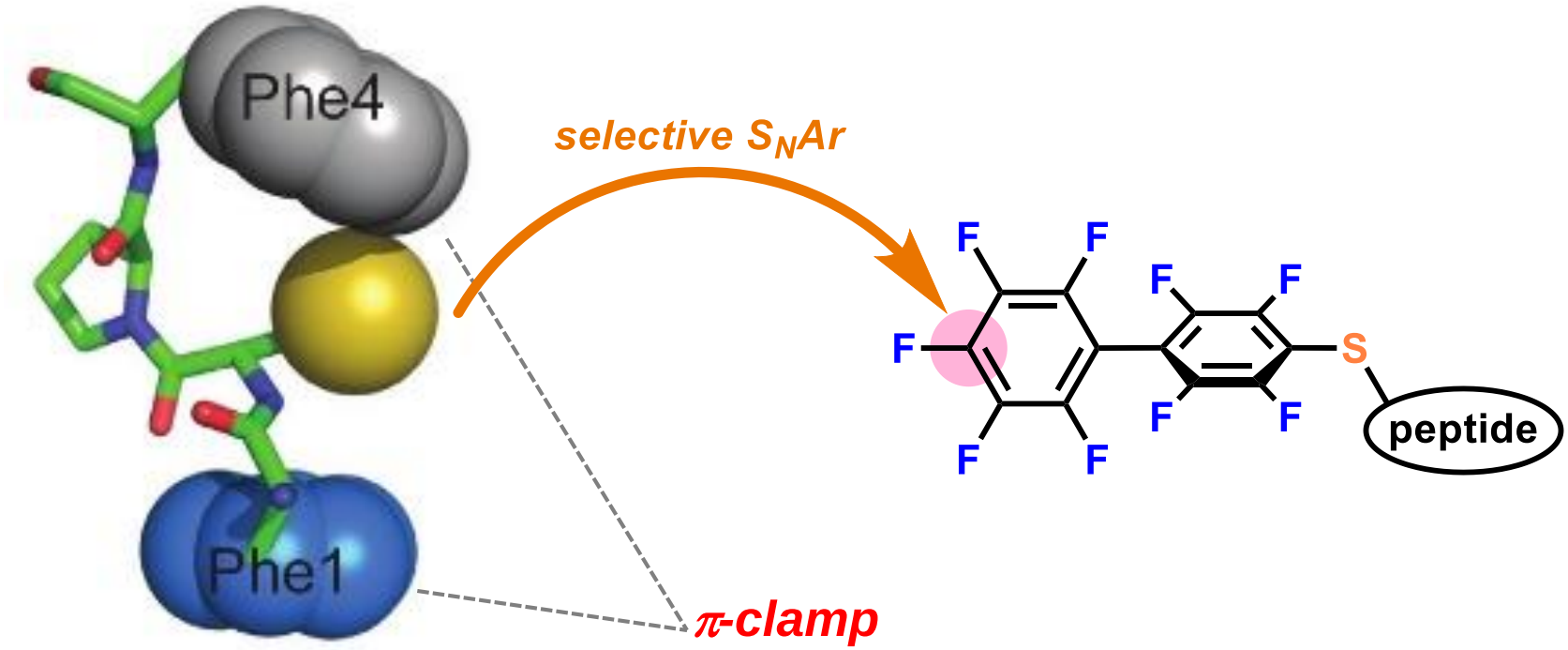
Protein bioconjugation using "π-clamp"

2016/6/4 Kotaro Tokumoto



Zhang, C.; Welborn, M.; Zhu, T.; Yang, N. J.; Santos, M. S.; Van Voorhis, T.; Pentelute, B. L. *Nat. Chem.* **2015**, *8*, 120.

contents

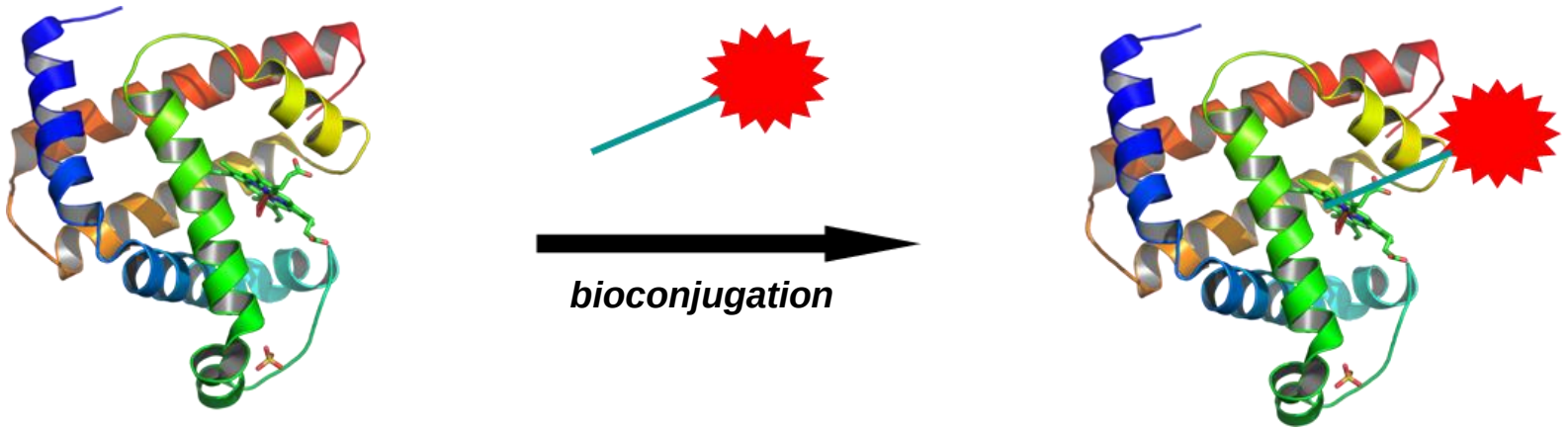
1. Introduction
   - Concept of bioconjugation
   - Motivation for bioconjugation

2. Existing methods for protein bioconjugation
   - Modification of Lysine, Tryptophan, Tyrosine and Cysteine
   - Modification of N-terminus
   - Modification using artificial amino acids
   - Problems in existing method

3. Main paper
   - Perfluoroaryl-cysteine S_NAr reaction
   - Discovery of π-clamp
   - Bioconjugation using π-clamp
   - Mechanistic study

1-1. Concept of bioconjugation

bioconjugation = "the covalent derivatization of biomolecules"

