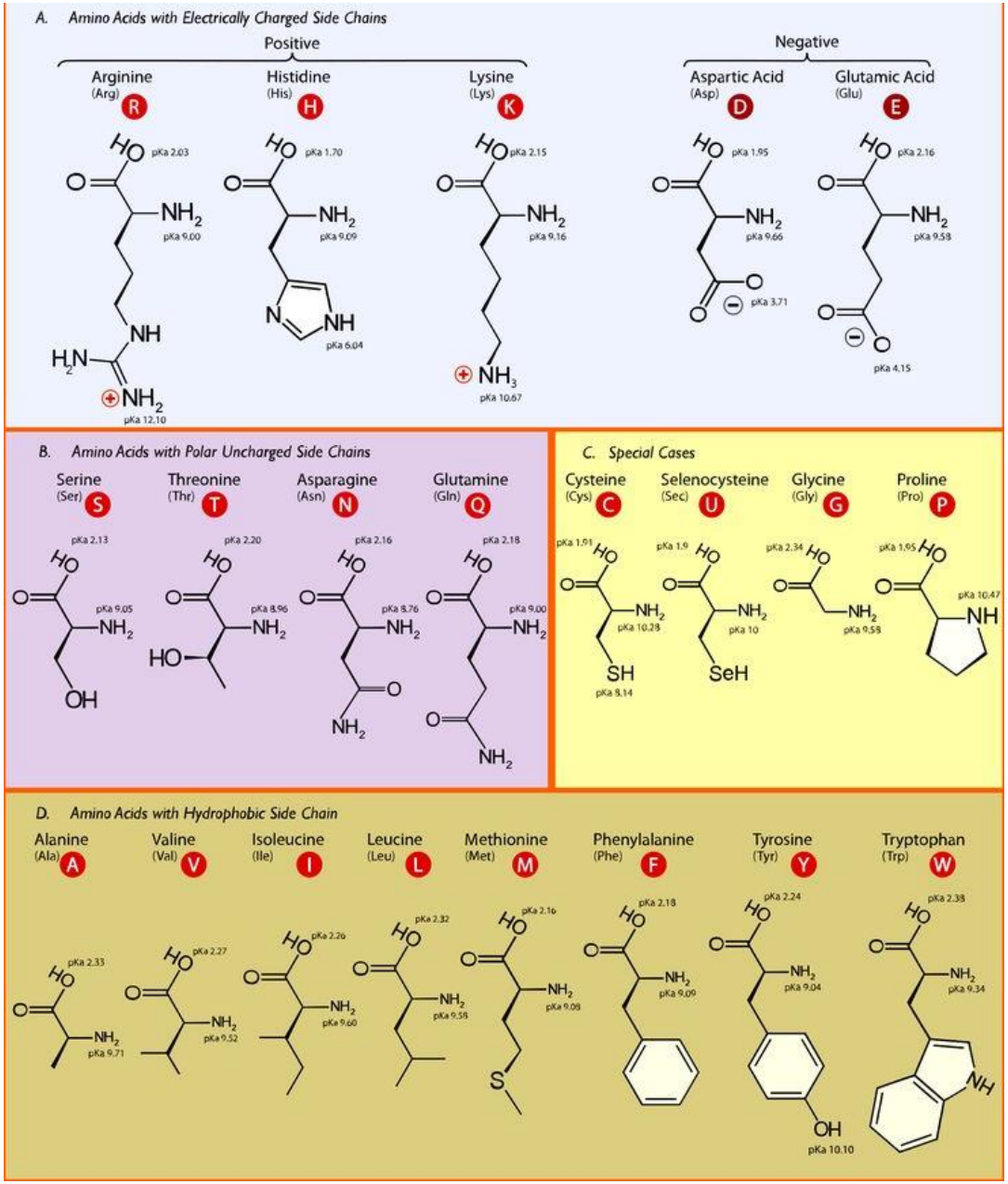


Scheme 2. Scope of peptide synthesis via histidine-promoted peptide ligation. [a] Isolated yield. [b] Reaction performed at room temperature. [c] Racemization observed.

- Under the optimized conditions, this ligation enabled the synthesis of a range of histidine-containing dipeptide, tripeptides and tetrapeptides in moderate to good yields without the need for protection of histidine sidechain.
- Amino acids such as serine, cysteine, lysine and glutamic acid, which contain either nucleophilic, basic or acidic sidechains, were not tested in histidine promoted ligation as the unprotected sidechains are highly likely to interfere with the action of imidazolate leading to poor ligation results.

Yuya. A. L *et al. Chem. Asian J.* **2018**, *13*, 400 – 403

Short Summary



- By investigating kind of chemical ligation, we greatly enlarged the scope of ligation site, both on the *N*-terminal and *C*-terminal.
- Some amino acid, with special substrates, can achieve ligation using more appropriate methods.

