

Site-selective bioconjugation to protein

Literature seminar

2016/01/23

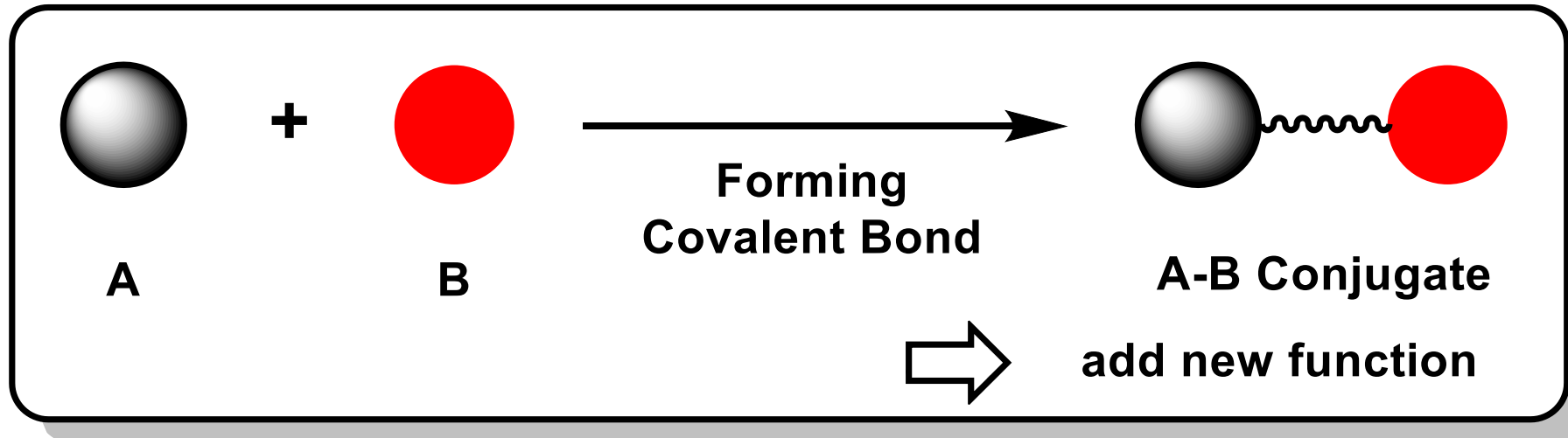
Takashi Ishiyama

Topics

1. Introduction
2. Importance control of bioconjugation
3. Conventional protein modification
4. Site-selective bioconjugation strategy
 - π -clamp-mediated cysteine bioconjugation
 - Ligand-directed selective bioconjugation
5. Summary

What is Bioconjugation?

Bio(生体)conjugation(共役)

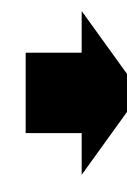
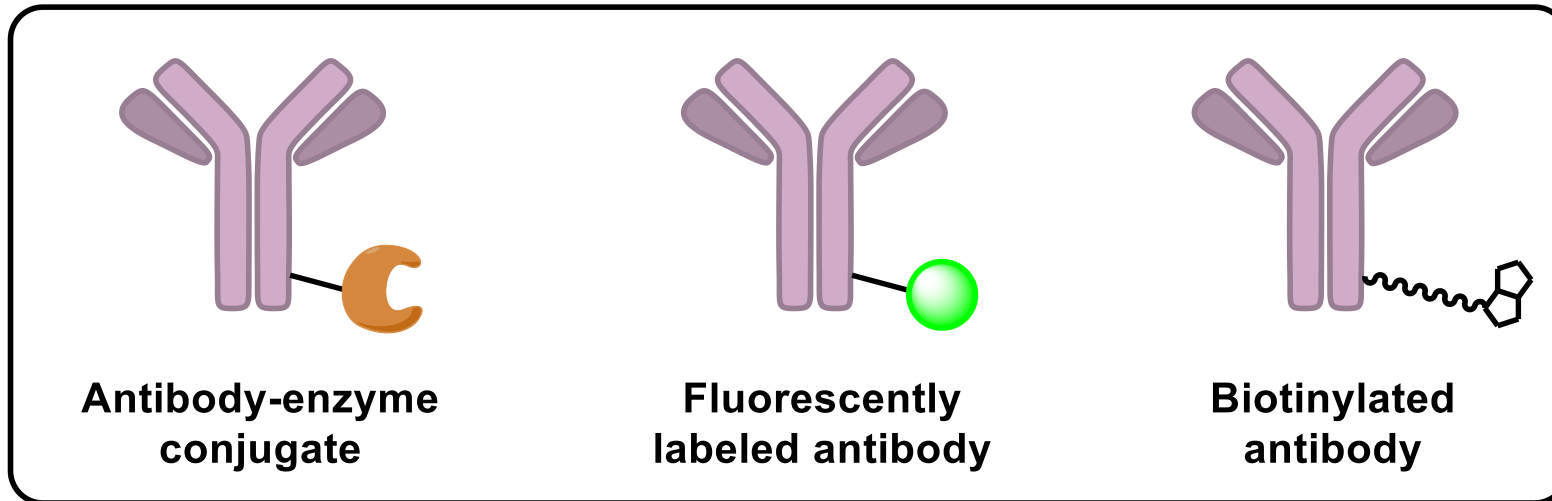


A : Biomolecules(mainly protein)

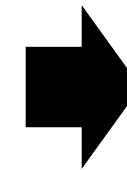
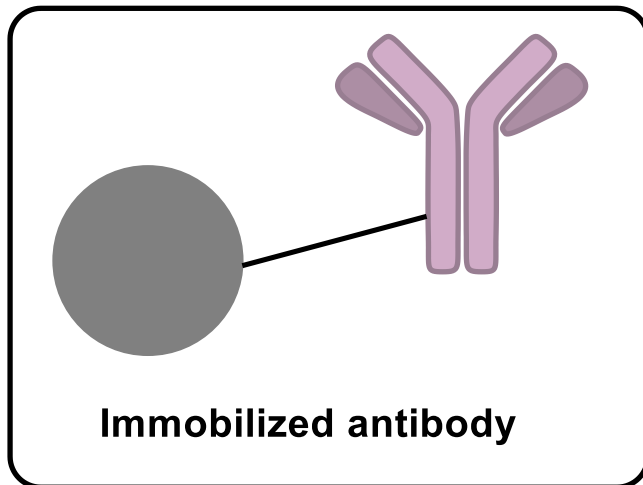
B : Molecules adding other function

Use of Bioconjugation

- Creating experimental tool



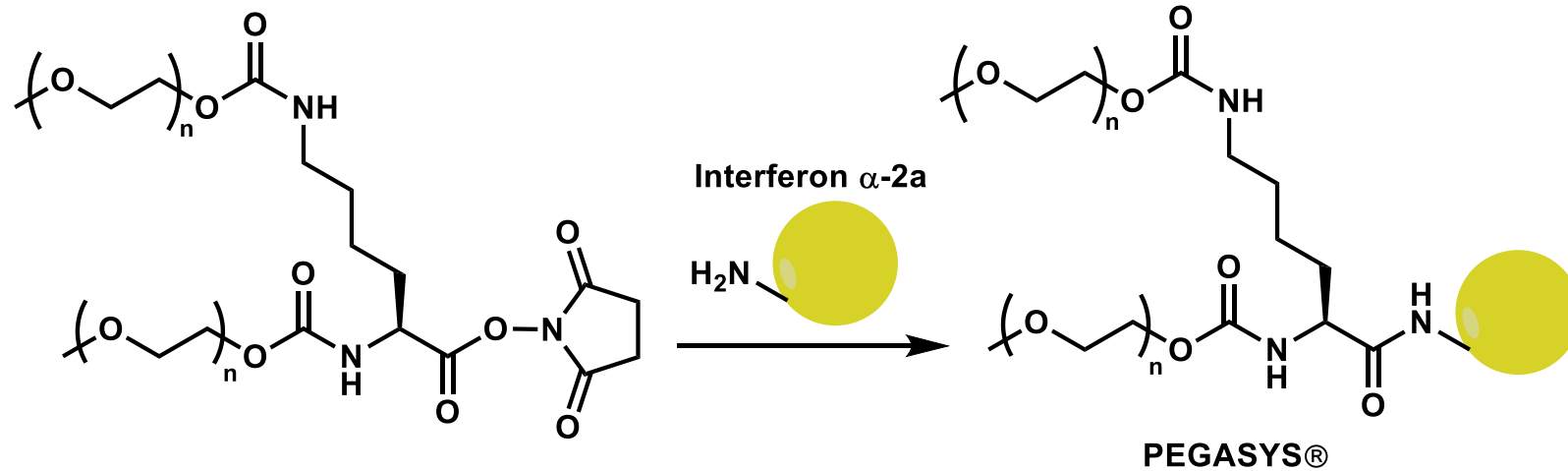
Immunoassay
Western blotting



Affinity chromatography

Use of Bioconjugation

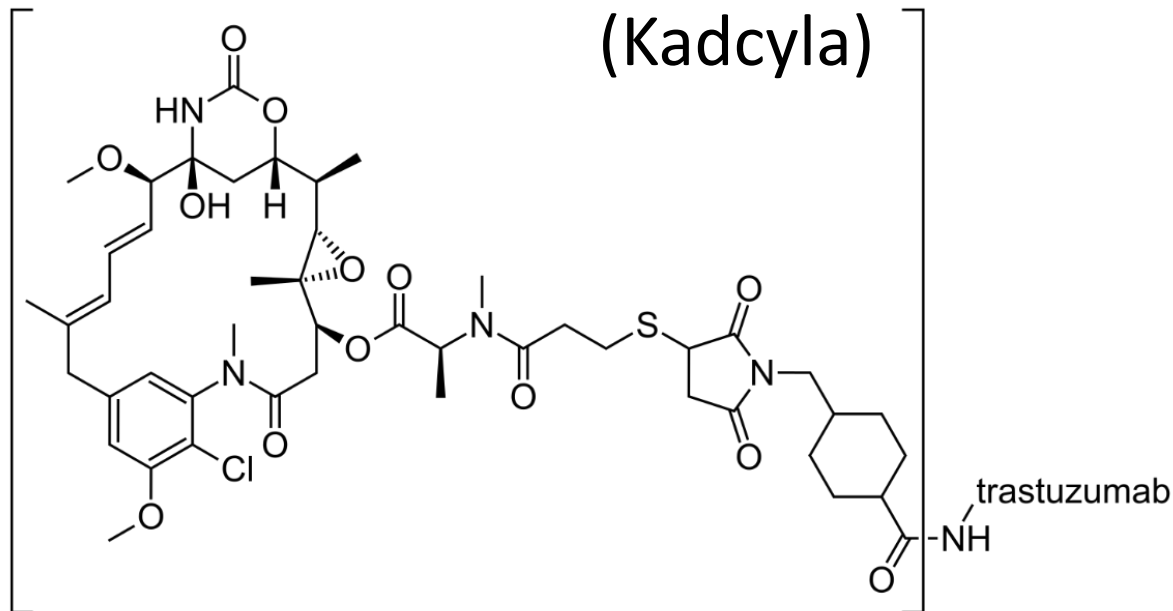
- Polyethylene glycol-protein conjugate drugs



	Native Interferon α -2a	PEGASYS	
Half-life	3-8 h	65 h	
Dosing interval	thrice-weekly	weekly	→ Improvement of patients' QoL
Sites of attachment		Lys 31, 121, 131 or 134	

Use of Bioconjugation

- ADC(Antibody-drug conjugate)



Trastuzumab(Herceptin)

Inhibition of HER2 signal



emtansine

Inhibition of tubulin polymerization

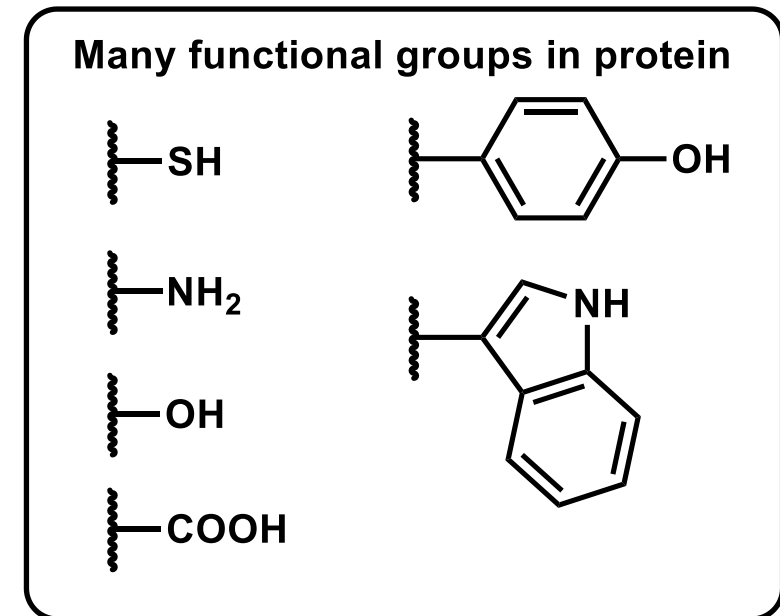
Stronger therapeutic effect and less side effects
than conventional chemotherapy

Conditions necessary for bioconjugation

- Physiological conditions not to interfere with proper protein folding and function

{
Temperature (<37°C)
pH(6-8)
Aqueous solvent

- Chemoselectivity(residue-selective reaction)
⇒ Distinction of different amino acids