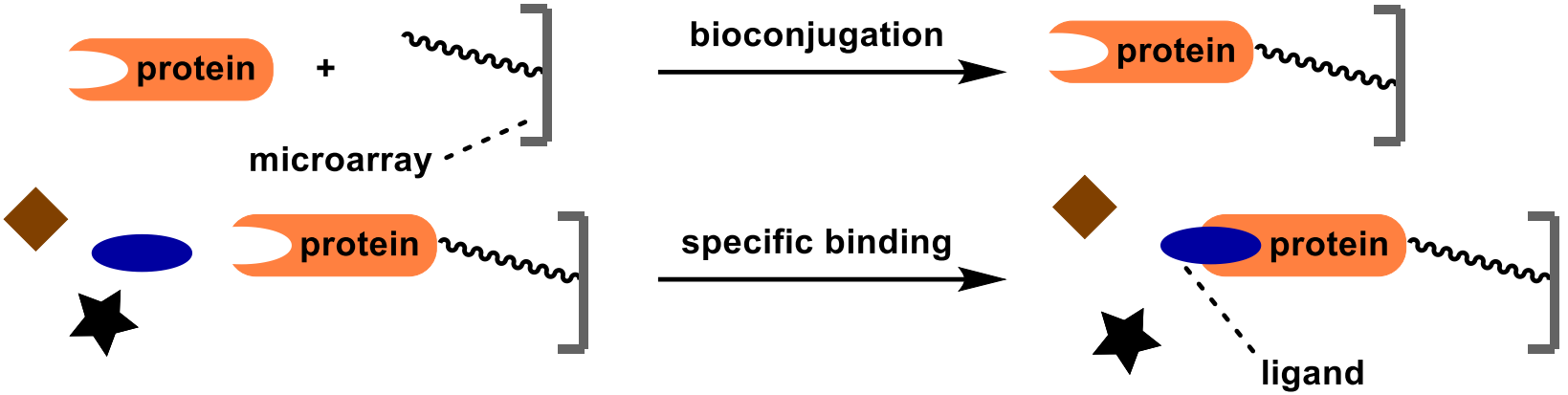


protein **derivatized protein**

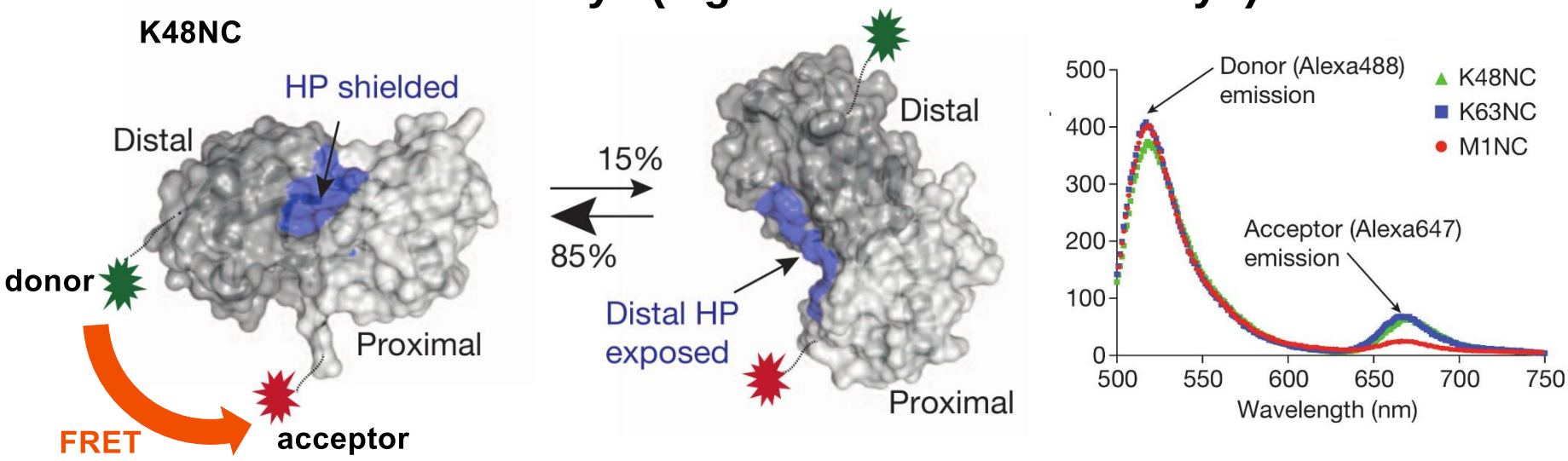
(a) Kalia, J.; Raines, R. *Curr. Org. Chem.* **2010**, *14*, 138.
(b) Stephanopoulos, N.; Francis, M. B. *Nat. Chem. Biol.* **2011**, *7*, 876.

1-2. Motivation for bioconjugation (1)

- discovery of biological interactions[1)]



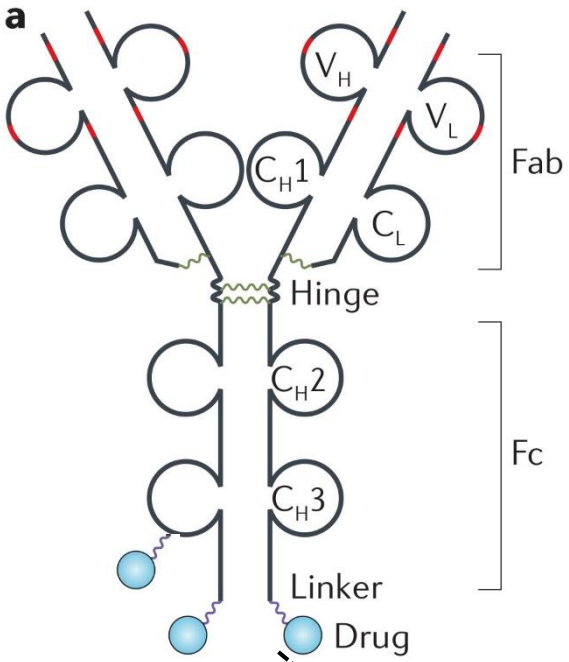
- biochemical assays (e.g. conformational study[2)])