Contents

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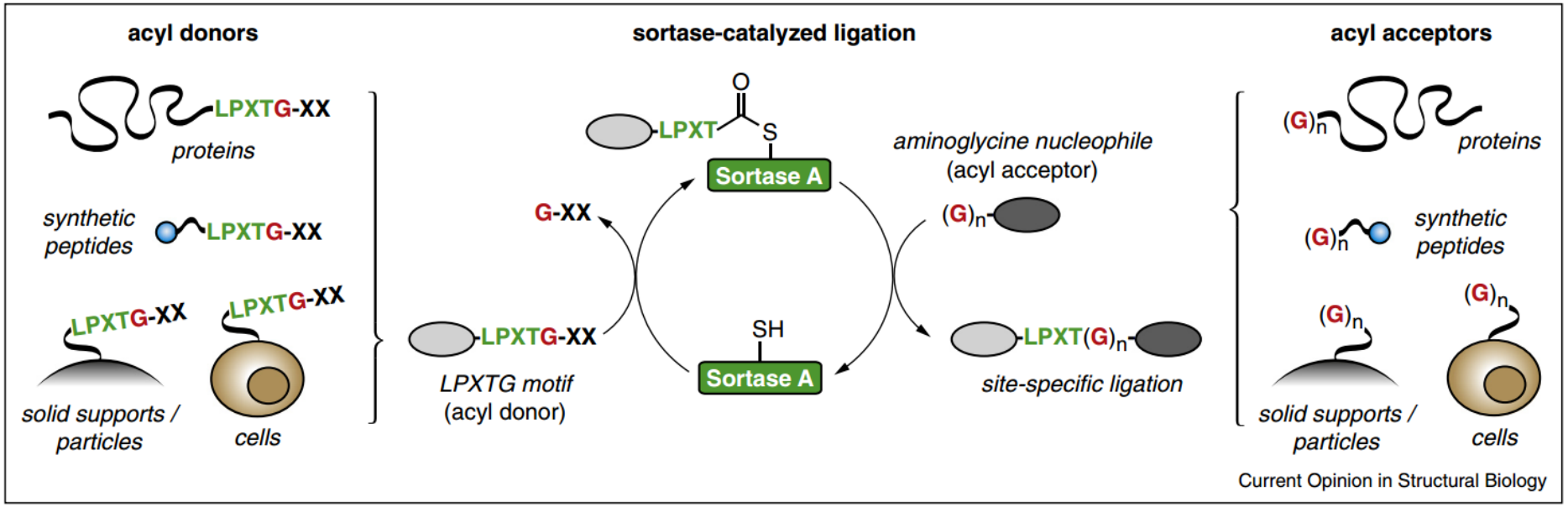
  - ✓ Butelase
  - ✓ Subtiligase
- Summary

Enzyme-Mediated Ligation

- Compared to chemical peptide ligation, enzymatic ligation has the advantages like mild reaction conditions, high regioselectivity, small toxicity, racemization-free.
- Enzymes, that mediate peptide and protein hydrolysis, also known as proteases are abundantly available from nature. On the contrary, peptide ligases, enzymes that catalyze the peptide bond formation are very rare.
- With the long-standing efforts, a few peptide ligases have been discovered from nature, such as sortases, butelase and etc.

Junfeng. Z *et al. Chin. Chem. Lett*, **2018**, *29,* 1009–1016
Chunmao. H *et al. Chin. Chem. Lett,* **2018,** *29,* 1017–1021

Sortase A



- Sortases are a class of transpeptidase enzymes that are responsible for 'sorting' and covalently anchoring virulence factors to the cell wall of Gram-positive bacteria, while the sortase A is the most widely used one among sortases.
- For sortase A, five amino acid recognition sequence LPXTG, X can be any amino acid, is necessary at the *C*-terminus.
- *N*-terminus consists of aminoglycine, G_n.