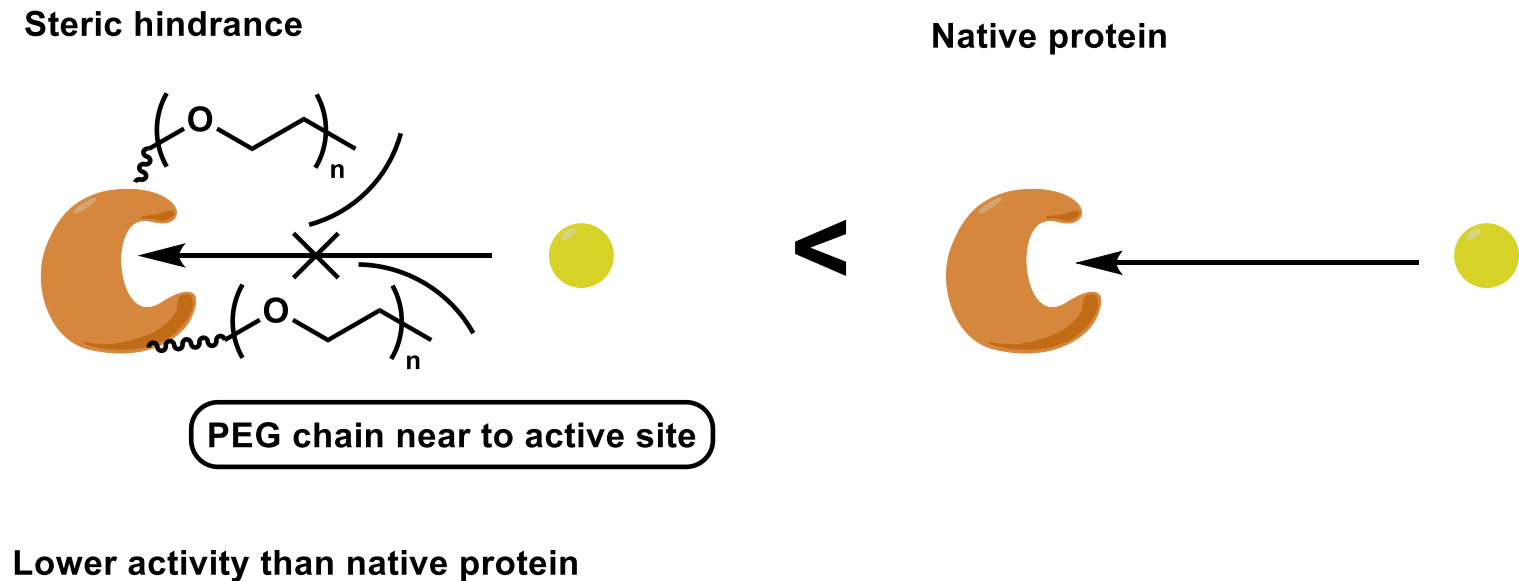
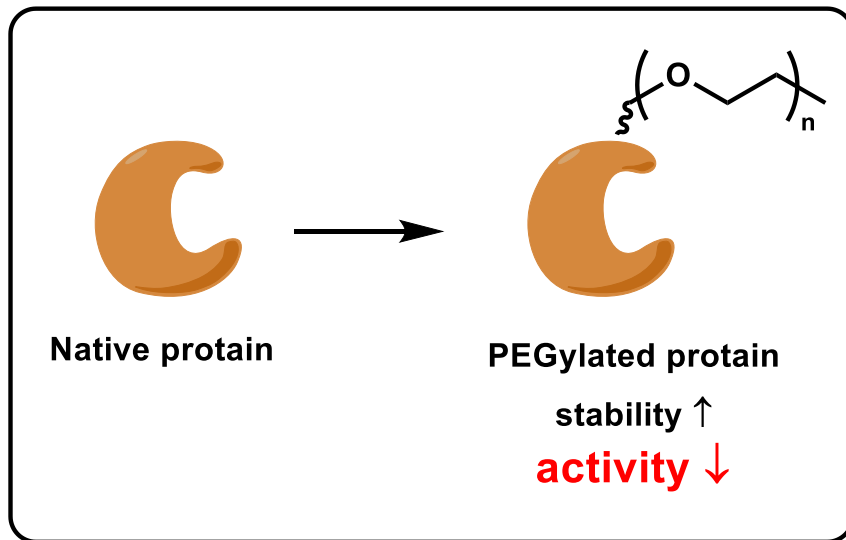
- Site-selectivity and control of the number of modifications
⇒ Distinction of local environment of amino acids

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Importance of control of modification

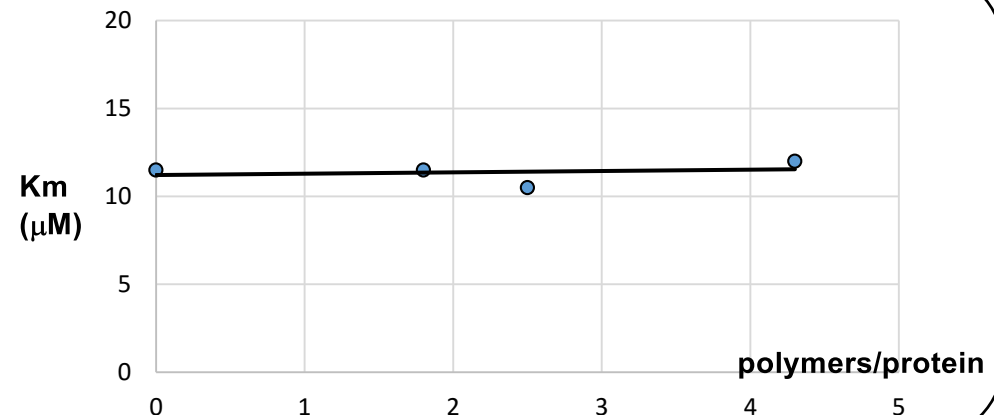
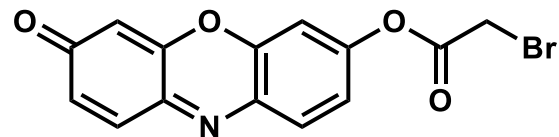
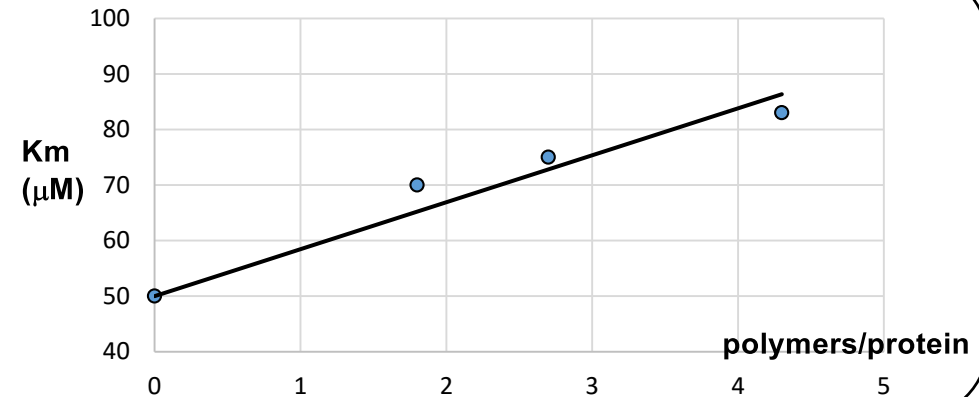
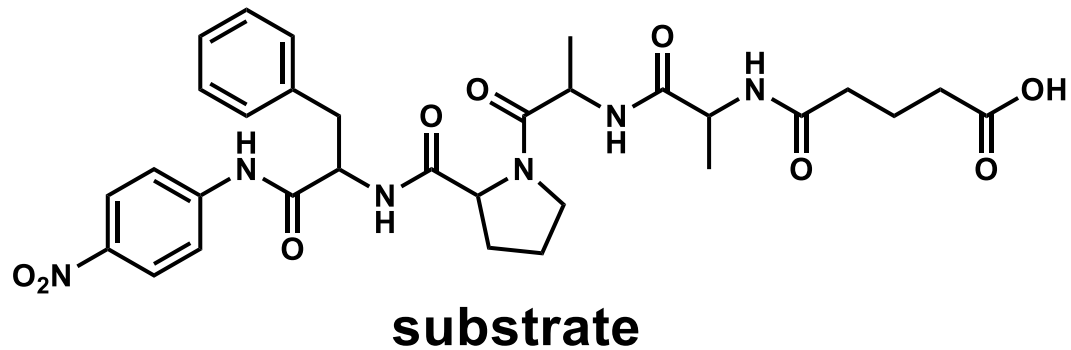
- PEGylation of proteins



Activity of proteins is reduced due to blocking active site by PEG chain

Importance of control of modification

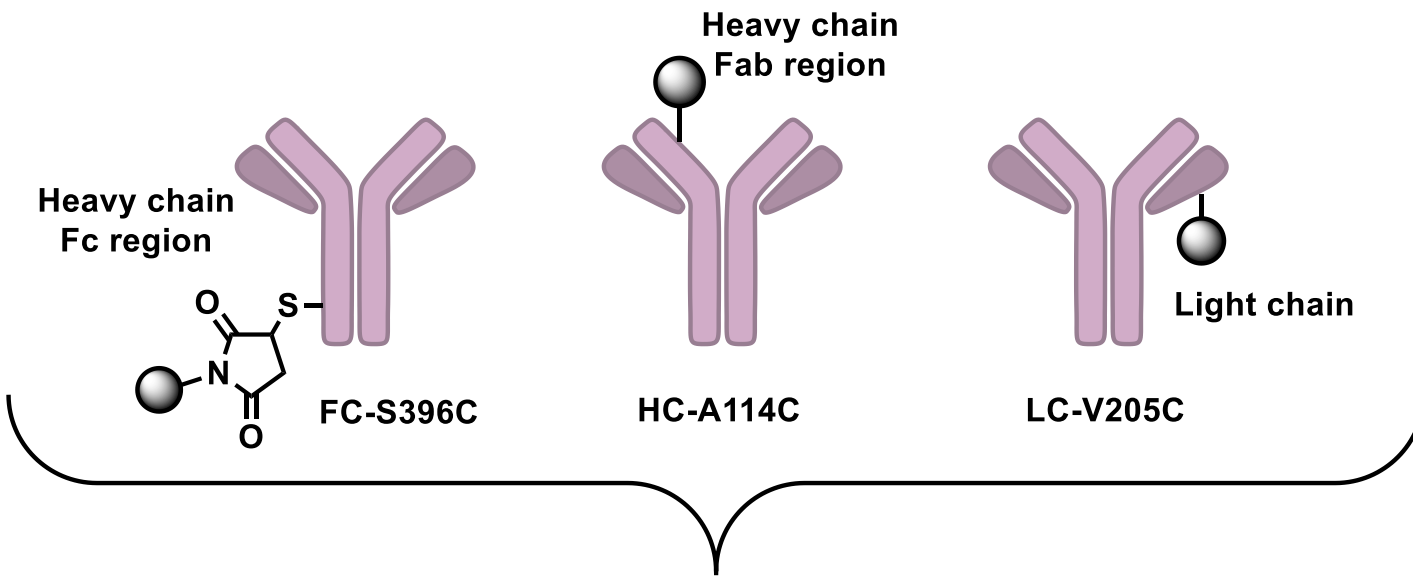
Activity of PEGylated chymotrypsin



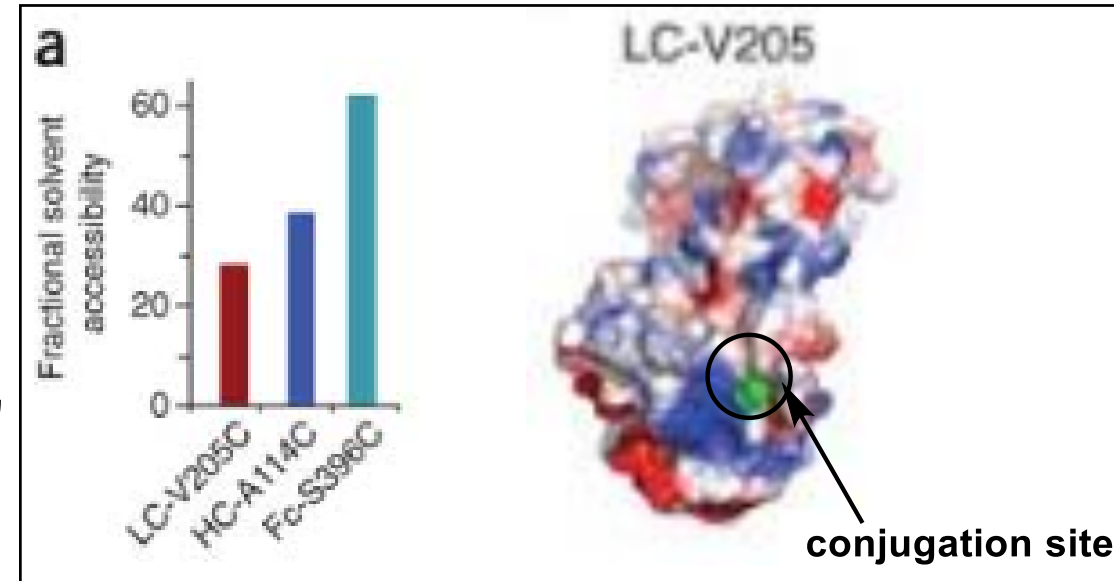
Need for controlled modification to the amino acid away from the active site