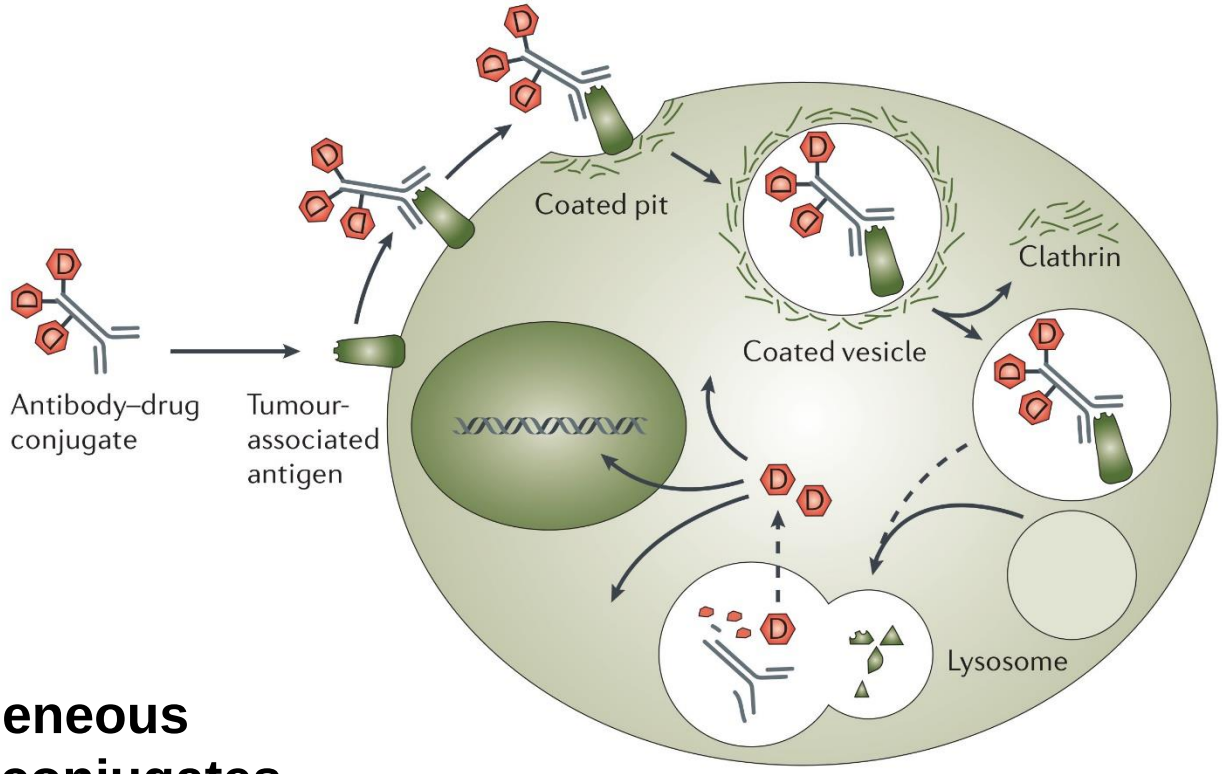
1) Jonkheijm, P.; Weinrich, D.; Schröder, H.; Niemeyer, C. M.; Waldmann, H. *Angew. Chem., Int. Ed.* **2008**, *47*, 9618.
2) Ye, Y.; Blaser, G.; Horrocks, M. H.; Ruedas-Rama, M. J.; Ibrahim, S.; Zhukov, A. A.; Orte, A.; Klenerman, D.; Jackson, S. E.; Komander, D. *Nature* **2012**, *492* , 266.

1-3. Motivation for bioconjugation (2)

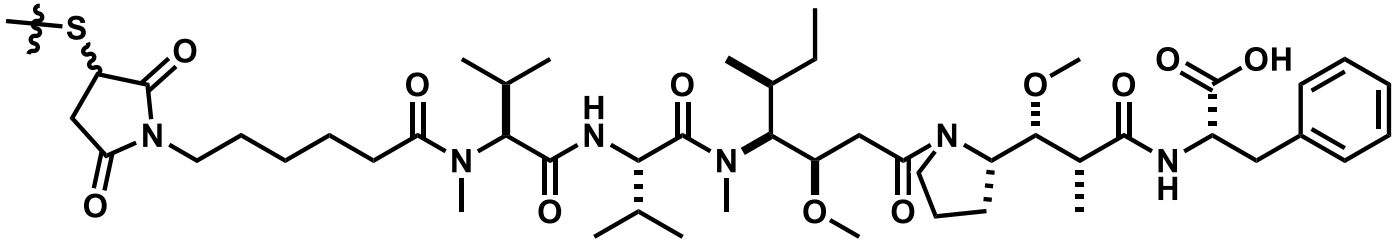
- **antibody-drug conjugate (ADC, 武装抗体)**



heterogeneous mixtures of conjugates



tumor-selective cytotoxicity



antibody-linked monomethyl auristatin F (MMAF)
tubulin inhibitor

Schrama, D.; Reisfeld, R. a; Becker, J. C. *Nat. Rev. Drug Discov.* **2006**, *5*, 147.

contents

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2. **Existing methods for protein bioconjugation**
   - **Modification of Lysine, Tryptophan, Tyrosine and Cysteine**
   - **Modification of N-terminus**
   - **Modification using artificial amino acids**
   - **Problems in existing method**

3. Main paper
   - Perfluoroaryl-cysteine S_NAr reaction
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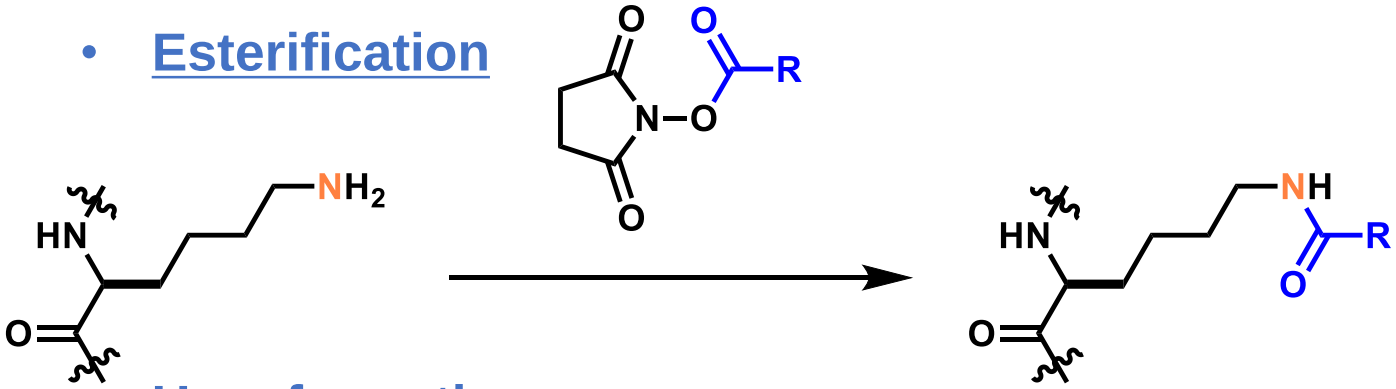
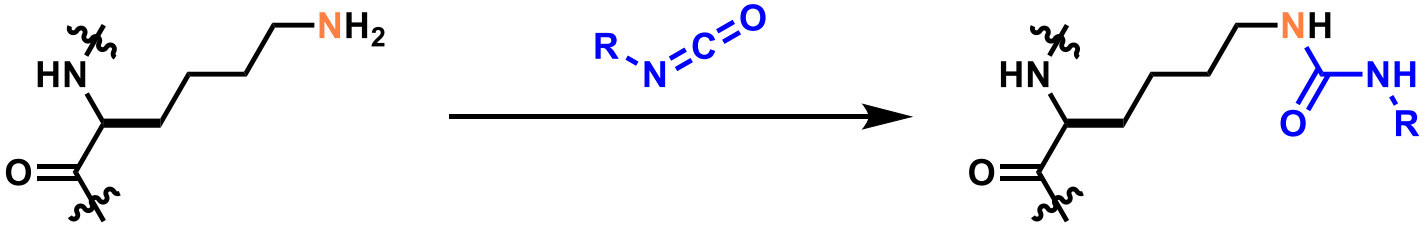
2-1. Modification of lysine