Ploegh. H. L *et al. Curr. Opin. Struc. Biol*, **2016,** *38*, 111–118

SrtA (Sortase A) with Modified Condition

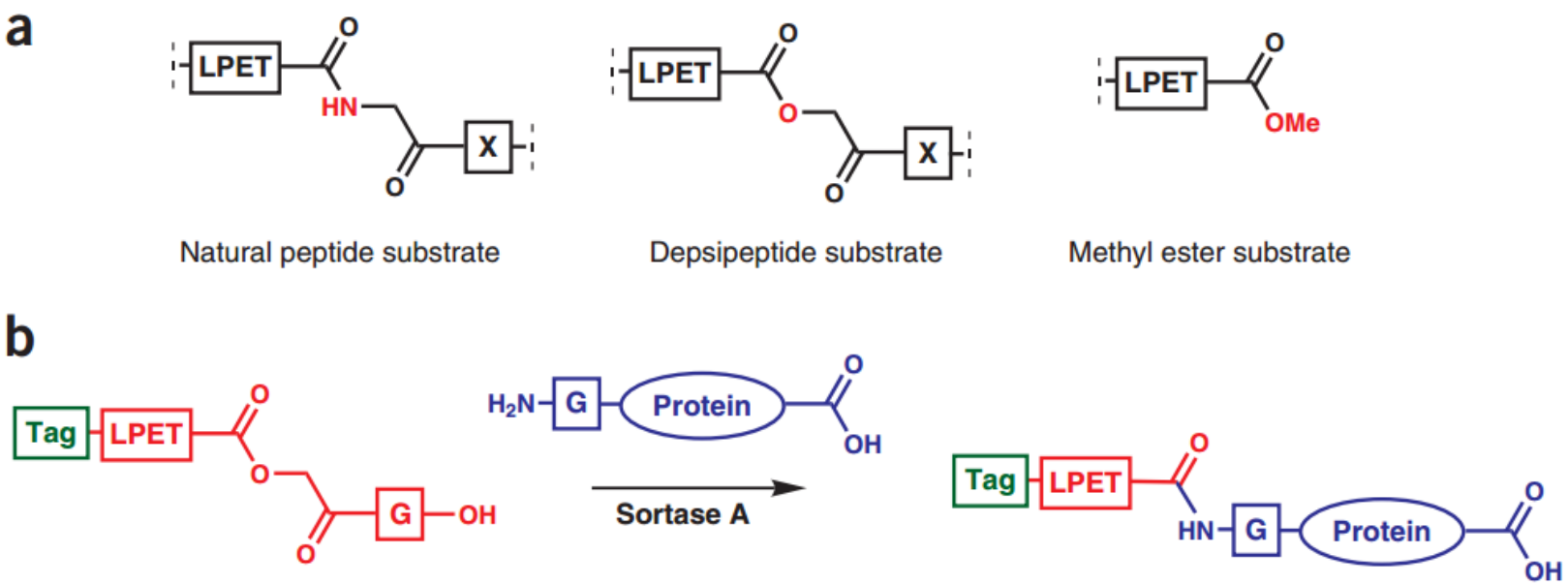
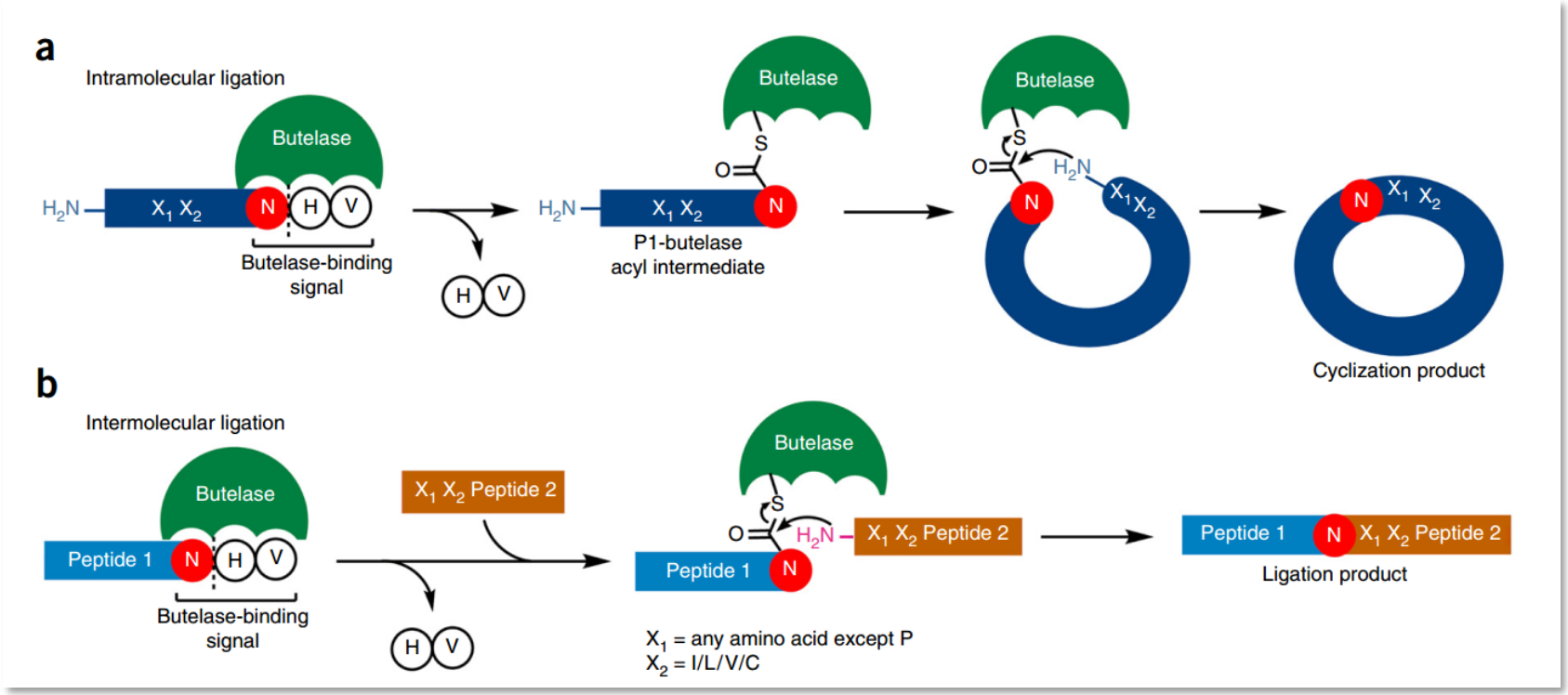


Figure 1 | Substrate variants and reaction with proteins. (**a**) SrtA substrate variants. X, any amino acid. (**b**) SrtA-mediated modification of proteins using depsipeptide substrates.

- The reaction can be rendered effectively irreversible if the scissile peptide bond (i.e., the amide bond between the threonine and glycine residues) is replaced by an ester linkage.
- Substoichiometric molar equivalents of SrtA required