Importance of control of modification

- Influence of conjugation site to ADC's stability and therapeutic activity



similar binding to HER2 overexpressing MCF7 cells

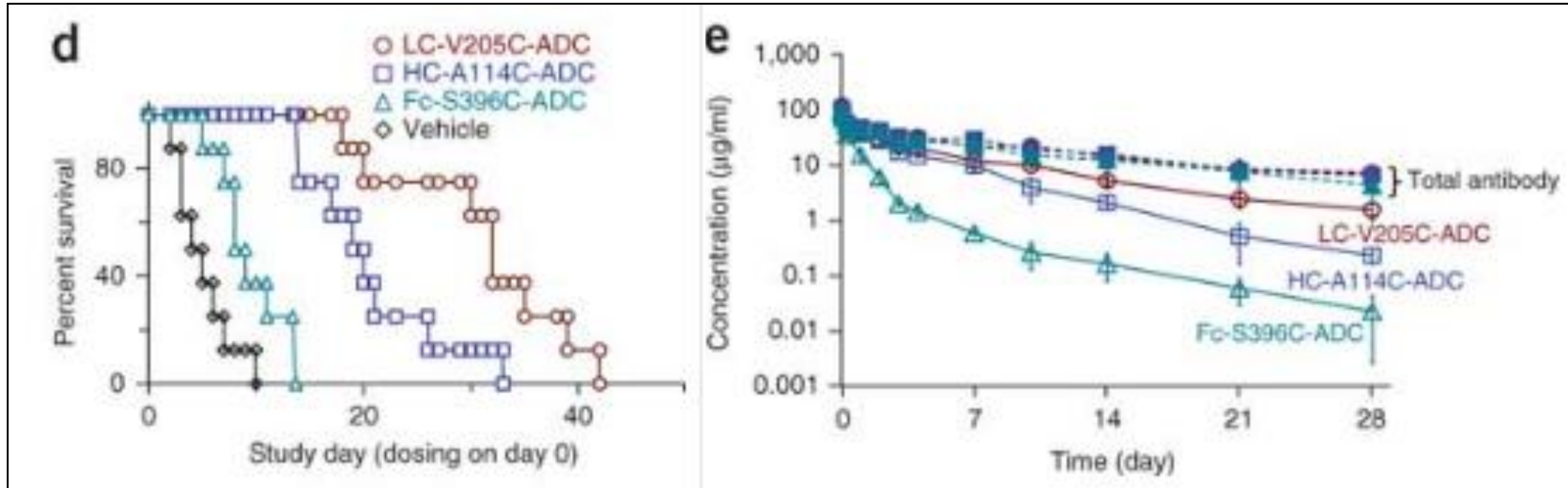


Shen, B.-Q. *et al. Nature Biotech.* **2012**, 30, 184

- Solvent accessibility : FC-S396C > HC-A114C > LC-V205C
- LC-V205C conjugation site is in a positively charged environment

Importance of control of modification

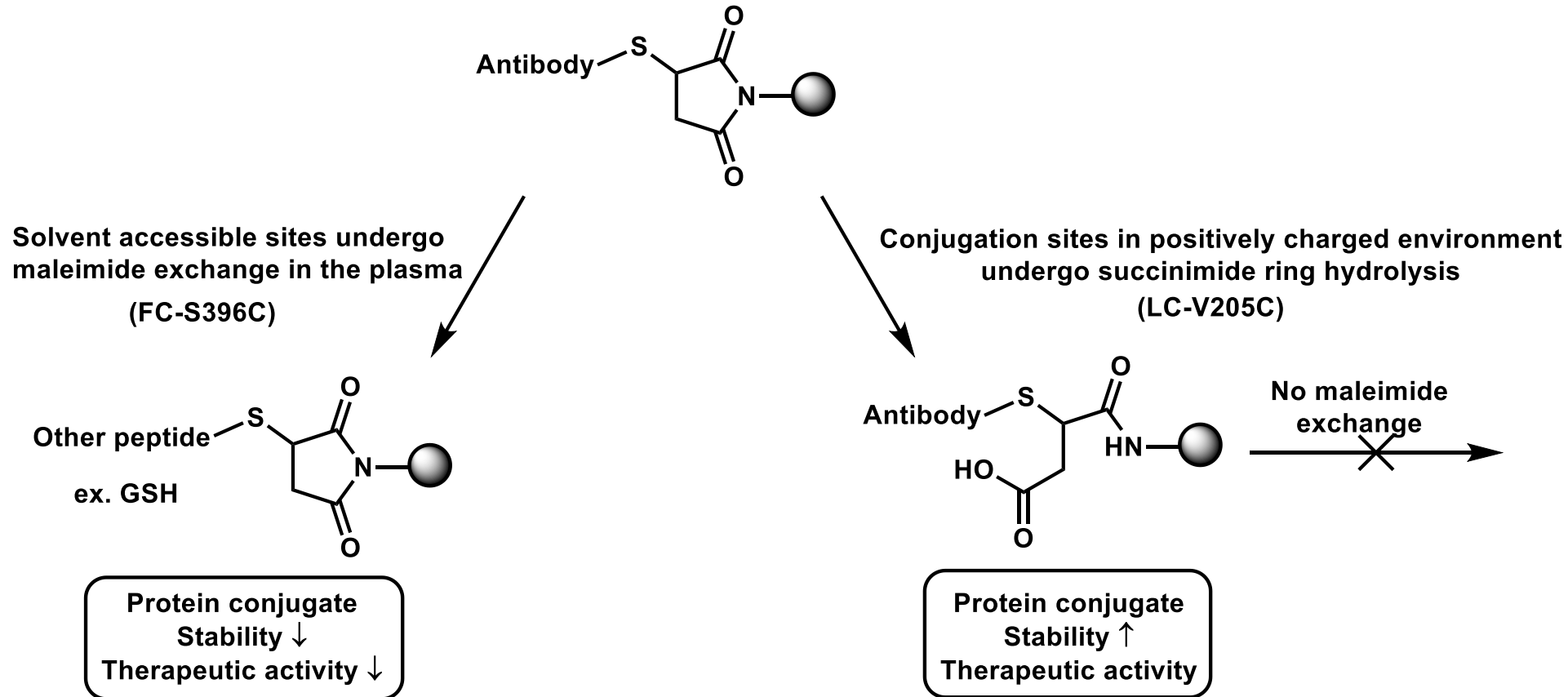
These ADCs' *in vivo* efficacies and pharmacokinetics properties



- In vivo activities and durabilities : LC-V205C > HC-A114C > FC-S396C
 - No differences in total antibody clearance rates
- ⇒ Differences in intact ADC levels from linker stability

Importance of control of modification

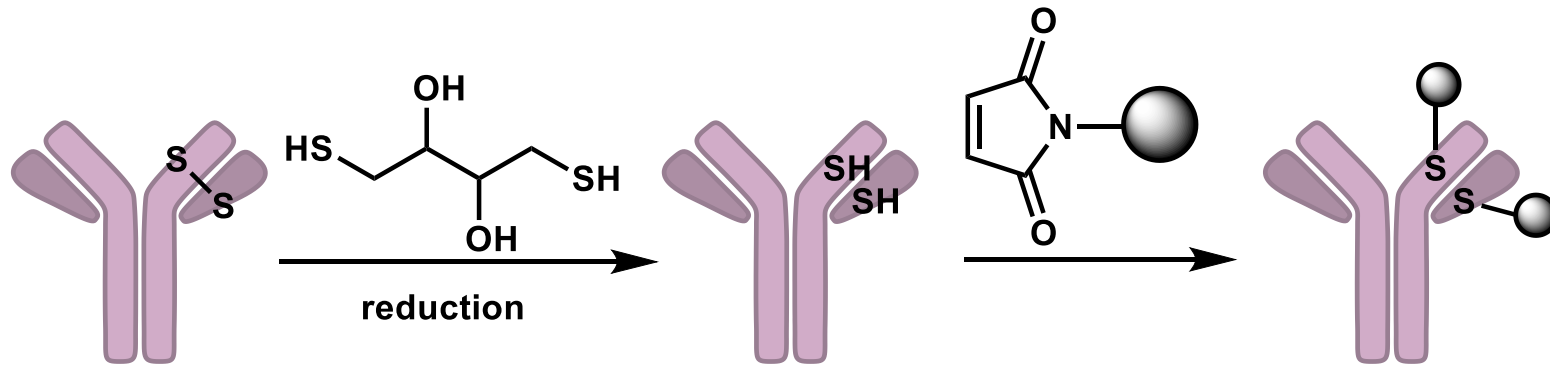
Difference of stability of antibody-drug bonding



⇒ Site-selective bioconjugation is important to improve the stability and activity of antibody-drug conjugate