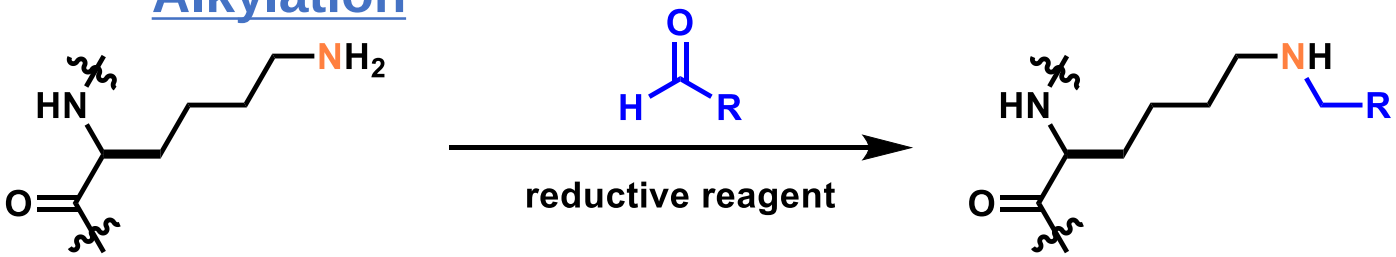
- **Esterification**



- **Urea formation**



- **Alkylation**

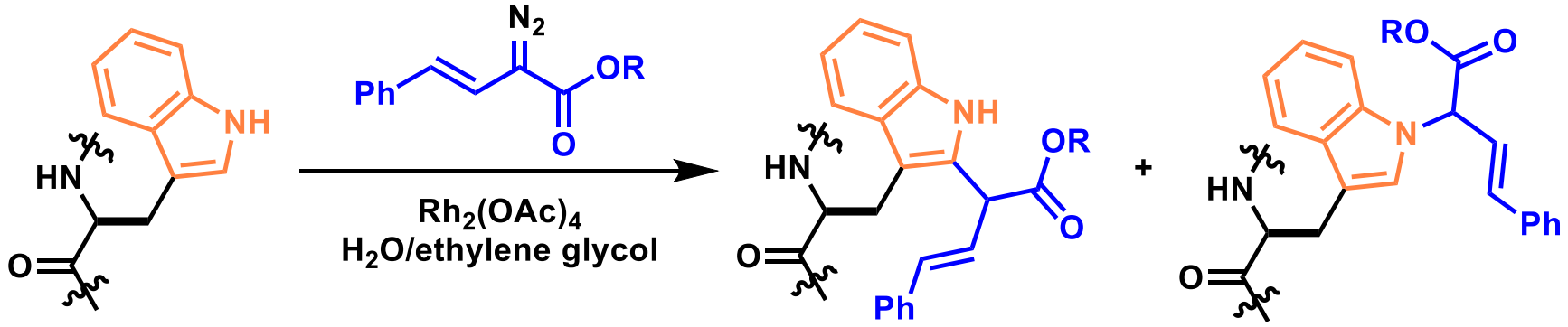


In most cases, control of the number of the reacted lysines is difficult.

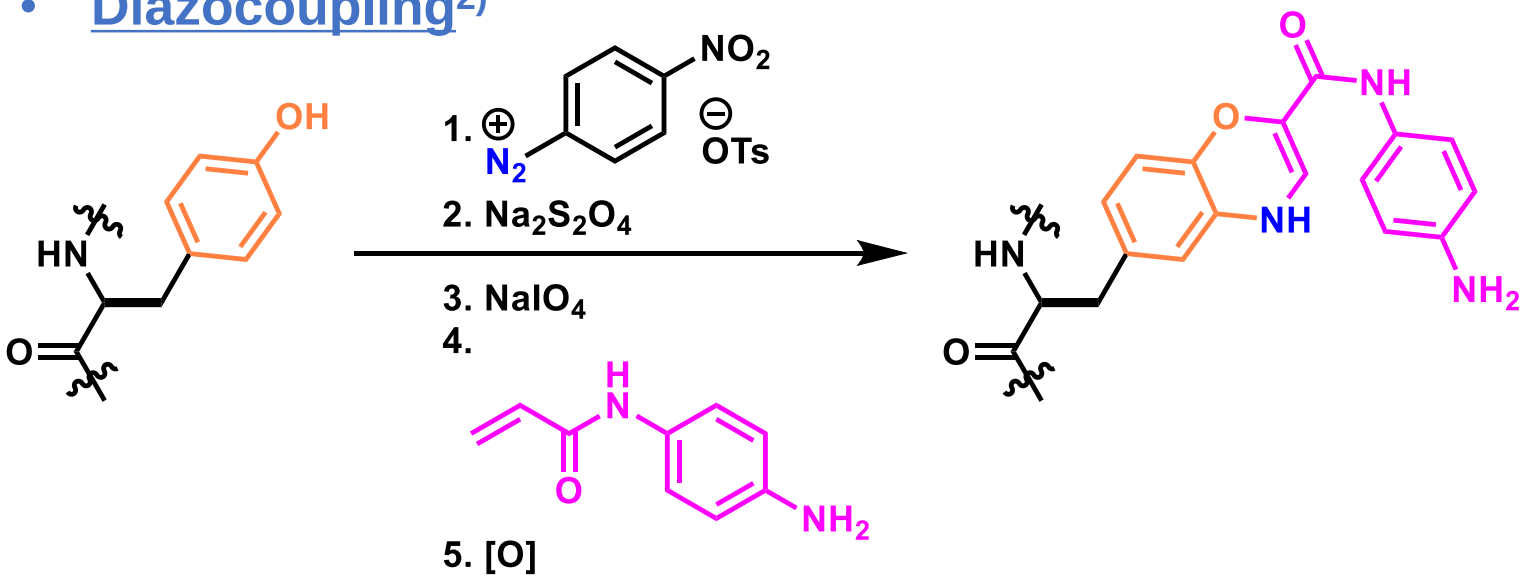
(a) Hermanson, G.T. *Bioconjugate Techniques*, 2nd edn. (Academic Press, 2008).
(b) Tilley, S.D., Joshi, N.S. & Francis, M.B. Proteins: chemistry and chemical reactivity. in *Wiley Encyclopedia of Chemical Biology* 1–16 (Wiley, 2008).