Turnbull. W. B *et al. Nature Protocols.* **2014,** *9,* 253-262

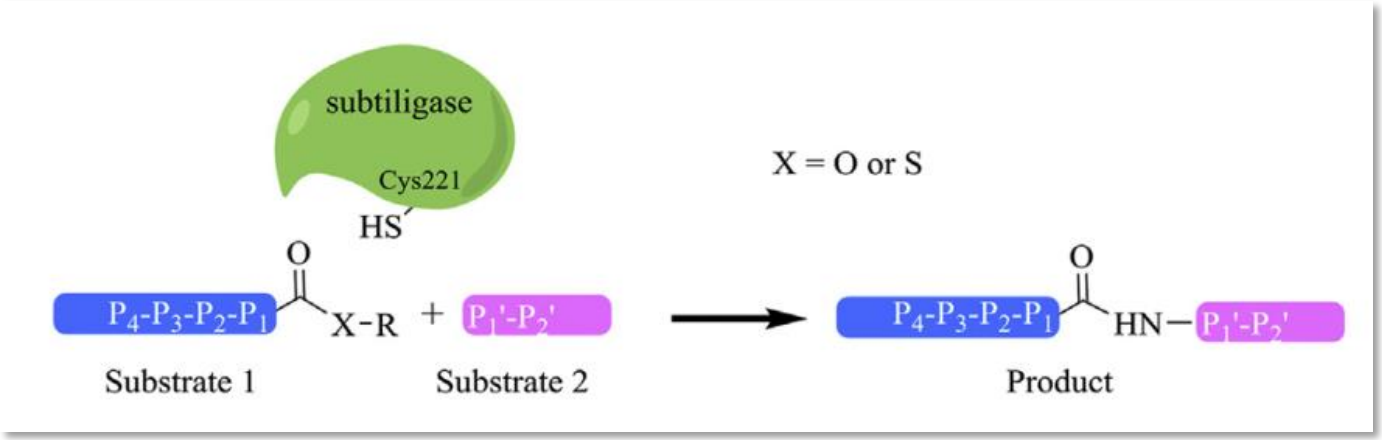
Butelases



- A tripeptide recognition sequence, Asx–His–Val, must be present at the *C*-terminus.
- For the *N*-terminus, butelase 1 accepts 20 natural amino acids at the X1 position, except for Pro, and acidic amino acids (Asp, Glu). And it highly favors bulky hydrophobic amino acids such as Ile, Leu, Val and, to some extent, Cys at the X2 position.
- The enzyme is usually added in very small quantities.

Tam. J. P *et al. Nature Protocols*, **2016**, *17*, 1977-1987

Subtiligase



- It does not require a certain recognition motif at the termini of any reaction partners.
- The sequence properties of the substrates do have great influence on the catalytic performance.

Liminations:

- The sequence of each target protein/ peptide and modifier pair has to be optimized to obtain satisfactory yields.
- A large excess of one ligation partner and the presence of Ca^{2+} is necessary.

Chunmao. H *et al. Chin. Chem. Lett,* **2018,** *29,* 1017–1021

Contents

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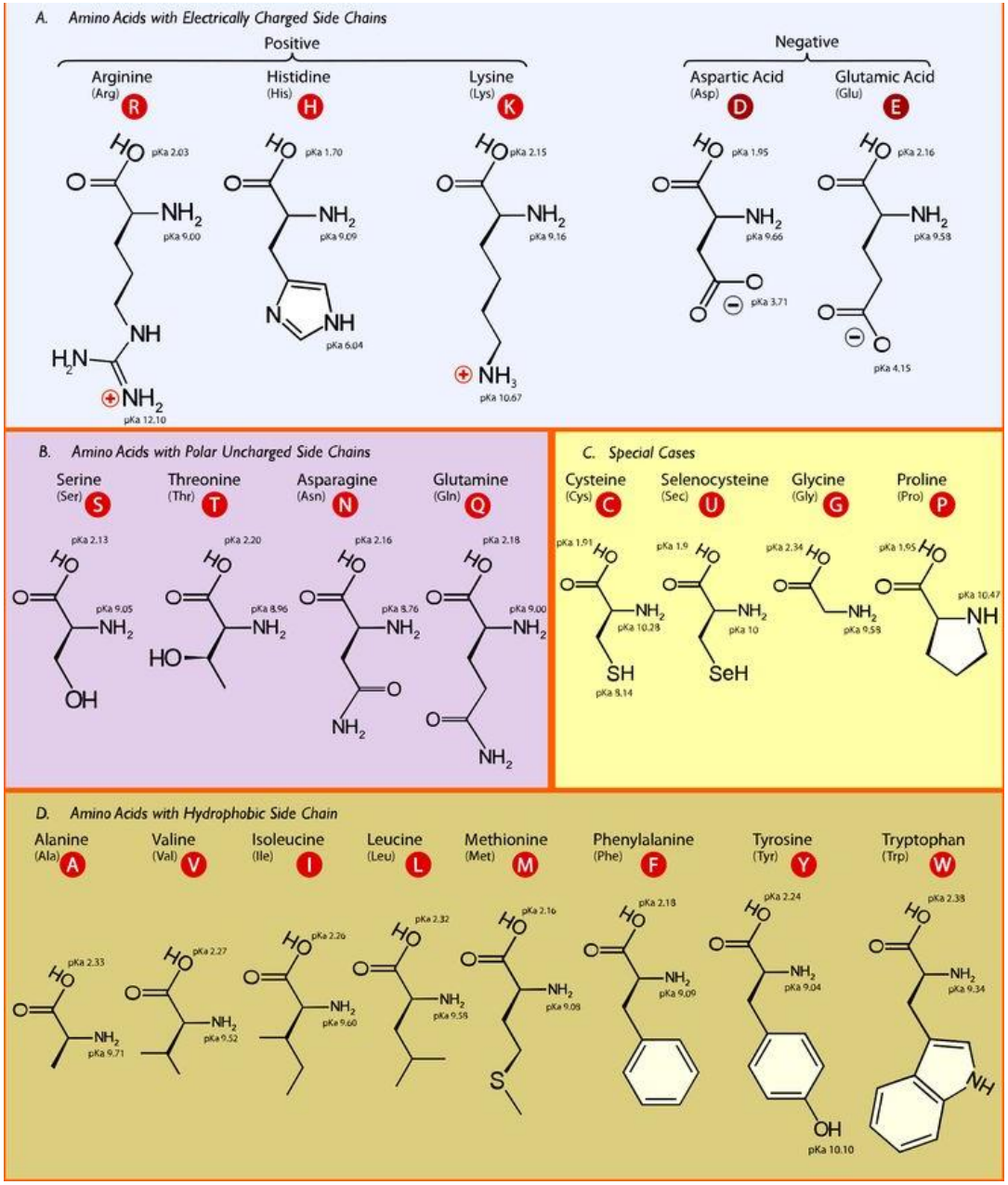
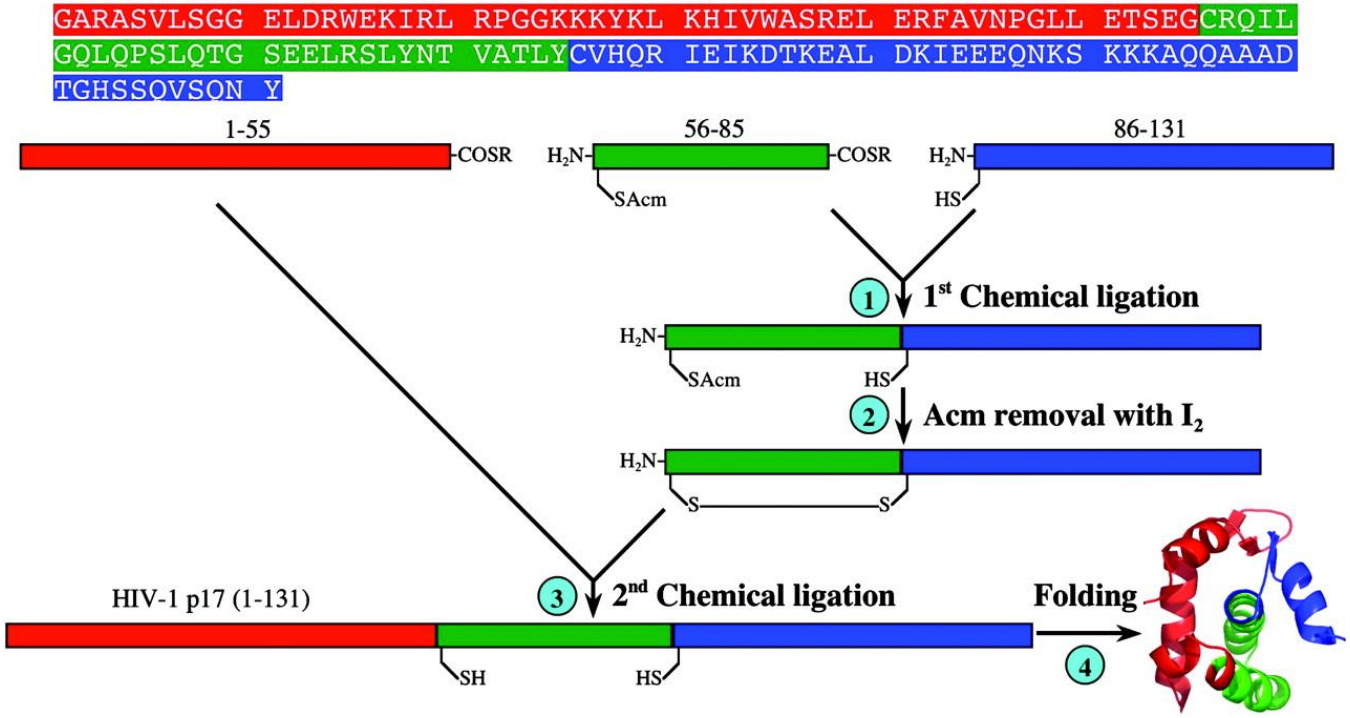
Summary