Importance of control of modification

- Influence of conjugation number to ADC's stability and therapeutic activity

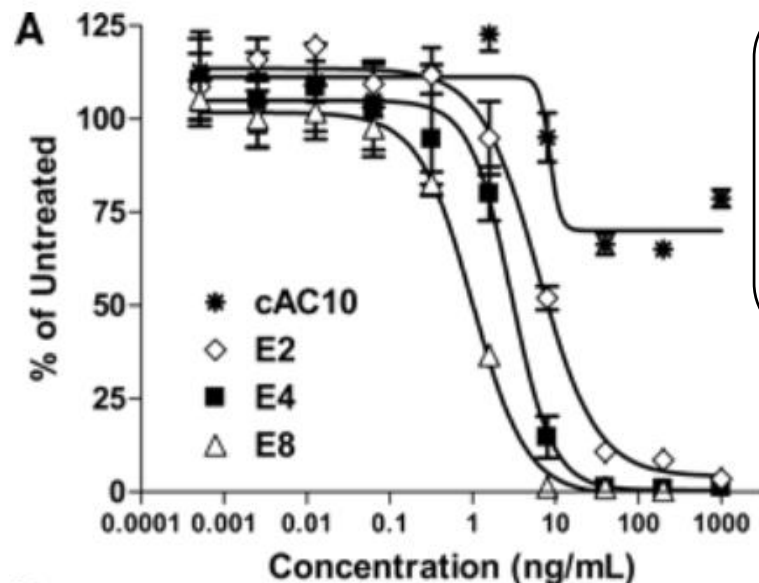


cAC10

DAR(drug-to-antibody ratio)=2,4,6,8

Hamblett, K. J. *et al. Clin. Cancer Res.* **2004**, 10, 7063

In vitro cytotoxic activities of ADC



IC50...E2 : 6.2 ng/mL

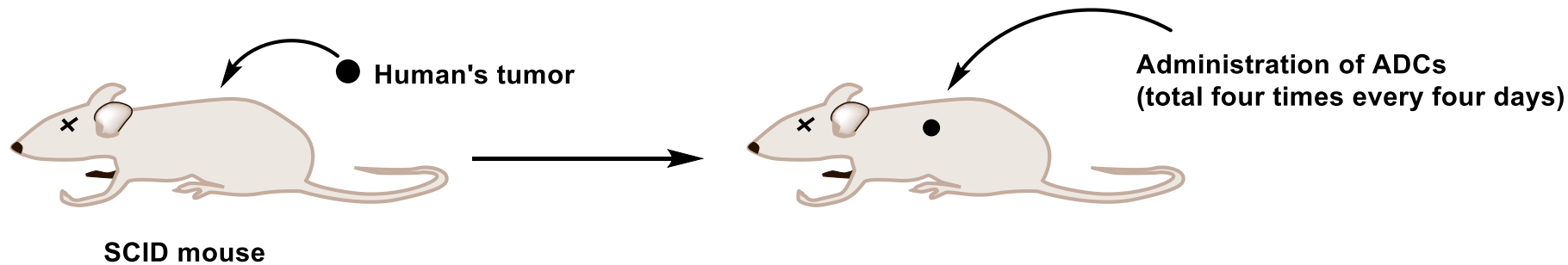
E4 : 2.9 ng/mL

E8 : 1.0 ng/mL

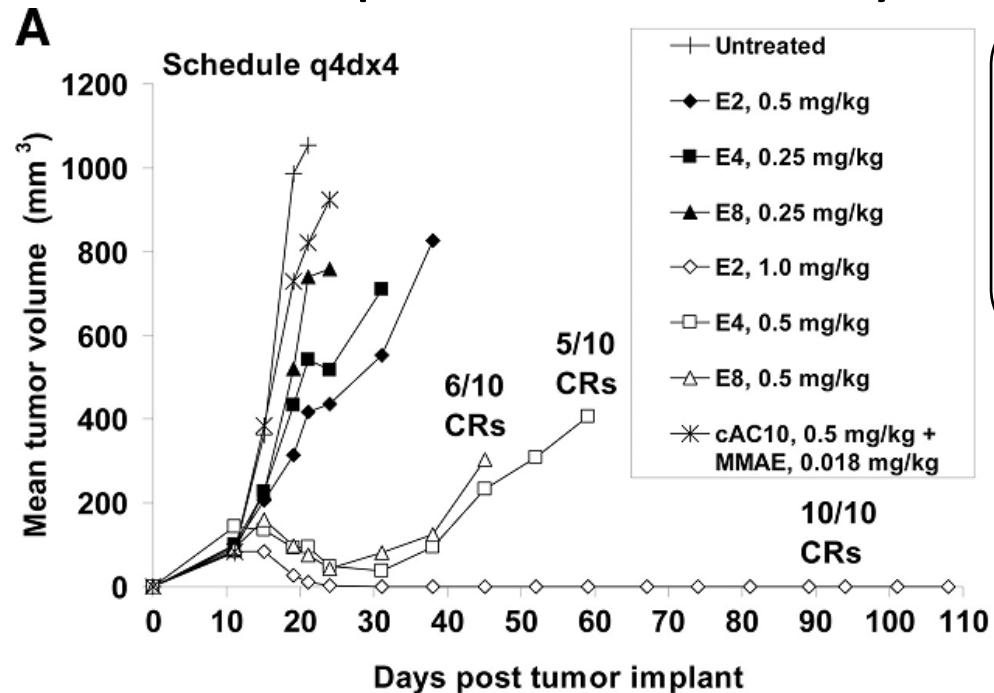
in vitro potency of ADC E2 < E4 < E8

Importance of control of modification

The effect of drug loading on in vivo antitumor activity



Relationship of ADCs' activity and DAR or dose



Therapeutic effect

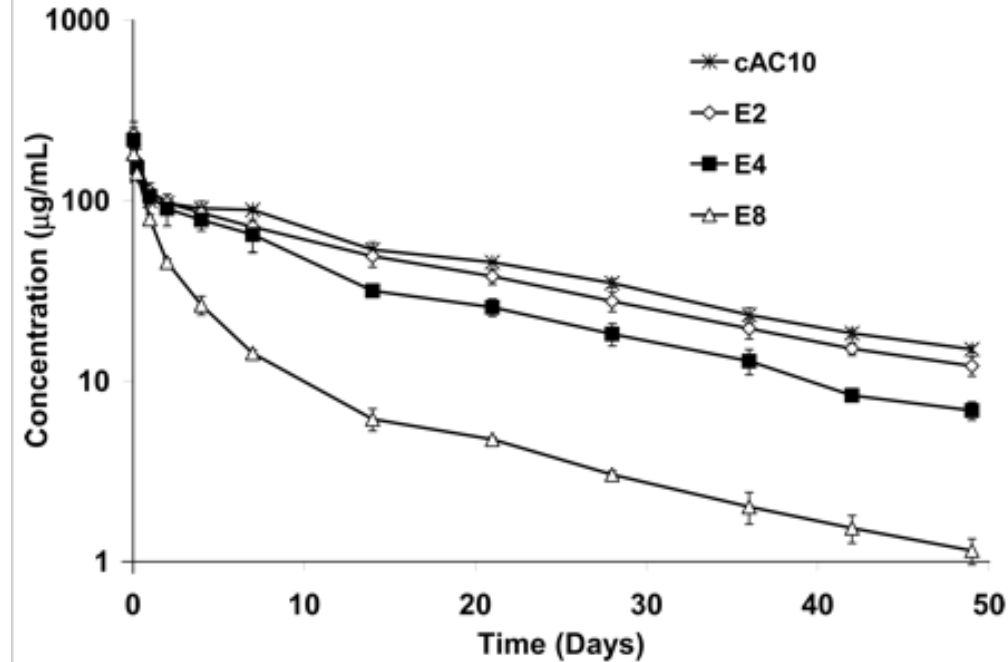
$E4\ 0.5\ \text{mg/kg} \cong E8\ 0.5\ \text{mg/kg}$

$E2\ 1.0\ \text{mg/kg} > E4\ 0.5\ \text{mg/kg} > E8\ 0.25\ \text{mg/kg}$

in vivo activity isn't proportional to drug number

Importance of control of modification

Relationship of conjugation number and pharmacokinetics properties



in vivo stability : E2 > E4 > E8

ADCs with higher DAR level are more sensitive to stress such as temperature.

Beckley, N. S., Lazzareschi, K. P., Chih, H.-W., Sharma, V. K. & Flores, H. L.
Bioconjugate Chem. **2013**, 24, 1674.

⇒ The number of bioconjugation is also important to improve the stability and activity of antibody-drug conjugate

Importance of control of modification

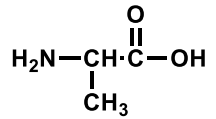
- Modification location and number of the protein are important for the its activity and stability
- ⇒ Site-selective bioconjugation reaction

Topics

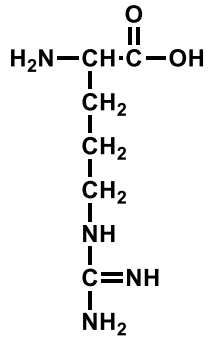
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Target of protein bioconjugation

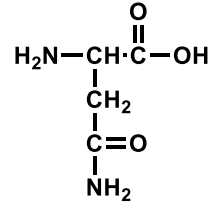
- 20 natural amino acids



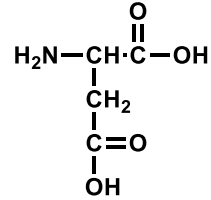
Ala



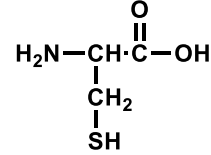
Arg



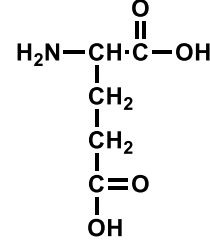
Asn



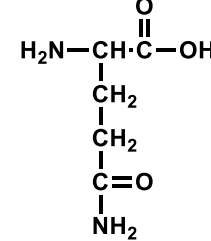
Asp



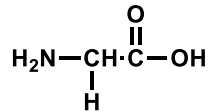
Cys



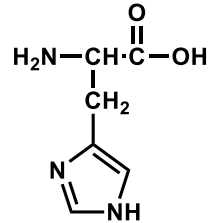
Glu



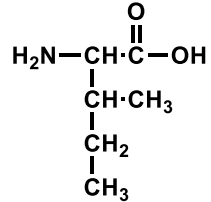
Gln



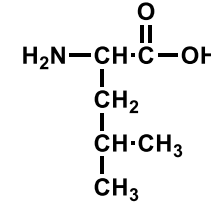
Gly



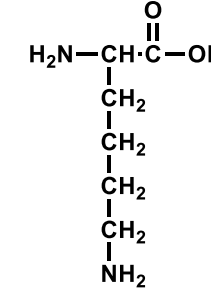
His



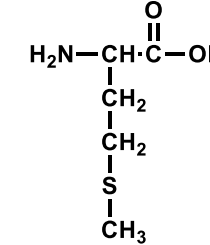
Ile



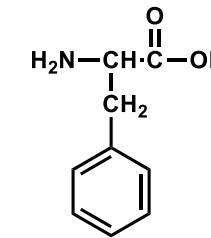
Leu



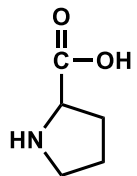
Lys



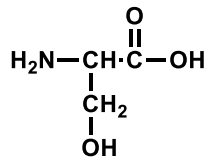
Met



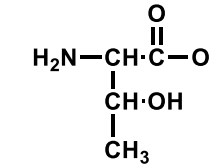
Phe



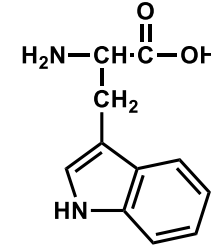
Pro



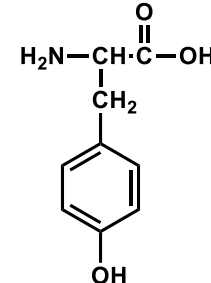
Ser



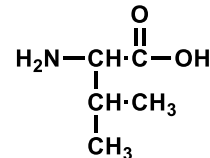
Thr



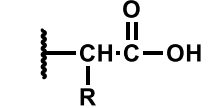
Trp



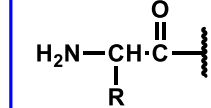
Tyr



Val



C-terminal



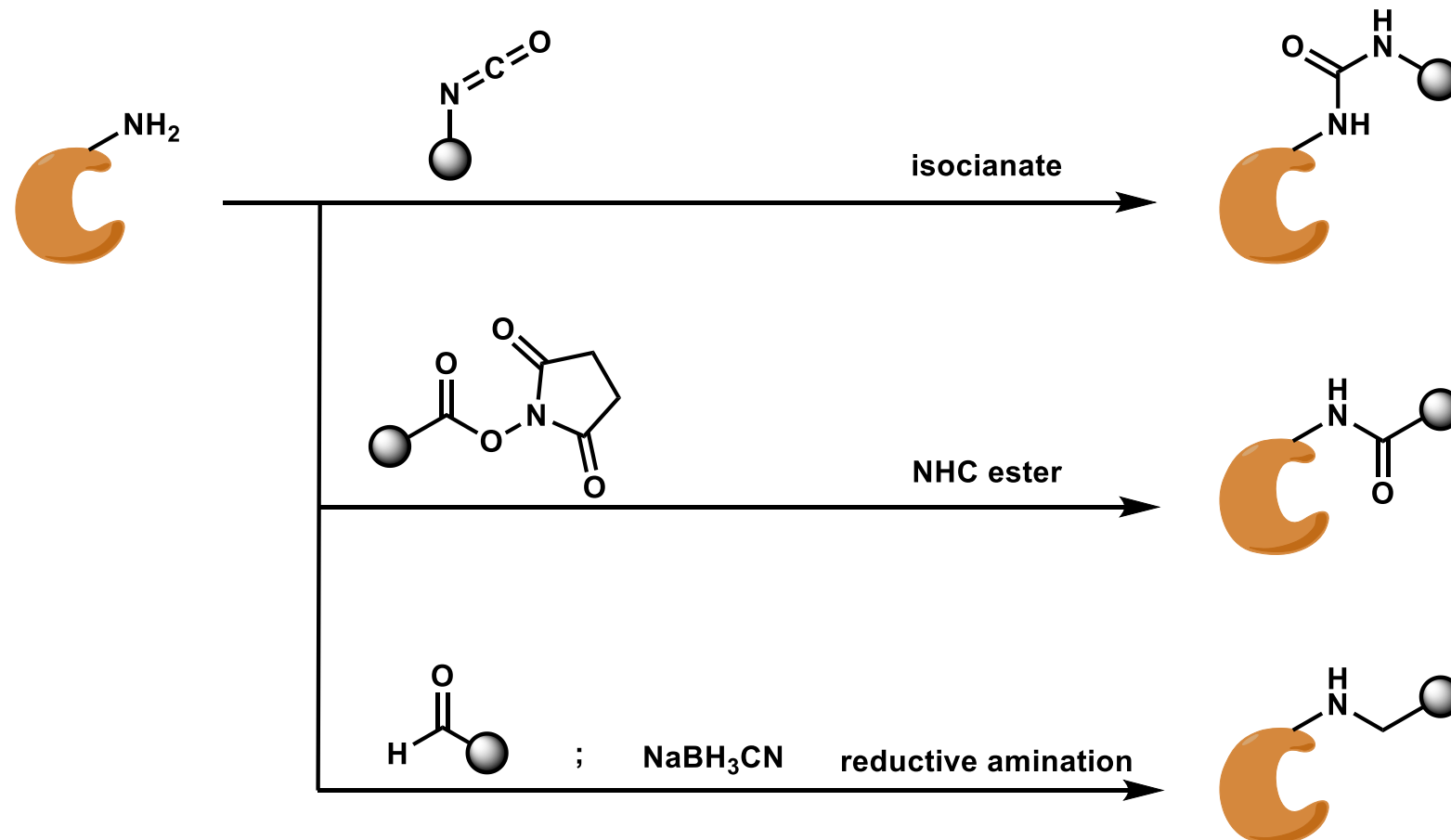
N-terminal

Conventional protein bioconjugation

1. Targeting natural lysine
2. Targeting *N*-terminal amine
3. Targeting natural cysteine

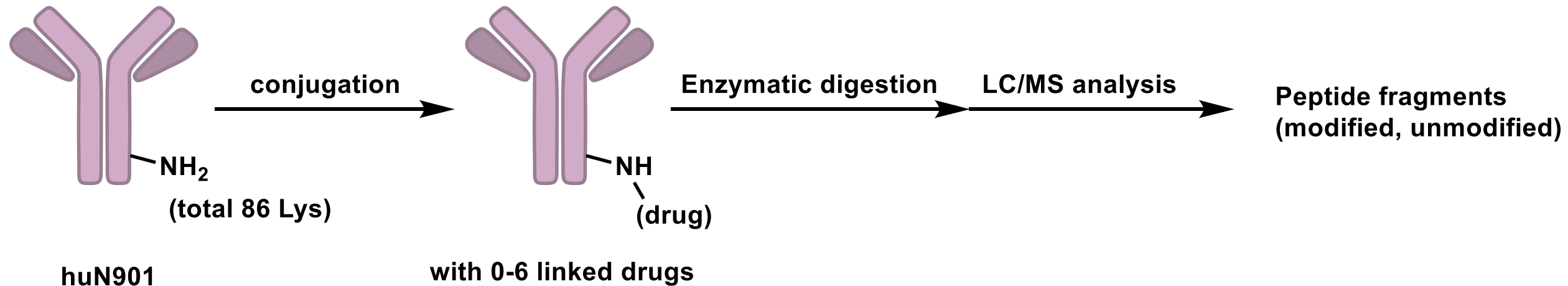
Targeting natural lysine

- One of the most nucleophilic functional group in a protein
 - Abundance on the solvent-exposed outside surfaces of protein
- ⇒ Most reactive amino acid