2-2. Modification of tryptophan and tyrosine

- **Metal carbenoid insertion**[1)]



- **Diazocoupling**[2)]



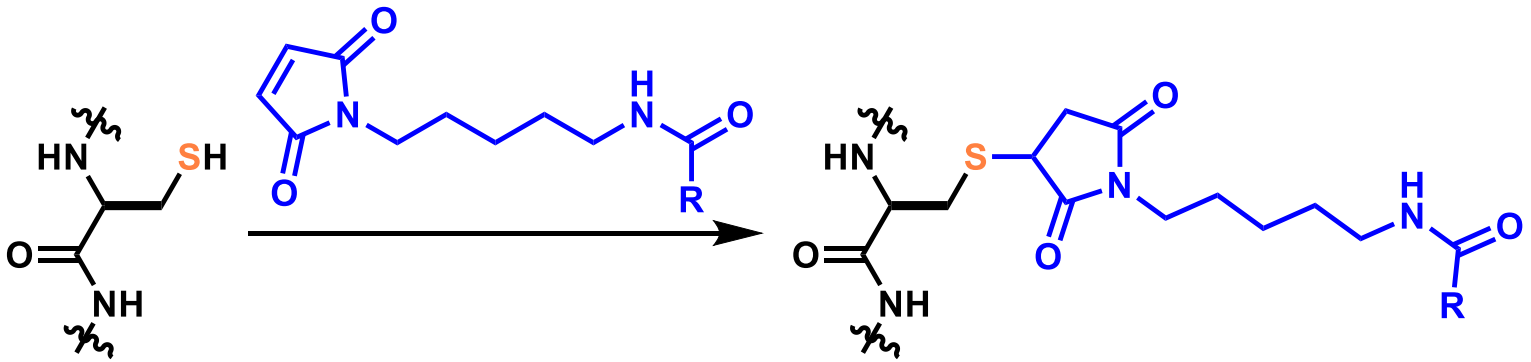
Harsh conditions.

Try and error are needed to conduct the reactions selectively.

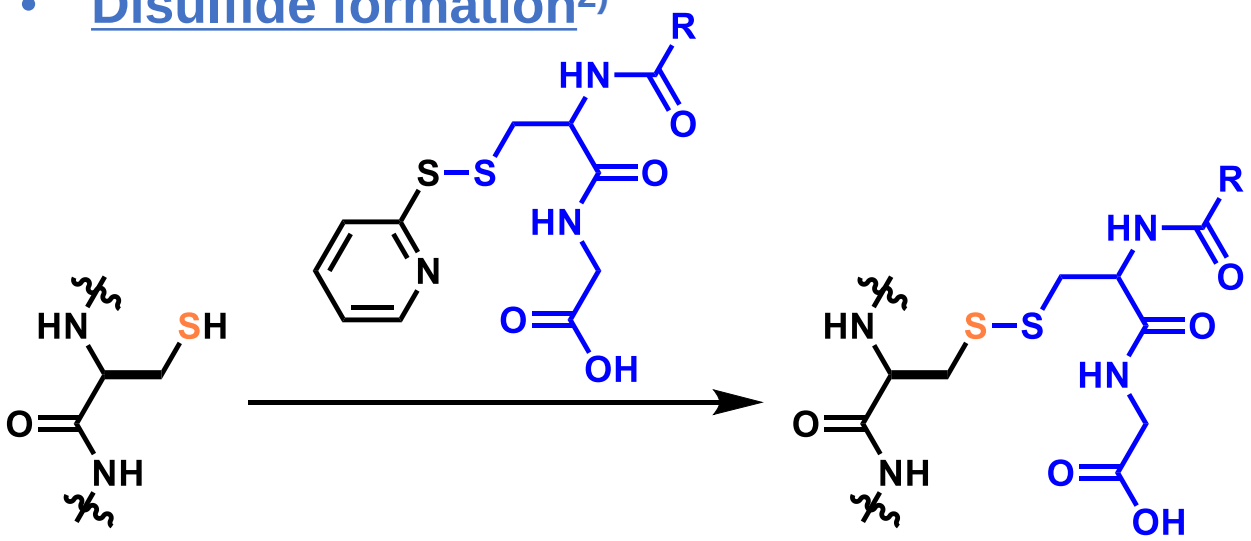
1) Antos, J. M.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 10256.
2) Hooker, J. M.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2004**, *126*, 3718.

2-3. Modification of cysteine (1)

- **Alkylation**[1)]



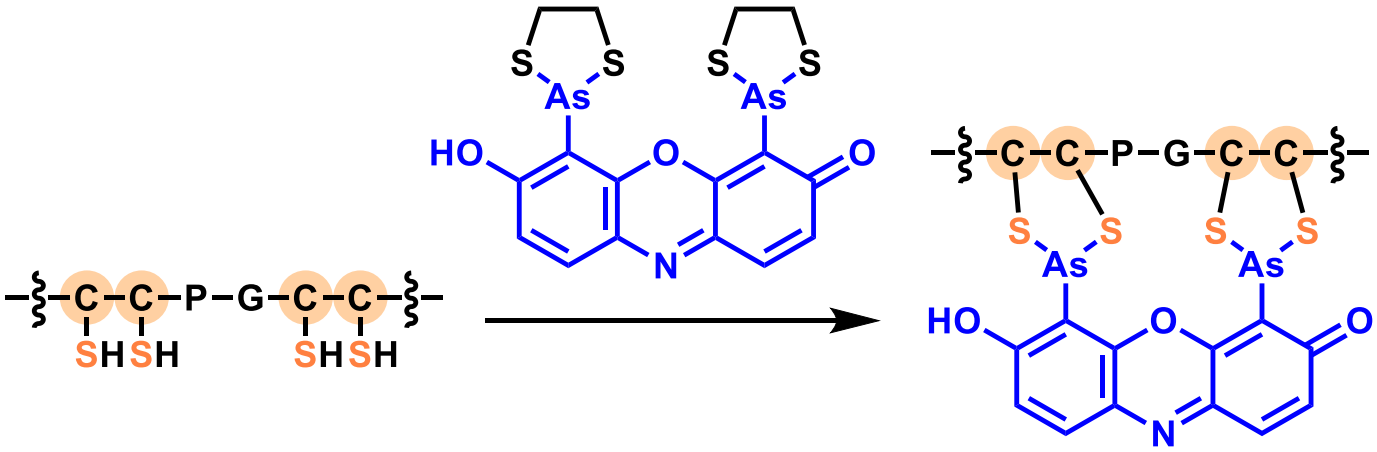
- **Disulfide formation**[2)]