- By investigating kind of chemical ligation and some enzyme-mediated ligation, we greatly enlarged the scope of ligation site.
- Some amino acid, with special substrates, can achieve ligation using more appropriate methods.
- Introduction of enzyme-mediated ligation gives a new version for peptide and protein ligation.

Appendix: Remove Acm group



Removal of the Acm group from the purified ligation product, H-Cys(Acm)-(57–131)-OH, was achieved quantitatively by dissolving the Cys-protected peptide at 0.5 mg/ml in 0.1 M citric acid/0.2 M HCl, to which 5 mM iodine (in MeOH) was added dropwise to a final concentration of 0.05 mM or until a stable yellow color was observed.

Zhibin. W *et al. PNAS*. **2004**, 101, 11587-11592

Appendix: Desulfurization with H_2-metal

1, Microcin J25

Table 1. Studies on the Desulfurization Conditions for Microcin J25

entry	metal reagent	reaction medium	yield (%)	advantage or disadvantage
1	Pd/Al_2O_3 (10%)	0.1 M phosphate, 6 M guanidine, pH 7.5	90	disulfide formation
2	Pd/Al_2O_3 (10%)	0.1 M acetate, 6 M guanidine, pH 4.5	>99	desalting required
3	Pd/Al_2O_3 (10%)	20% aqueous AcOH	>99	directly lyophilizable
4	Pd/Carbon (10%)	20% aqueous AcOH	>99	directly lyophilizable
5	$Pd/BaSO_4$ (10%)	20% aqueous AcOH	>90	directly lyophilizable
6	PdO	20% aqueous AcOH	<30	reaction incomplete
7	Raney nickel	20% aqueous AcOH	>99	directly lyophilizable

Dawson. P. E *et al. J. Am. Chem. Soc*. **2001**, *123*, 526-533

Appendix: β-thiol Val Ligation

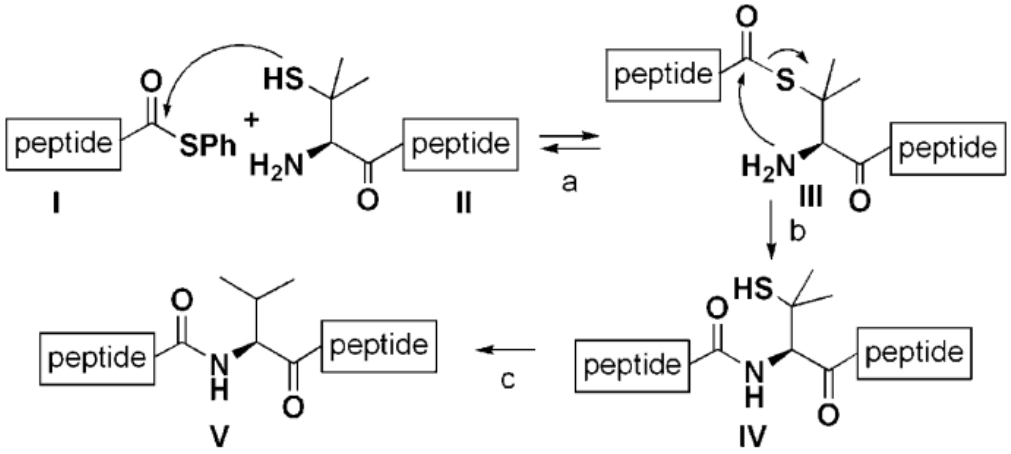
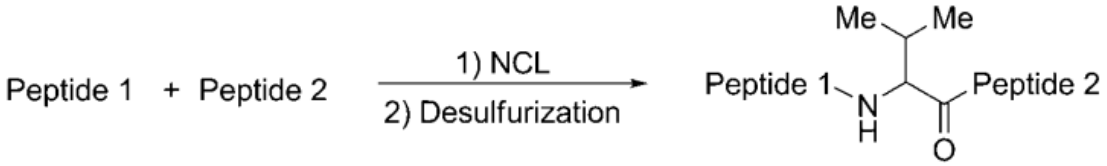