Targeting natural lysine

- Random modification of Lys residues



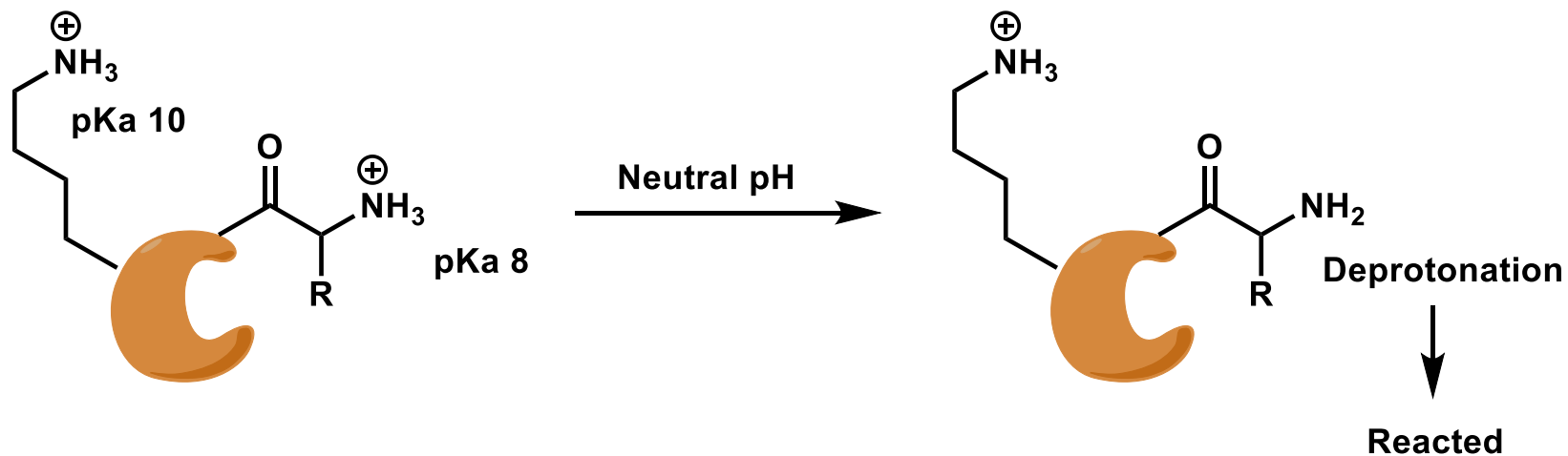
40 of identified modification sites \Rightarrow millions of types of products

Modified lysine residues either are located on the protein surface or have relatively large structural flexibilities.

But site-selectivity is remarkably low

Targeting N-terminal amine

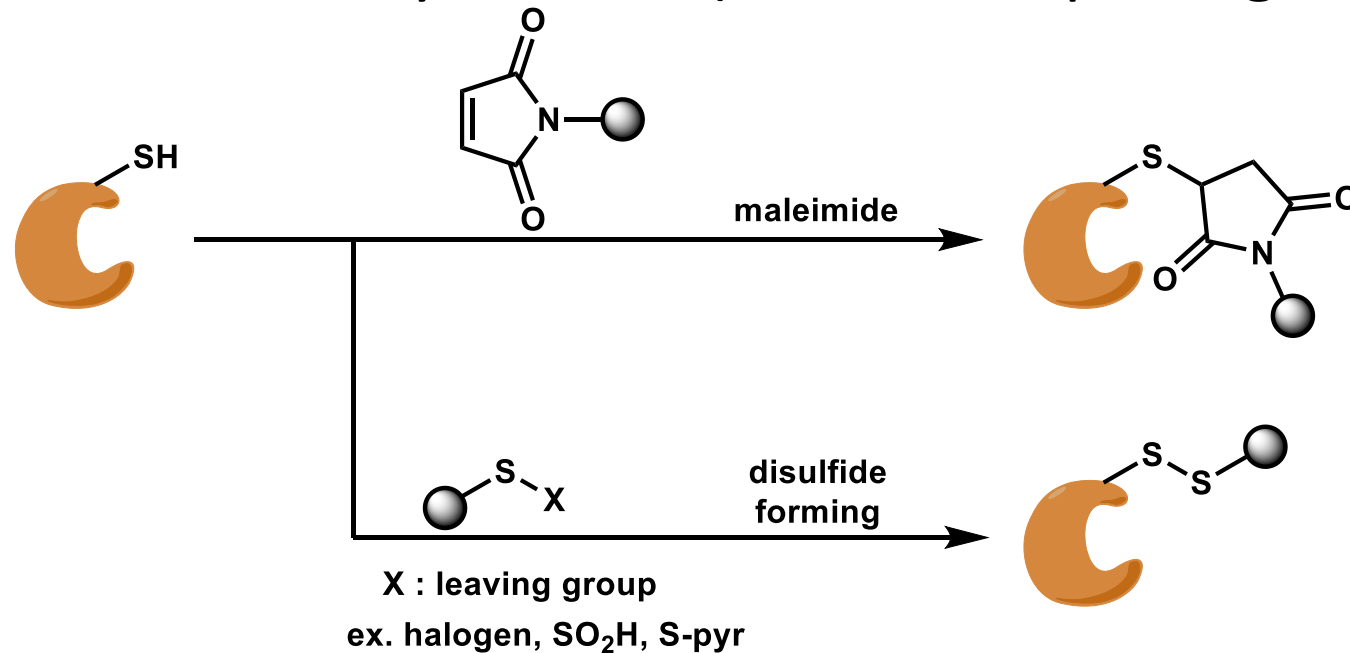
- Only one N-terminal amine
⇒ No diversity of conjugation products
- Possible to distinguish between N-terminal α amine and Lysine ϵ amine



But impossible to introduce multiple modifications

Targeting natural cysteine

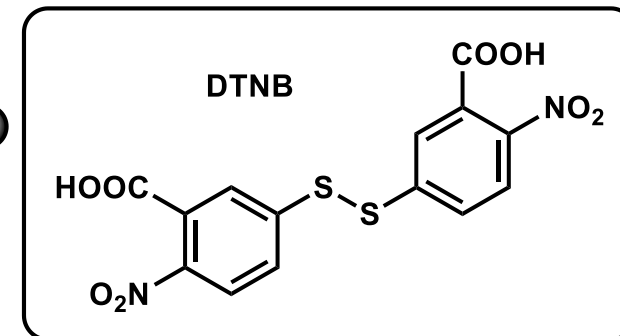
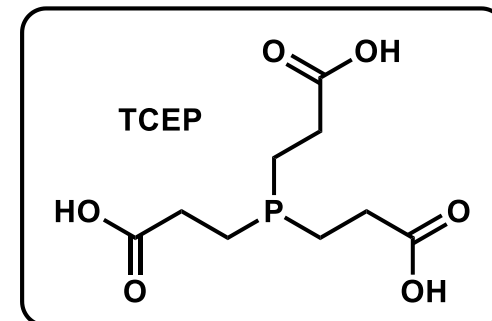
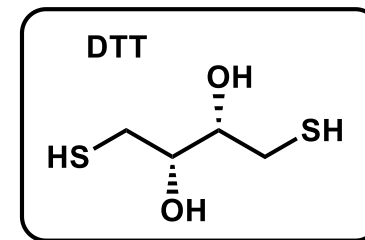
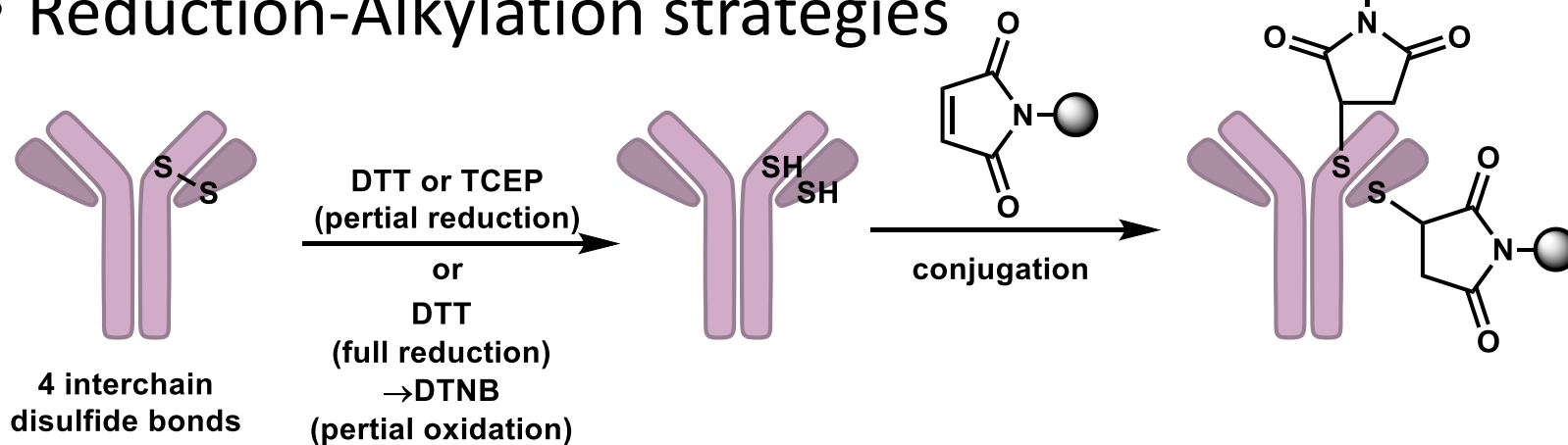
- Rarity of cysteine in proteins
⇒ Low heterogeneous products
- Thiol also has high nucleophilicity
- Different reactivity of Thiol (soft nucleophilic group)



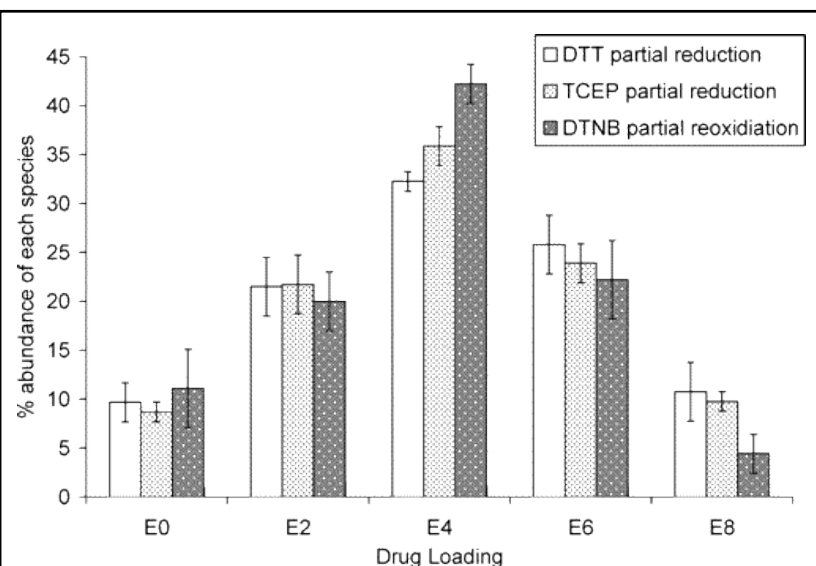
But Cys in protein form disulfide bonds ⇒ Necessity to reduction of S-S bonds

Targeting natural cysteine

- Reduction-Alkylation strategies



25/46



Intermolecular and intramolecular disulfide bonds were distinguished (intermolecular S-S only reduced)
Partial reduction can't distinguish intermolecular S-S bonds \Rightarrow various reduction product
 \Rightarrow various conjugation product

Site-selective Cys reduction-modification was difficult

Strategy of site-selective protein bioconjugation

- Impossible to distinguish same amino acids

Lys and Lys, Cys and Cys

⇒ Recognize other than the target amino acids

➤ Local environment around the reaction point

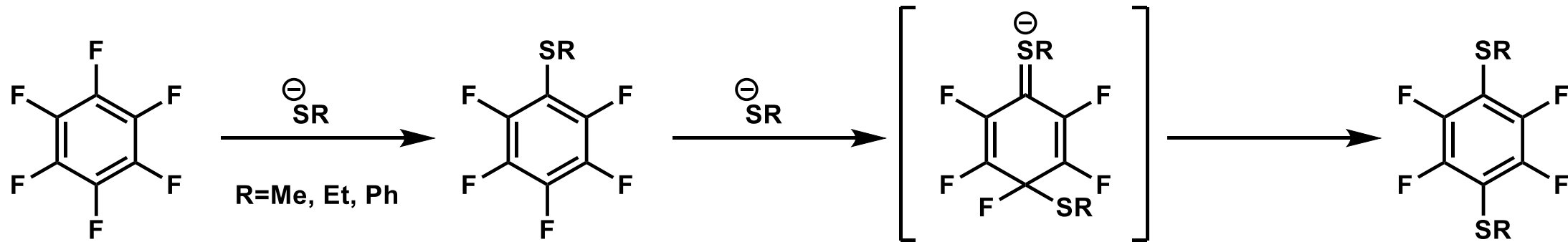
➤ Ligand recognition site away from reaction point