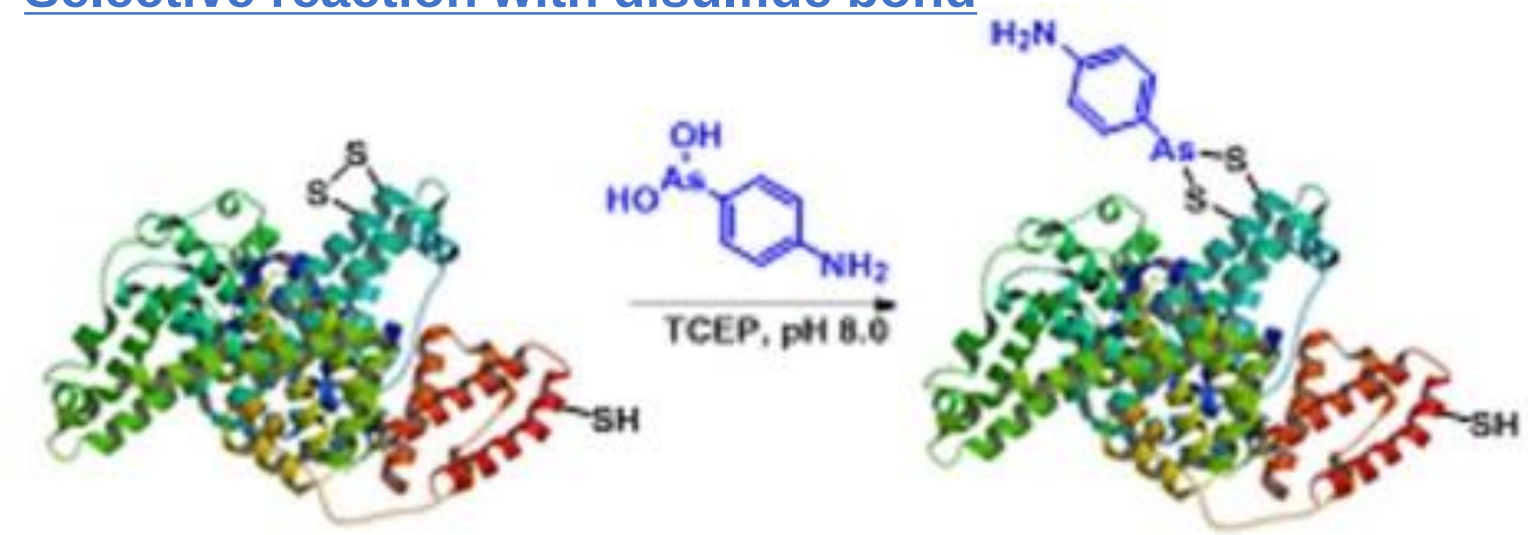
1) Ghosh, S. S.; Kao, P. M.; McCue, a W.; Chappelle, H. L. *Bioconjug. Chem.* **1990**, *1*, 71.
2) Ulbrich-Hofmann, R.; Arnold, U.; Mansfeld, J. *J. Mol. Catal. - B Enzym.* **1999**, *7*, 125.

2-4. Modification of cysteine (2)

- **Selective reaction with tetra-cysteine motifs**[1)]



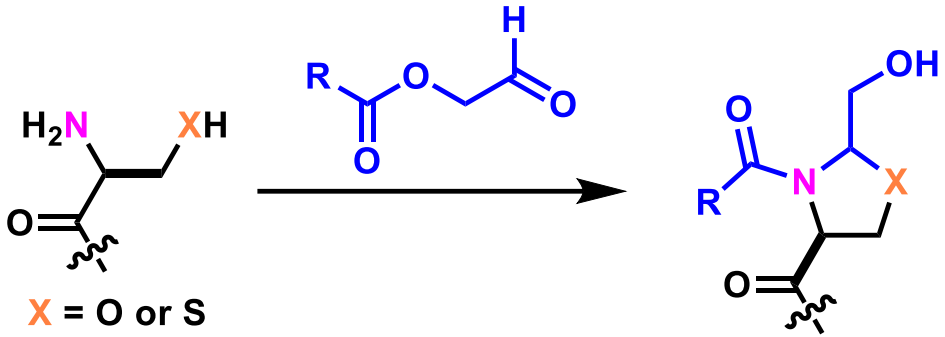
- **Selective reaction with disulfide bond**[2)]



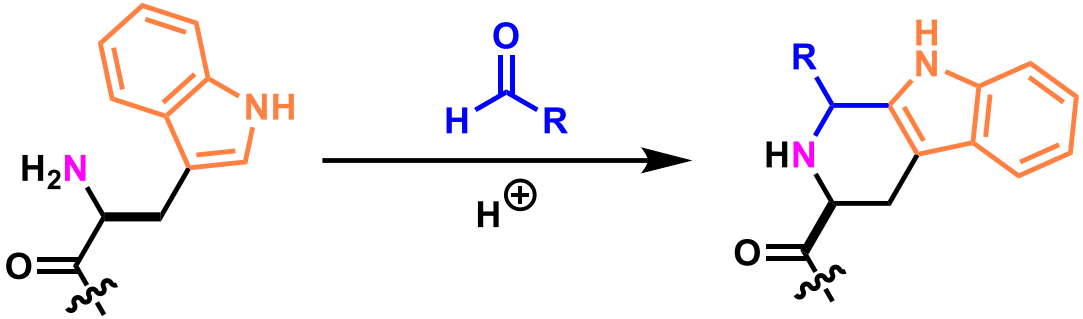
1) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063.
2) Wilson, P.; Anastasaki, A.; Owen, M. R.; Kempe, K.; Haddleton, D. M.; Mann, S. K.; Johnston, A. P. R.; Quinn, J. F.; Whittaker, M. R.; Hogg, P. J.; Davis, T. P. *J. Am. Chem. Soc.* **2015**, *137*, 4215.

2-5. Modification of N-terminal amino acids

- **Oxazolidine (thiazolidine) formation**[1)]



- **Pictet-Spengler reaction**[2)]