Table 1: Yields of the ligations and the desulfurization.

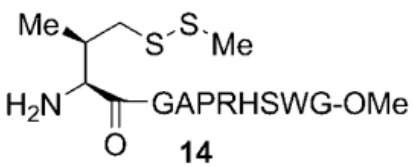
Penicillyl peptide	Peptide thioester	Ligation	Desulfurization product	Desulfurization yield [%]	
		Product/yield [%]/reaction time [h]		metal-based	metal-free
1	**2**	LYKAG**Pen**RAEYS **11**/87/12	LYKAG**V**RAEYS **17**	61	98
1	**3**	LYKAH**Pen**RAEYS **12**/70/24	LYKAH**V**RAEYS **18**	–	93
1	**4**	LYKAM**Pen**RAEYS **13**/65/24	LYKAM**V**RAEYS **19**	–	77
1	**5**	LYKAL**Pen**RAEYS **14**/70[a] and 82[b]/48	LYKAL**V**RAEYS **20**	–	79
7	**6**	TLQNREHETNG**Pen**AKSDQKQEQL **15**/78[c]/24	LKKPFNRPQG**V**QPKTGPFEDLK **21**	0[d]	72
9	**8**	LKKPFNRPQG**Pen**QPKTGPFEDLK **16**/87[c]/24	TLQNREHETNG**V**AKSDQKQEQL **22**	54	91
10	**2**	LYKAGVRAEYS **17**/0[e]	–	–	–

Seitz. O *et al. Angew. Chem. Int. Ed*. **2008,** *47*, 6807-6810

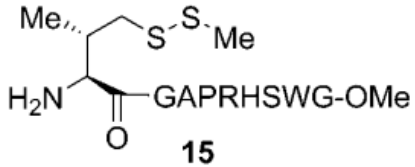
Appendix: γ-thiol Val Ligation



Peptide 2:



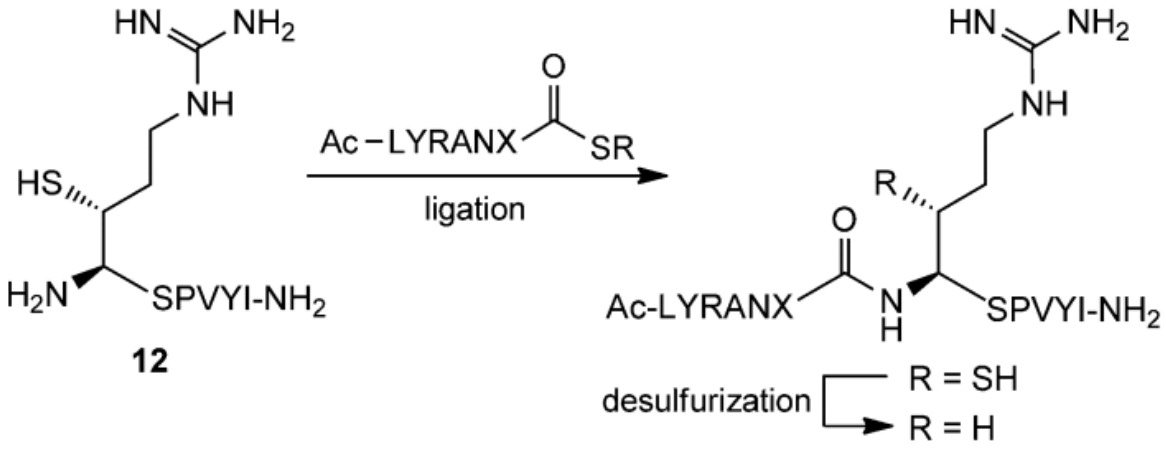
or



Entry	Peptide 1	Peptide 2	Ligation Product/Yield[b]/Time	Desulfurization Product/Yield[b]/Time
1	FmocThzRGDSCys(Acm)RPGQ-O-(SSEt) **1**	**14**	FmocThzRGDSCys(Acm)RPGQ(SH)VGAPRHSWG-OMe **16'** / 78% / 1 h	FmocThzRGDSCys(Acm)RPGQVGAPRHSWG-OMe **16** / 84% / 3 h
2	FmocKYDSRGF-O-OPh-*p*CN **12**	**14**	FmocKYDSRGF(SH)VGAPRHSWG-OMe **17'** / 80% / 1 h	FmocKYDSRGFVGAPRHSWG-OMe **17** / 81% / 3 h
3	FmocRTGDSAGT-O-SPh **4**	**14**	FmocRTGDSAGT(SH)VGAPRHSWG-OMe **18'** / 87% / 4 h	FmocRTGDSAGTVGAPRHSWG-OMe **18** / 89 % / 3 h
4	FmocRTGDSAGT-O-SPh **4**	**15**	FmocRTGDSAGT(SH)VGAPRHSWGOMe **18''** / 87% / 9 h	FmocRTGDSAGTVGAPRHSWG-OMe **18** / 90% / 3 h
5	FmocVRSYTAGP-O-OPh-*p*NO_2 **13**	**14**	FmocVRSYTAGP(SH)VGAPRHSWG-OMe **19'** / 55% / 10 h	FmocVRSYTAGPVGAPRHSWG-OMe **19** / 98% / 3 h