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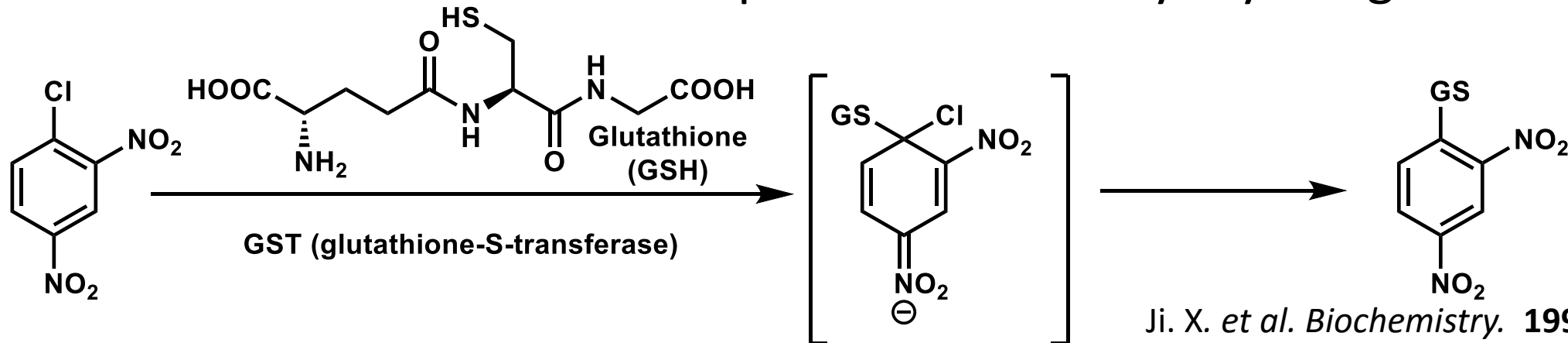
π -clamp-mediated cysteine bioconjugation

- Thiol's Nucleophilic aromatic substitution (S_NAr) reactions



Birchall, J. M. *et al. Chem. Comm.* **1967**, 338

- Glutathione-S-transferase are capable of selectivity arylating

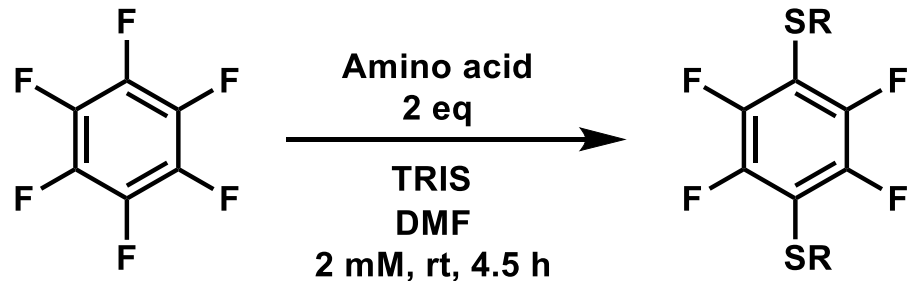


Ji, X. *et al. Biochemistry.* **1993**, 32, 12949

⇒ Arylation of cysteine can be used to Cys-selective bioconjugation.

π -clamp-mediated cysteine bioconjugation

• Cys-perfluoroarylation reaction



Cys-selective

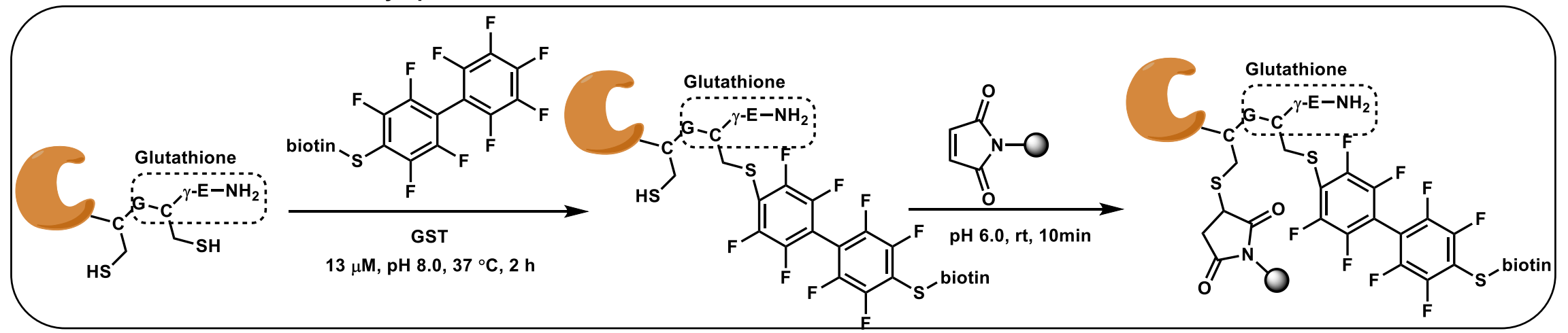
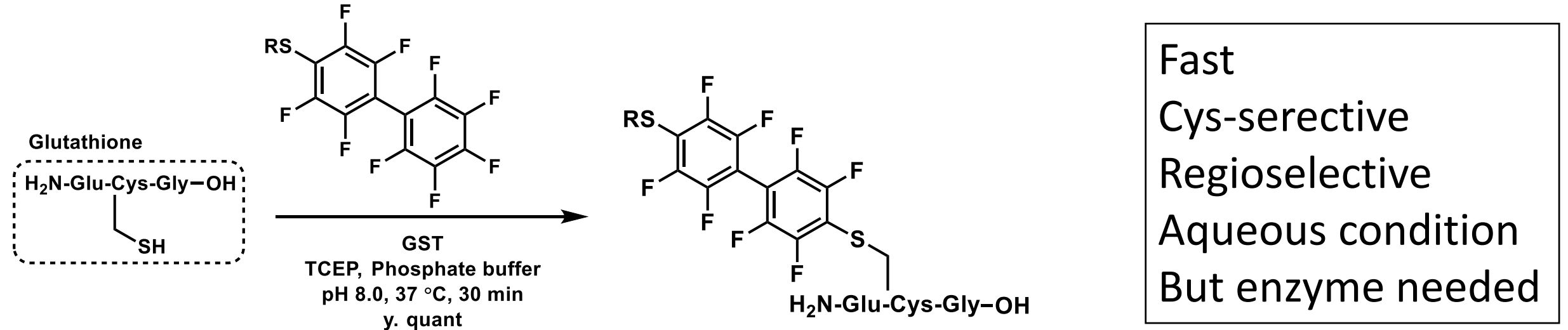
Room temperature

But organic solvent needed

Amino acid	Yield(¹⁹ F NMR)
	> 95%
	> 95%
	No reaction
	No reaction

π -clamp-mediated cysteine bioconjugation

• Enzyme-catalyzed Cys-bioconjugation



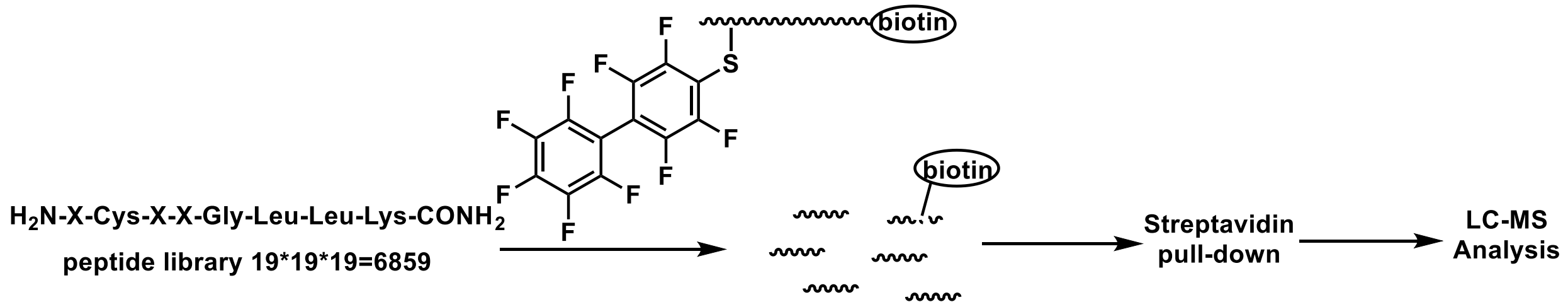
π -clamp-mediated cysteine bioconjugation

• Cysteine bioconjugation using without

1. Organic solvent

2. Enzyme (GST)

⇒ Search of substrates reacting in water



⇒ Phe-Cys-Pro-Trp-... reacted in water

⇒ Aromatic amino acids activated the cysteine thiol and interact with the perfluoroaryl group?

π -clamp-mediated cysteine bioconjugation

- π -clamp mediated conjugation

peptide	$K_2(\text{M}^{-1}\text{s}^{-1})$	yield(%)
Gly-Cys-Pro-Gly-	N/A	<1
Gly-Cys-Pro-Phe-	0.09	50
Phe-Cys-Pro-Phe-	0.73	>99
Tyr-Cys-Pro-Tyr-	0.08	47
Trp-Cys-Pro-Trp-	0.20	79



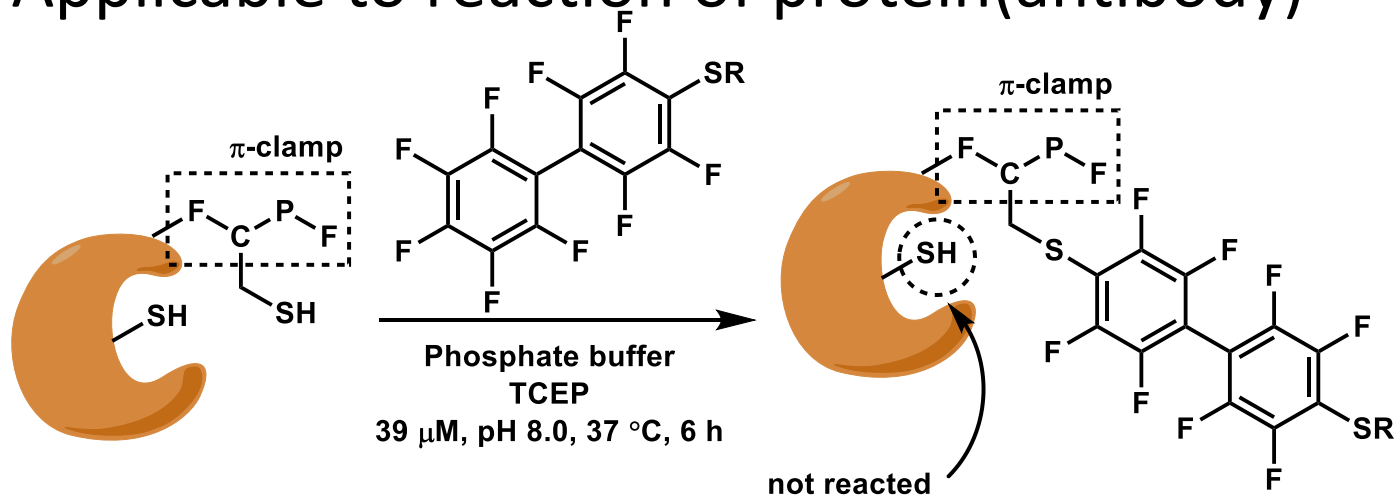
X=Phe, Tyr, Trp

Aromatic amino acids accelerated reaction

Only Phe-Cys-Pro-Phe reacted quantitatively

Zhang, C. *et al. Nature. Chemistry. 2015*, 2413

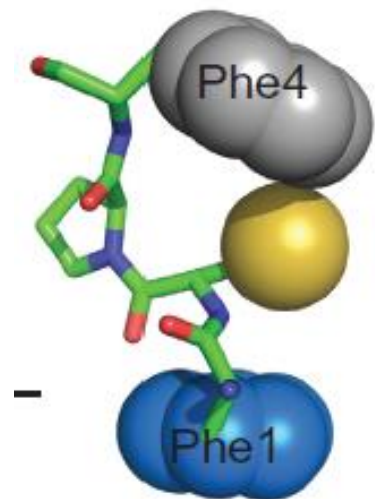
- Applicable to reaction of protein(antibody)



π -clamp-mediated cysteine bioconjugation

- Mechanism of π -clamp mediated cysteine bioconjugation
- ◆ Activation of thiol of Cysteine (stabilization of thiolate)