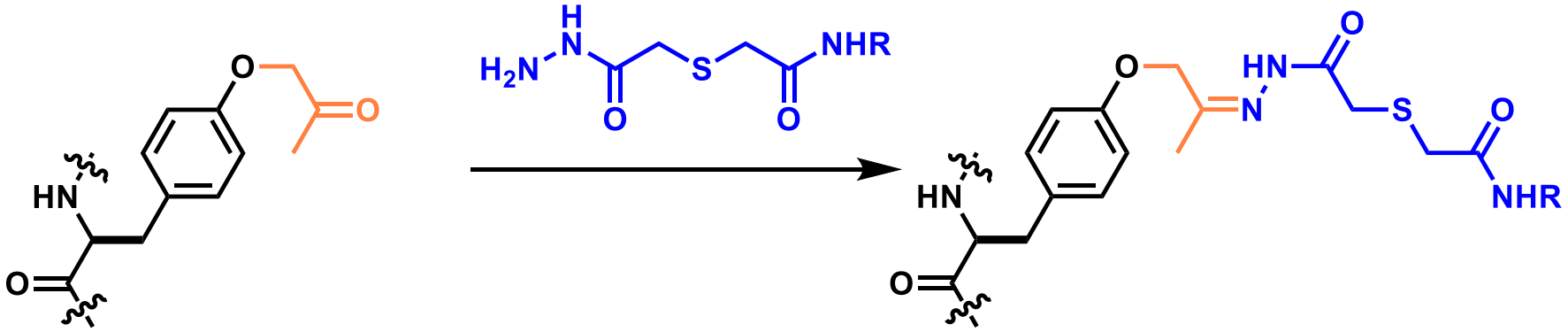


Unable to be used for N-terminally acylated proteins or other amino acids

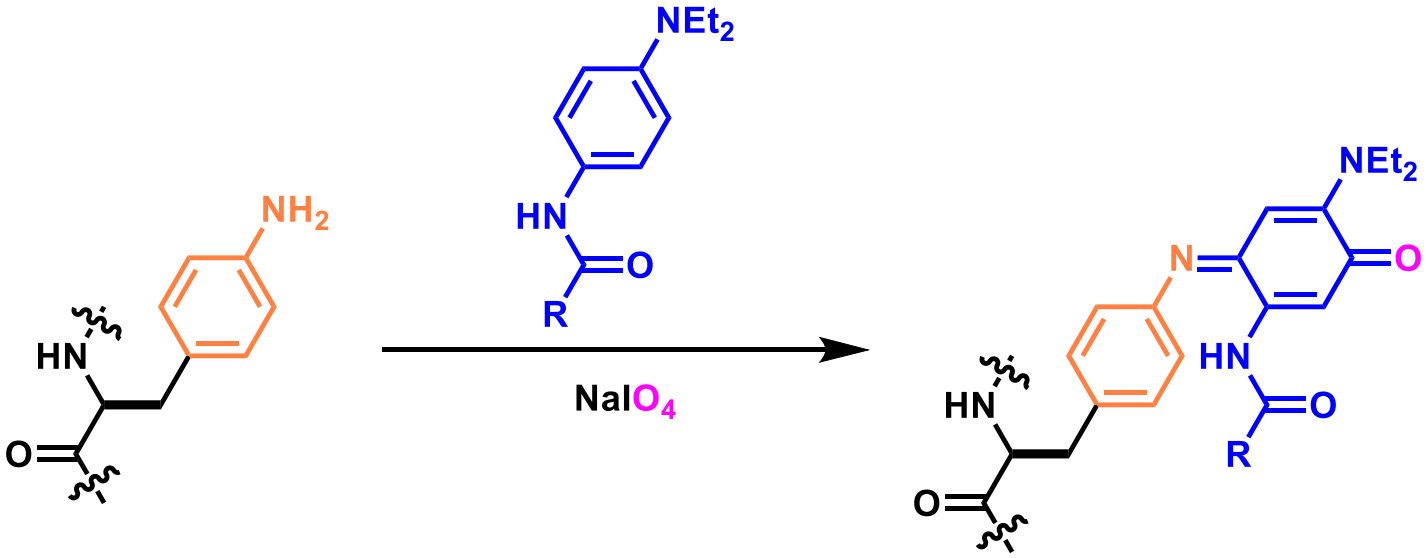
1) Tam, J. P.; Yu, Q.; Miao, Z. *Biopolymers* **1999**, *51*, 311.
2) Li, X.; Zhang, L.; Hall, S. E.; Tam, J. P. *Tetrahedron Lett.* **2000**, *41*, 4069.

2-6. Modification using artificial amino acids (1)

- **Hydrazone formation**[1)]



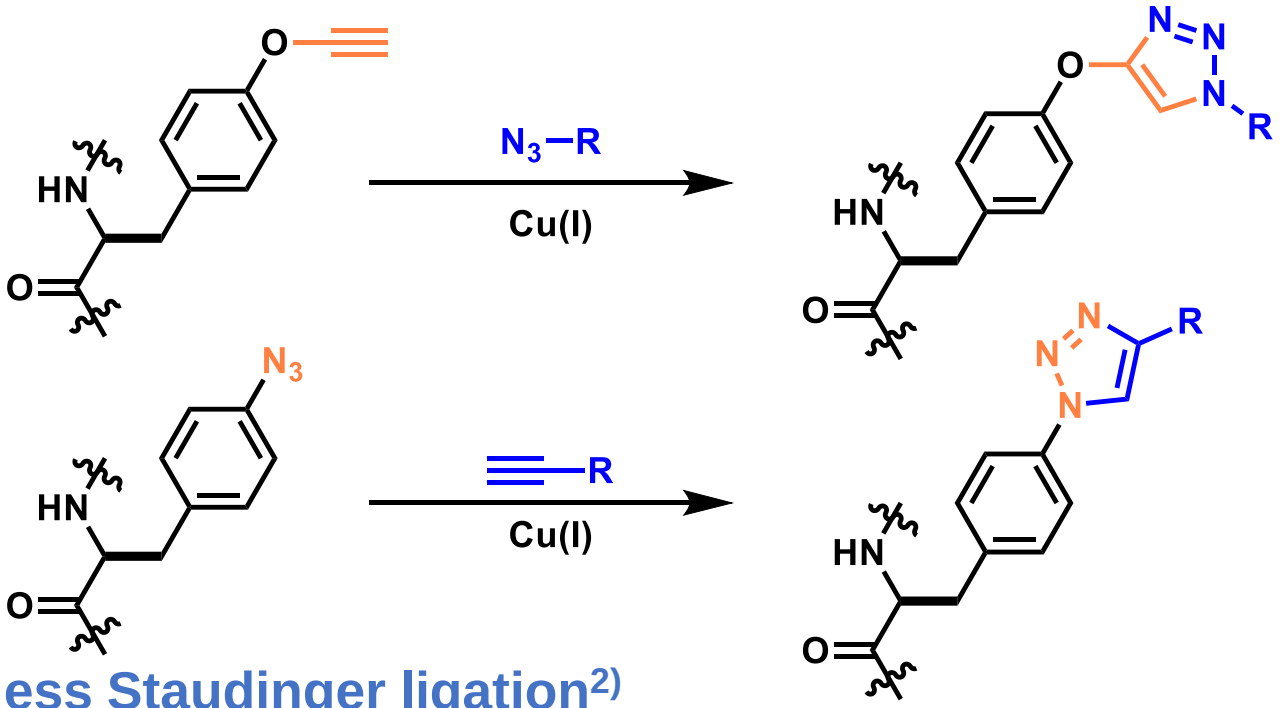
- **Oxidative coupling using aniline**[2)]



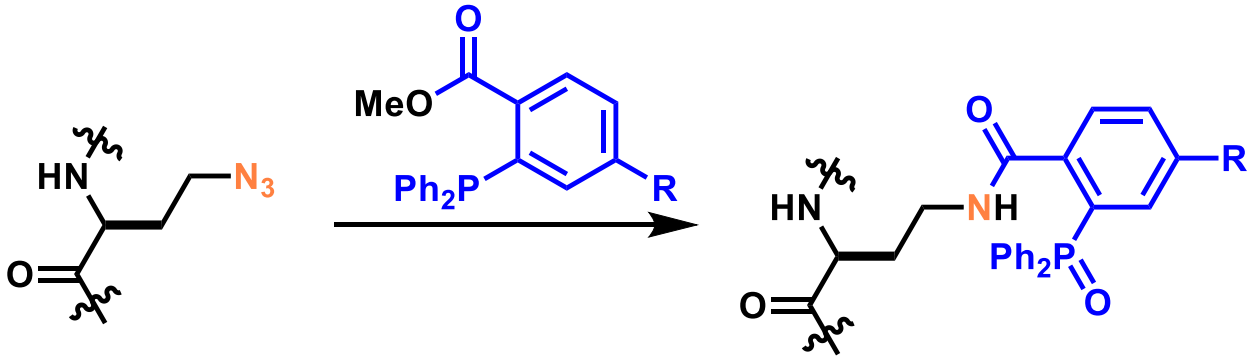
1) Cornish, V. W.; Hahn, K. M.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 8150.
2) Carrico, Z. M.; Romanini, D. W.; Mehl, R. A; Francis, M. B. *Chem. Commun. (Camb.)* **2008**, 1205.