Danishefsky. S. J *et al. Angew. Chem. Int. Ed*. **2008,** *47*, 8521 –8524

Appendix: β-thiol Arg Ligation

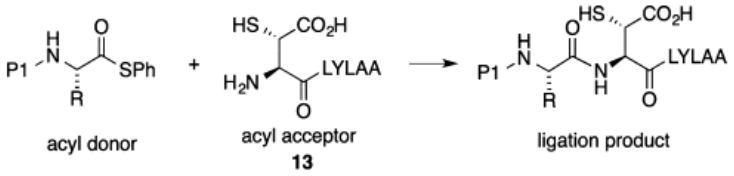


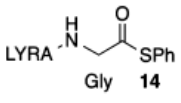
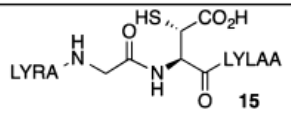
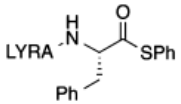
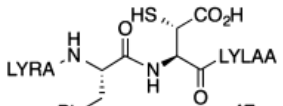
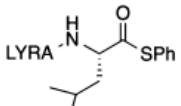
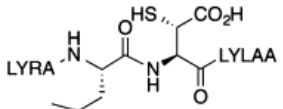
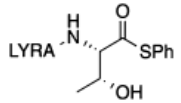
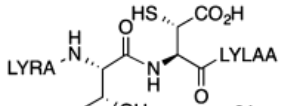
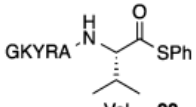
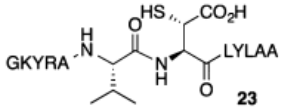
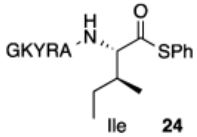
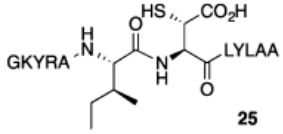
	Peptide thioester (X =)	Ligation yield[a] [%]	Second-order rate constant, k [$M^{-1} s^{-1}$]	Desulfurization yield[a] [%]
1	G	94	6.50 ± 0.53	72
2	A	75	0.67 ± 0.12	91
3	F	77	3.73 ± 0.82	75
4	S	81	0.36 ± 0.06	69
5	V	79	0.0053 ± 0.0007	66

Payne. R. J *et al. Chem. Bio. Chem.* **2013**, *14,* 559 – 563

Appendix: β-thiol Asp Ligation

Table 1. Substrate Scope of Aspartic Acid Ligation[a]

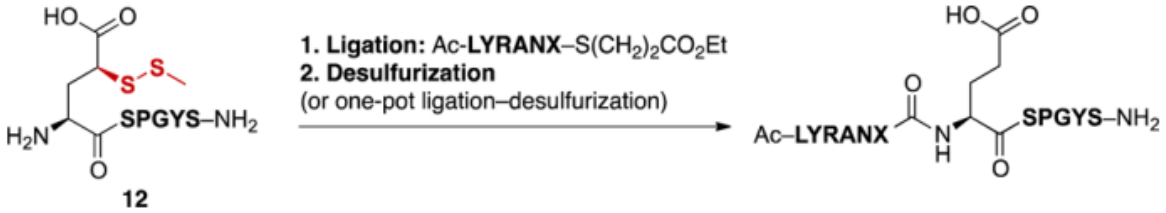


	acyl donor	ligation product	yield [%]	*t* [h]
1			85	2
2			75	2
3			89	2
4			84	2
5			77	10
6			69	10