Structure of Phe-Cys-Pro-Phe



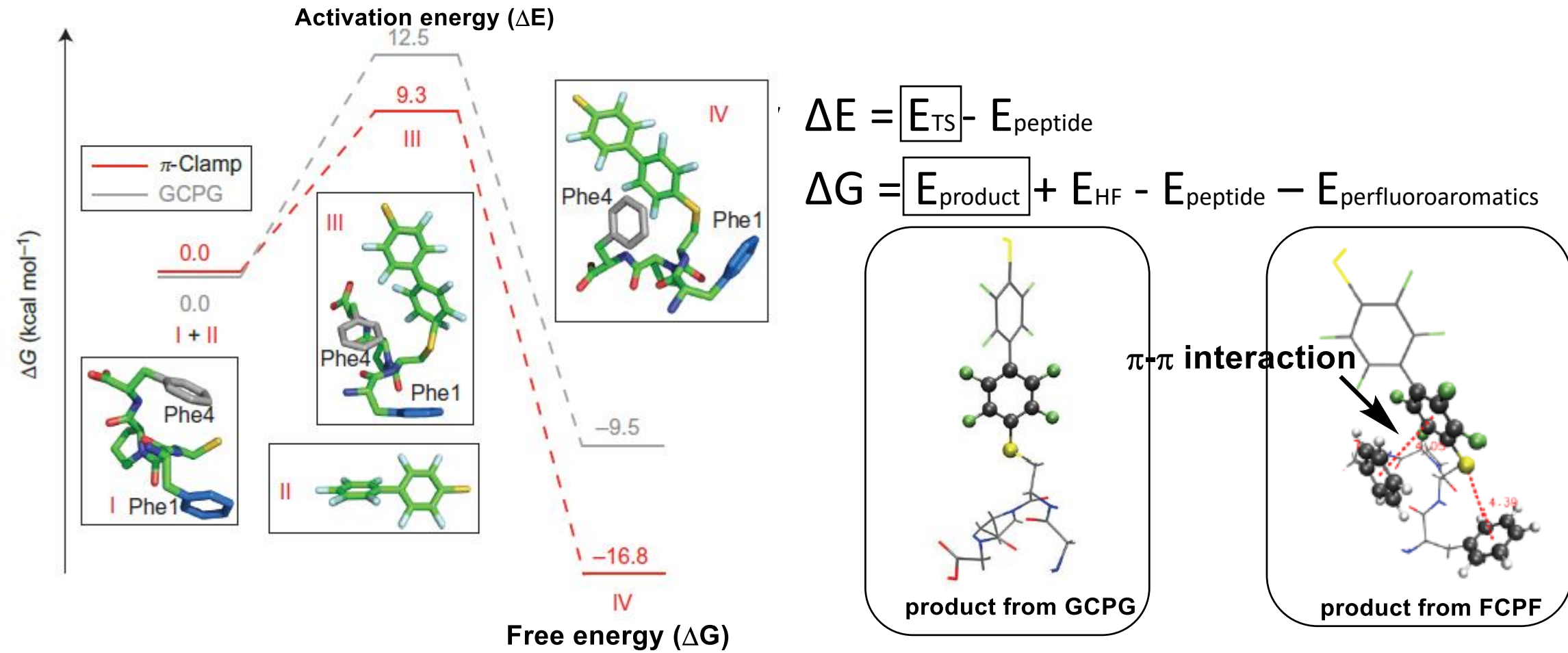
←-SH ($pK_a=7.69 \pm 0.09$)

More acidic than thiol of Gly-Cys-Pro-Gly ($pK_a=8.30 \pm 0.05$)

⇒ Nucleophilicity of thiol increases

π -clamp-mediated cysteine bioconjugation

◆ Lower activation energy and product free energy



π - π interaction stabilizes transition state and product \Rightarrow reaction acceleration

π -clamp-mediated cysteine bioconjugation

Interaction between reagent and amino acids around target

Cysteine increases site-selectivity of bioconjugation

- Problem

- ◆ Necessity of structure X-Cys-Pro-X to drive bioconjugation reaction

 - ⇒ Introduction of X-Cys-Pro-X to target protein by genetic manipulation

- Next step

- ◆ Recognition of native amino acids around target residue

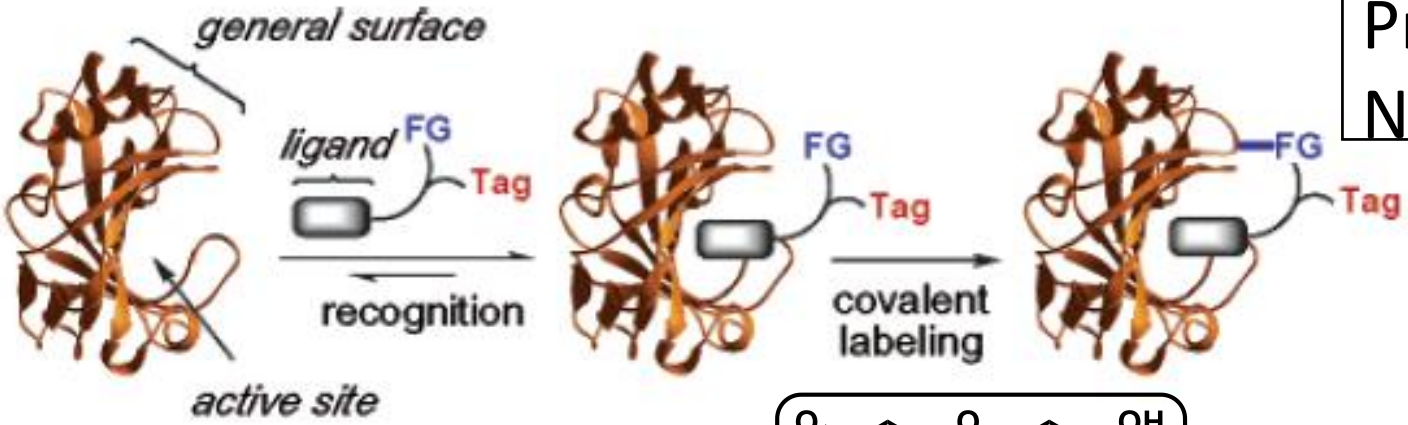
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 - Ligand-directed selective bioconjugation
5. Summary

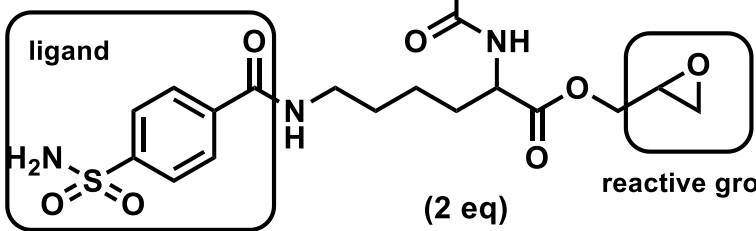
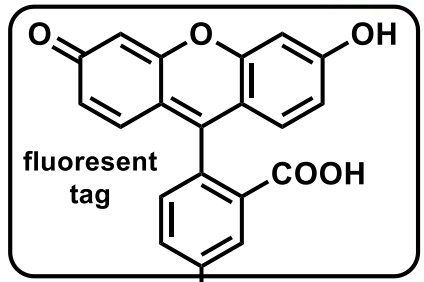
Ligand-directed selective bioconjugation

- Concept of an affinity labeling probe

Site-selective
 Protein-selective
 Not react to amino acids in active-site



ex.



Human carbonic anhydrase II (HCA II)

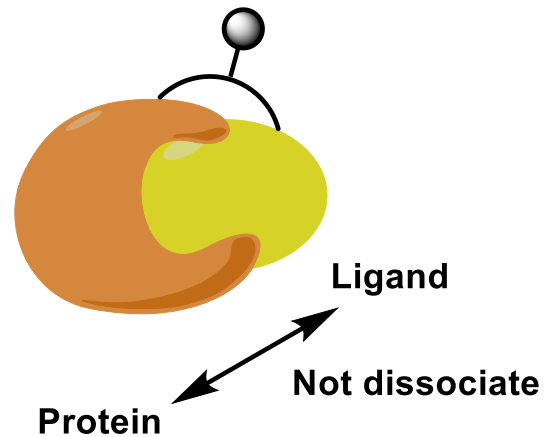
Tris buffer (pH 7.4)
rt, 20 h

Chen, G. *et al.* *J. Am. Chem. Soc.* **2003**, 125, 8130

High yield (>90%)
 HCA II selective
 His-64 selective
 Not reacted with inhibitor of HCA II
 Not reacted without ligand of HCA II

Ligand-directed selective bioconjugation

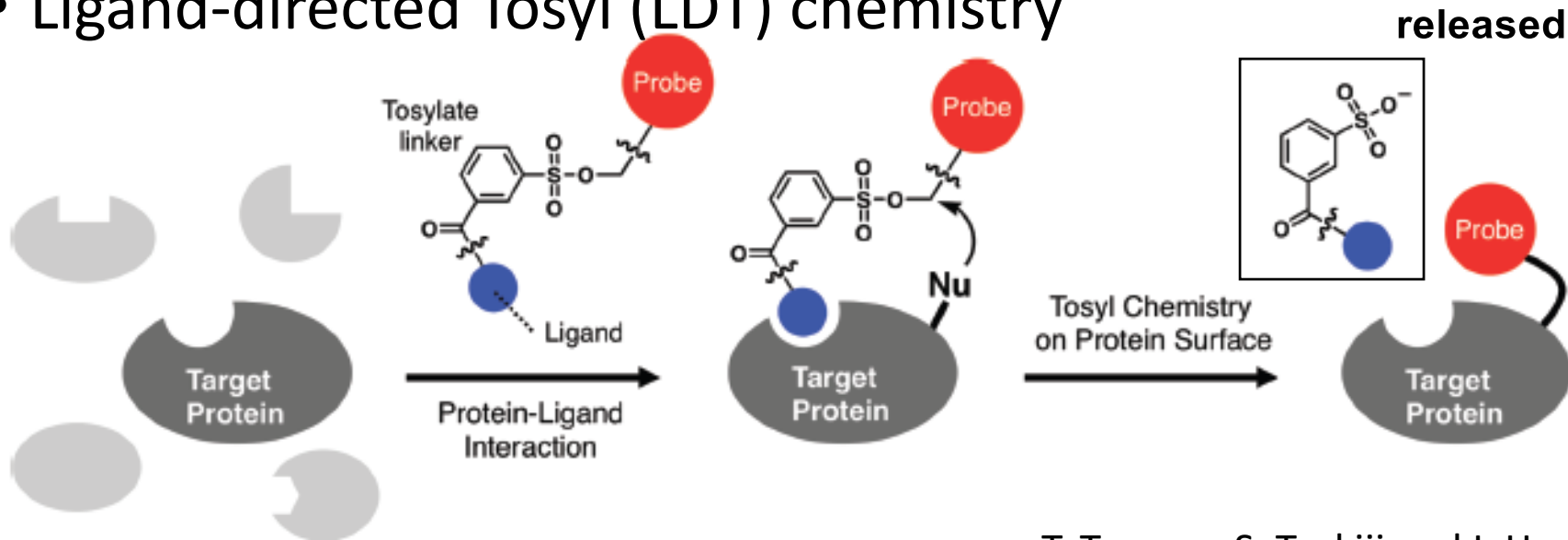
- Problem



⇒ Active site was blocked, reactivity ↓

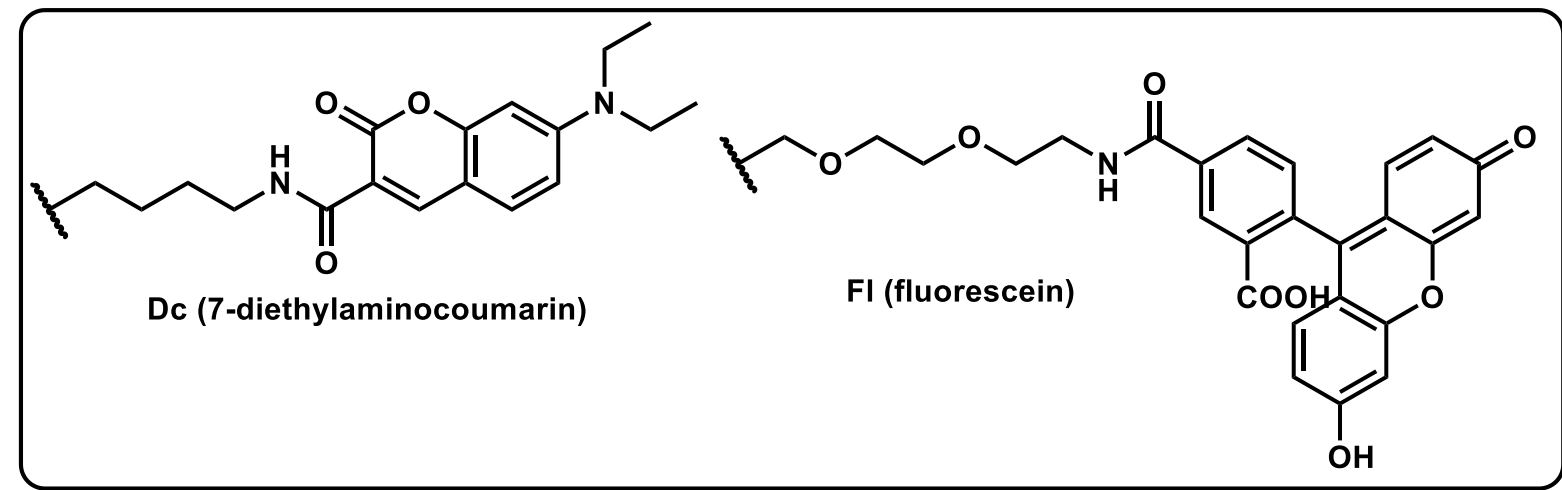
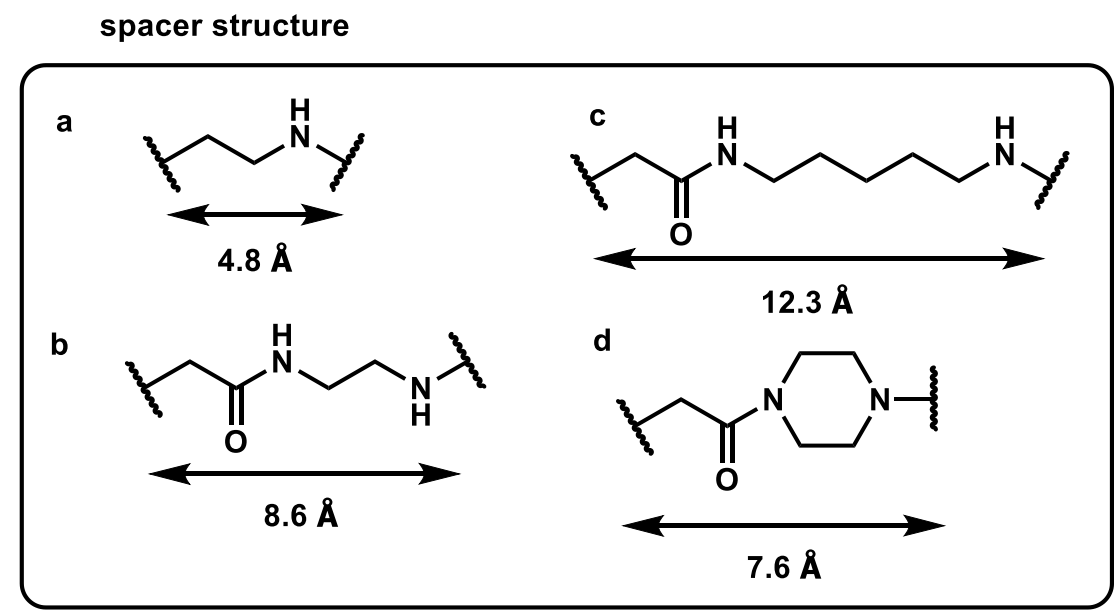
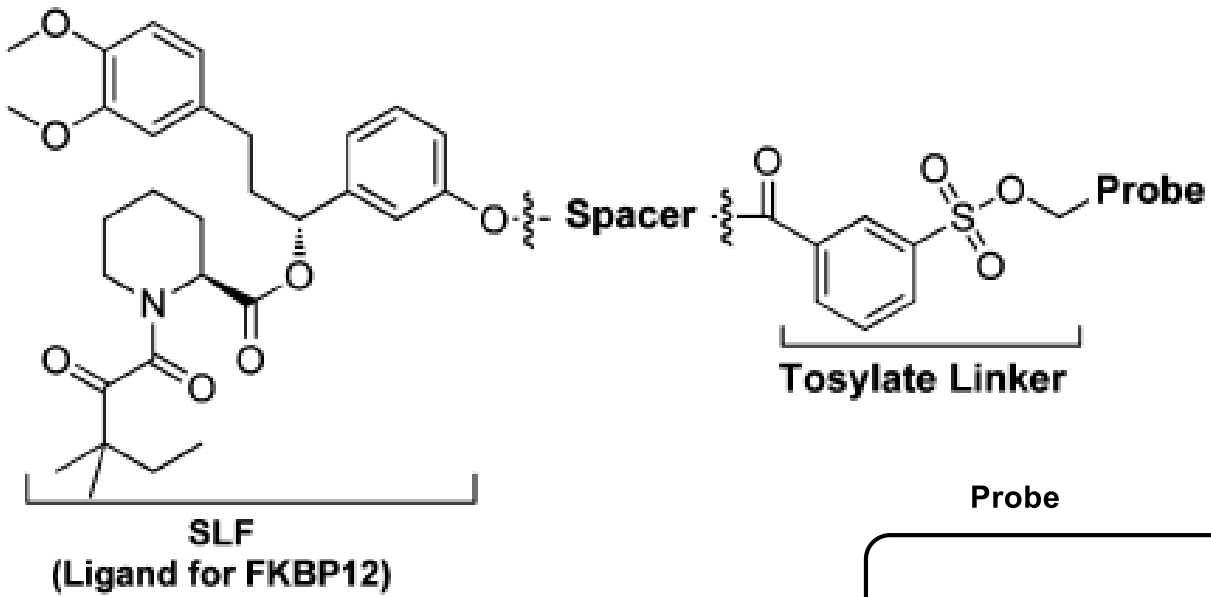
⇒ Reagents not yield covalent protein-ligand adducts

- Ligand-directed Tosyl (LDT) chemistry



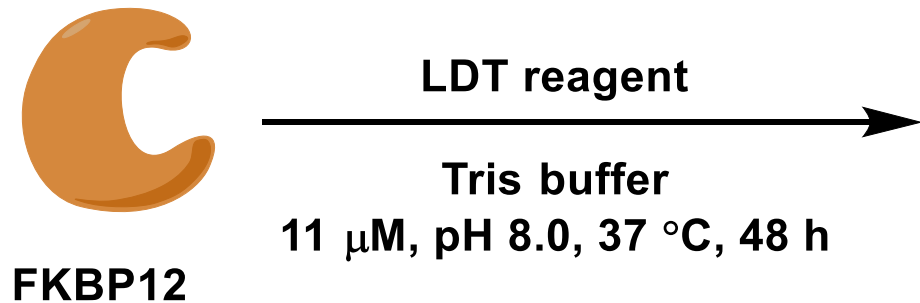
Ligand-directed selective bioconjugation

- Design of LDT reagents (spacer structure)



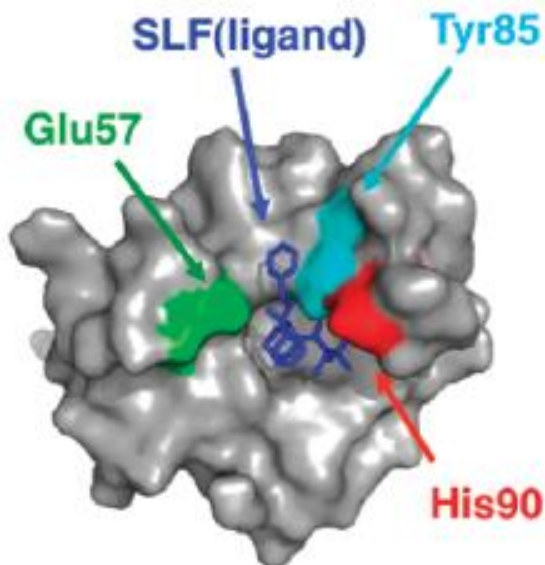
Ligand-directed selective bioconjugation

- Reactivity of reagent



entry	spacer	probe	yield
1	a	Dc	3%
2	b	Dc	19%
3	c	Dc	6%
4	b	Fl	21%
5	d	Fl	71%

- Site-selectivity of reagent

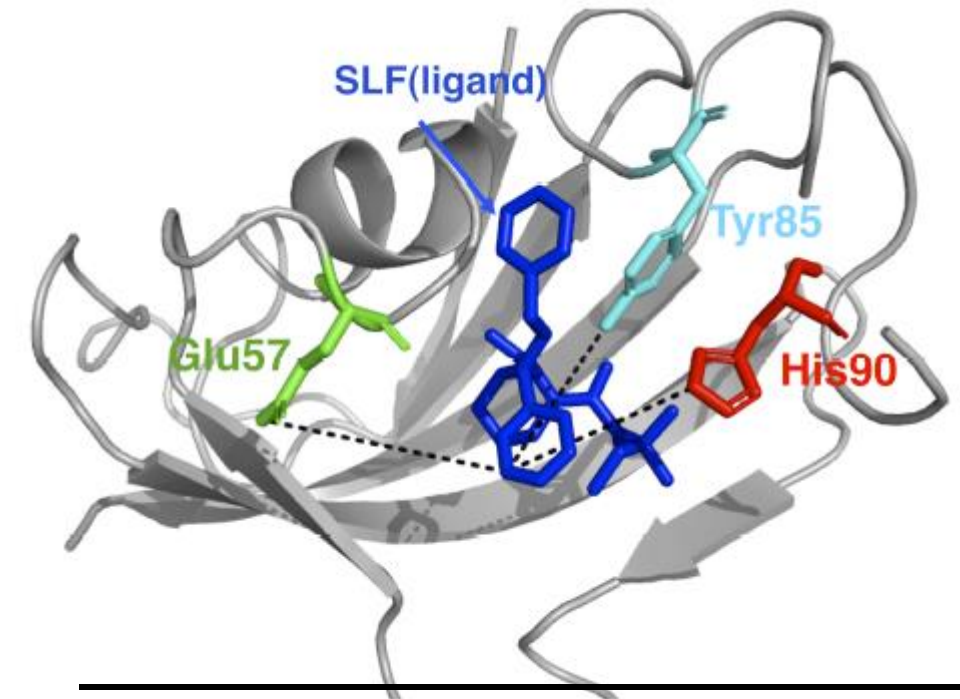


Entry	His90	Tyr85	Glu57
4	43%	4%	53%
5	4%	0%	96%

⇒ Spacer D increases reactivity and site-selectivity

Ligand-directed selective bioconjugation

- Consideration of high reactivity and site-selectivity



- ◆ Length of spacers b, d is close to the distance between ligand and target amino acids
- ◆ Piperazine type spacer may fix probe towards Glu57

Labeling site	Distance from ligand
His90	8.51 Å
Tyr85	6.74 Å
Glu57	6.88 Å

⇒ Hard spacer with appropriate length is important to reactivity and selectivity

Ligand-directed selective bioconjugation

Binding of ligand and active site accelerate bioconjugation reaction

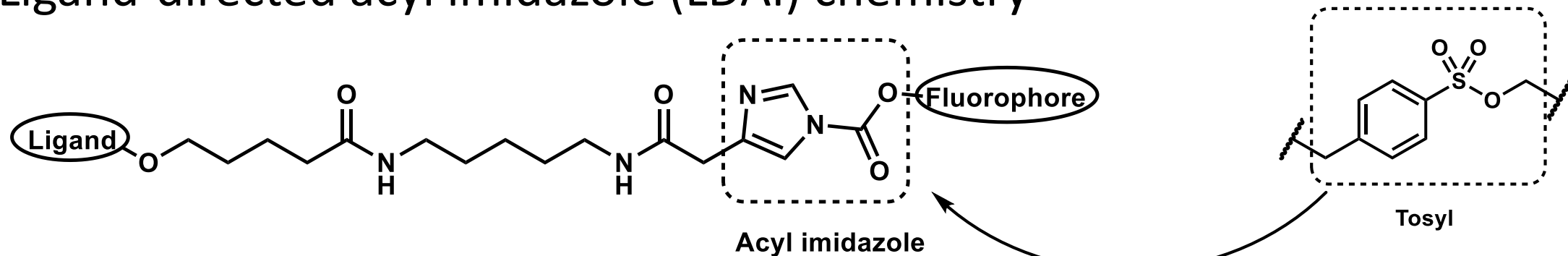
- Problem

- ◆ Slow reaction rate and low labeling efficiency of ligand-directed tosylate chemistry

⇒ More reactive reagent

Ligand-directed selective bioconjugation

- Ligand-directed acyl imidazole (LDAI) chemistry

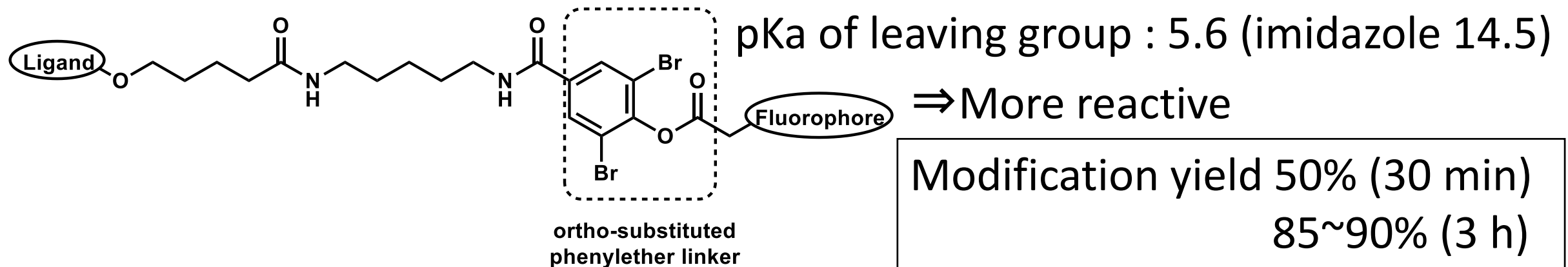


Acyl transfer is faster than S_N2 reaction?

Much more reactive than LDT reagent

Matsuo. K. *et al.*, *Chem. Sci.*, **2013**, 4, 2573

- Ligand-directed dibromophenyl benzoate (LDBB) chemistry



Modification yield 50% (30 min)
85~90% (3 h)

Takaoka. J. *et al.*, *Chem. Sci.*, **2015**, 6, 3217

Ligand-directed selective bioconjugation

- Problem

- ◆ Modification point is limited to around active site

indicated that the original molecular recognition ability of CAII is fully retained.

(S. Tsukiji, *et al.* *J. Am. Chem. Soc.*, **2009**, 131, 9046)

⇒ Not applicable to modification of amino acids away from active site

- Next step

- ◆ Recognition of structure of protein surface other than the active site

Topics

1. Introduction
2. Importance control of bioconjugation
3. Conventional protein modification
4. Site-selective bioconjugation strategy
 - π -clamp-mediated cysteine bioconjugation
 - Ligand-directed selective bioconjugation
5. **Summary**

Summary

- ◆ Importance of modification location and number of the protein
- Resent study
- ◆ Transferring the microenvironment of the protein surface to the site-selectivity of bioconjugation
 - π -clamp-mediated cysteine bioconjugation
 - Ligand-directed selective bioconjugation
- Next step
- ◆ Development of technique for recognition of protein surfaces