Zhongping. T *et al. Org. Lett.* **2013**, *15, 6128-6131*

Appendix: γ-thiol Glu Ligation

Table 1. Scope of γ-Thiol-Glu Ligation–Desulfurization Chemistry

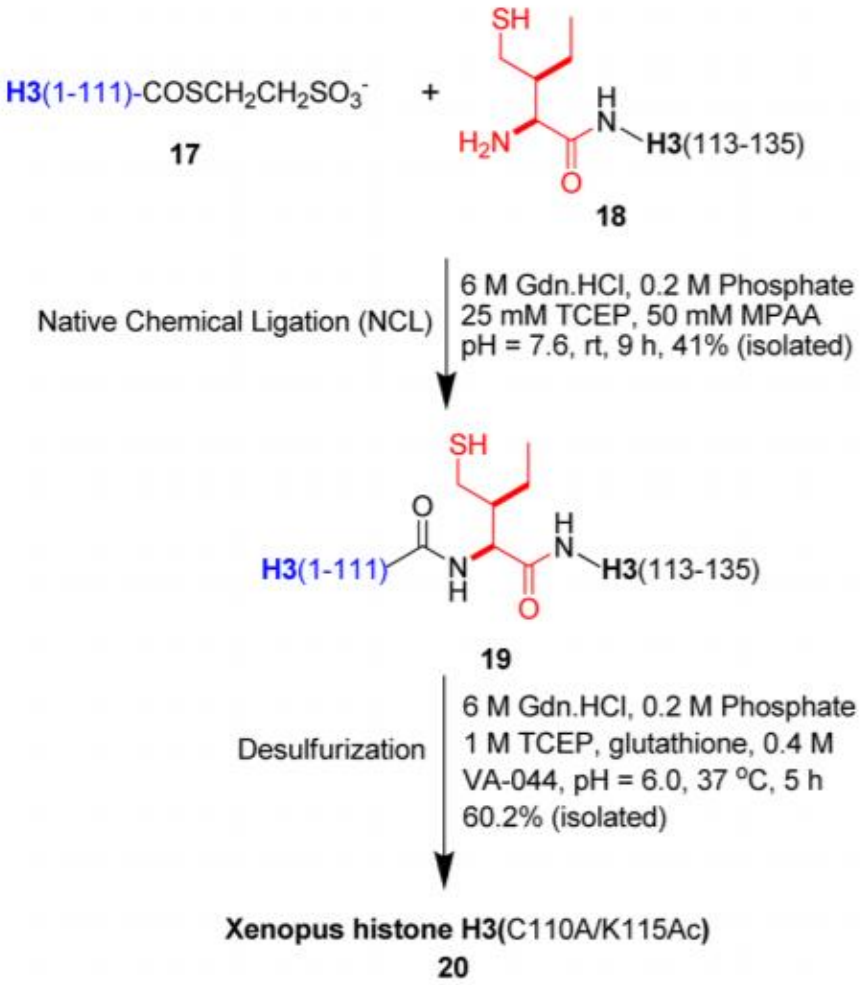


entry	thioester (X =)	ligation yield[a] (%)	desulfurization yield[a] (%)	one-pot yield[c] (%)
1	Gly	72	89 (64)[b]	73
2	Ala	77	91 (70)[b]	67
3	Met	83	98 (81)[b]	72
4	Phe	80	84 (67)[b]	74
5	Val	68	98 (67)[b]	56

[a]Isolated yields after HPLC purification. Ligation: 5 mM **12** in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP), PhSH (2 vol %), 37 °C, pH 7.2–7.4, 16 h. Desulfurization: 500 mM TCEP in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4), reduced glutathione (40 mM), VA-044 (200 mM), pH 6.5–6.8, 65 °C, 16 h. [b]Yield over two steps. [c]Desulfurization reactions were carried out at 37 °C in the one-pot protocol.

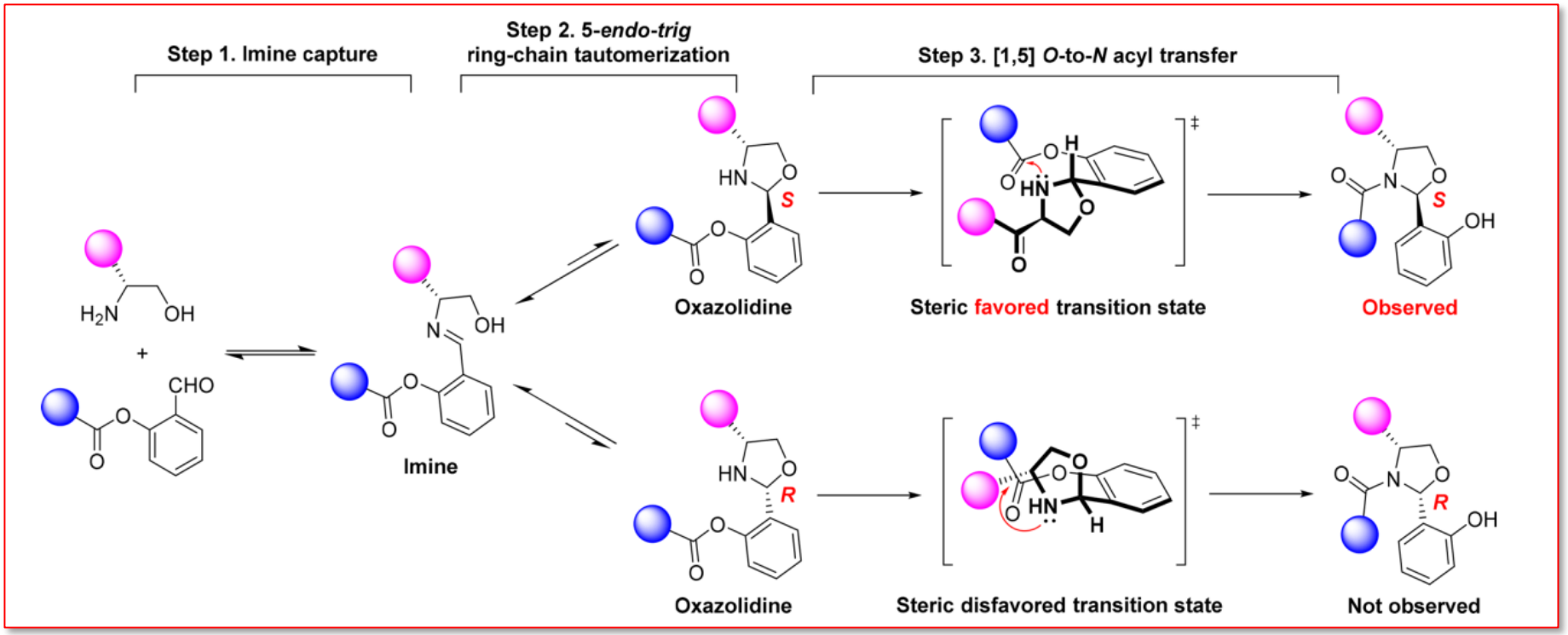
Payne. R. J *et al. Org. Lett.* **2014,** *16*, 290–293

Appendix: Isoleucine Ligation Site



Chuanfa. L *et al. Org. Lett.* **2016**, *18,* 2696−2699

Appendix: Serine/Threonine Ligation



- Product has the nature peptide ligation site, which is generated from *S*-five-member intermediate.

Xuechen. L *et al. Chinese. Chem. Lett.* **2018,** *29,* 1119–1122

Acc. Chem. Res. **2018**, DOI: 10.1021/acs.accounts.8b00151