2-7. Modification using artificial amino acids (2)

- **Click chemistry**[1)]



- **Traceless Staudinger ligation**[2)]



1) Deiters, A.; Cropp, T. A.; Mukherji, M.; Chin, J. W.; Anderson, J. C.; Schultz, P. G. *J. Am. Chem. Soc.* **2003**, *125*, 11782.
2) Kiick, K. L.; Saxon, E.; Tirrell, D. a; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 19.

2-8. Problems in existing methods

1. Lysine
   - ○ particularly general approach
   - × no site specificity
2. Tryptophan and Tyrosine
   - × harsh condition
     (Fine-tuning of the reaction condition is necessary.)
3. Cysteine
   - ○ relatively high site selectivity
   - × possible deactivation of protein
4. N-terminus
   - ○ possible chemoselective modification among many serine, cysteine or tryptophan residues
   - × requirement for free N-terminal amine
5. Artificial amino acid
   - ○ high site selectivity
   - × not genetically encodable amino acids

orthogonal reaction with encodable amino acids?

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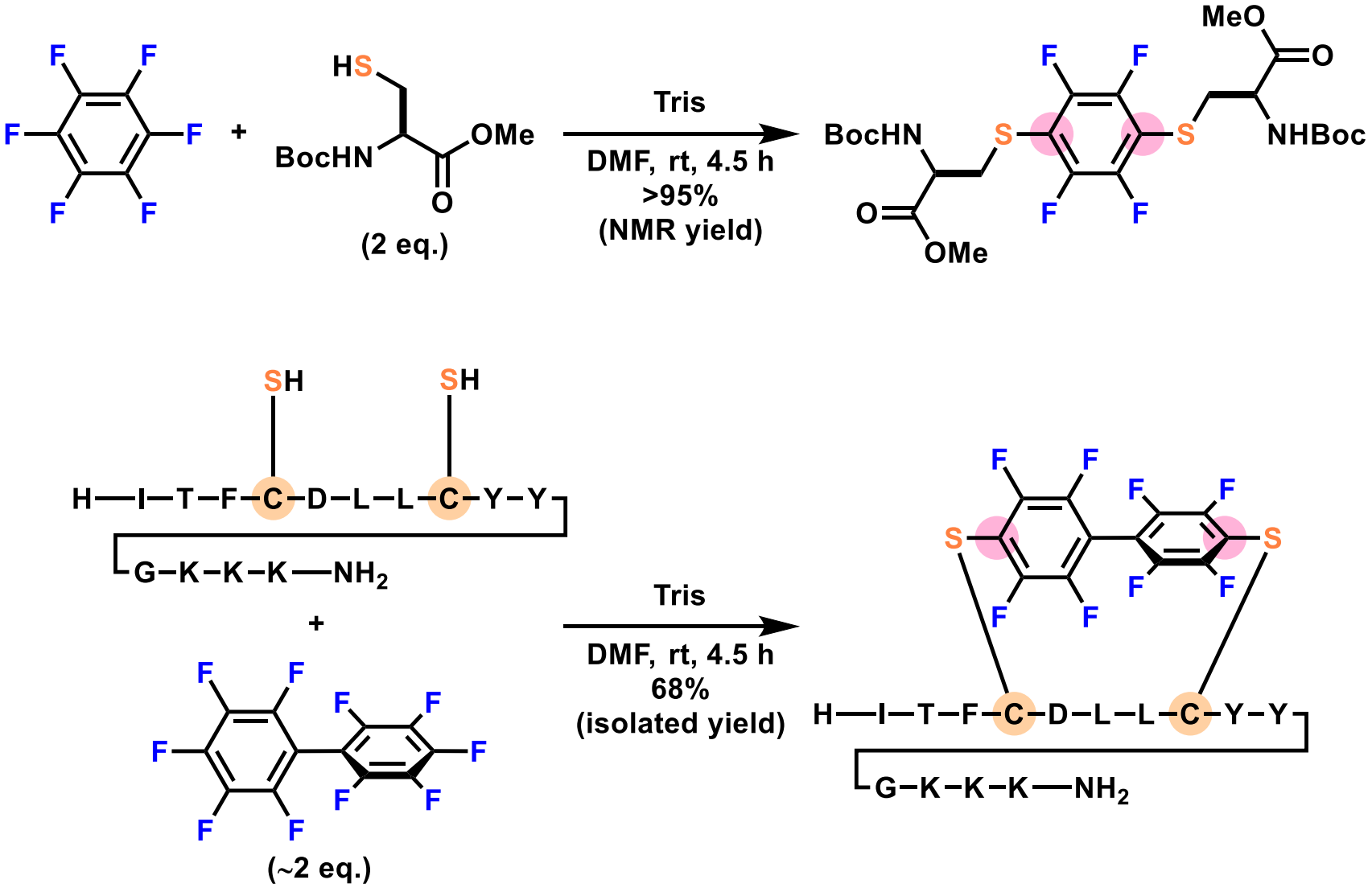
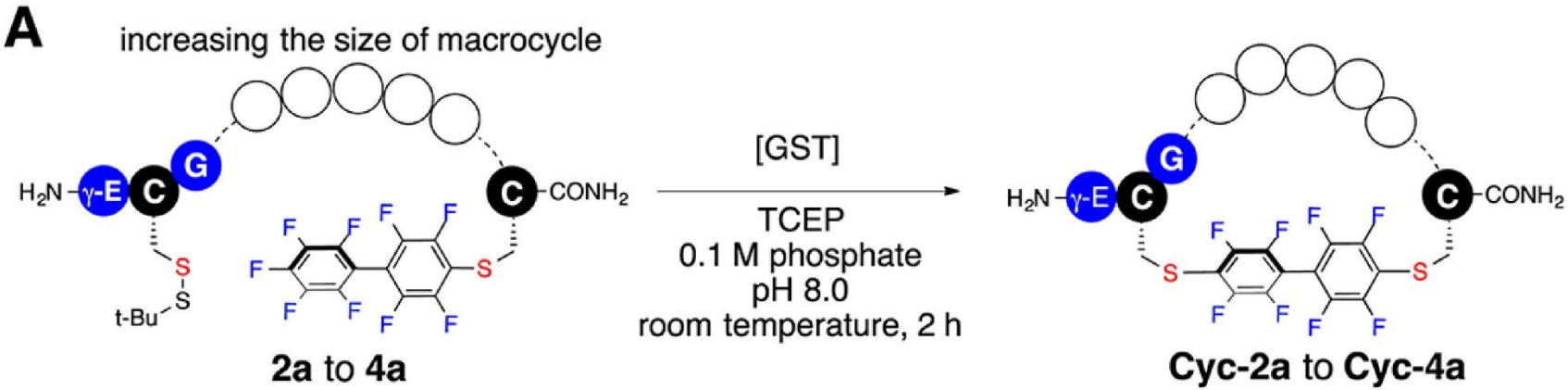
3. **Main paper**
   - **Perfluoroaryl-cysteine S_NAr reaction**
   - **Discovery of π-clamp**
   - **Bioconjugation using π-clamp**
   - **Mechanistic study**

3-1. Perfluoroaryl-cysteine S_NAr reaction



3-2. Reactivity in water

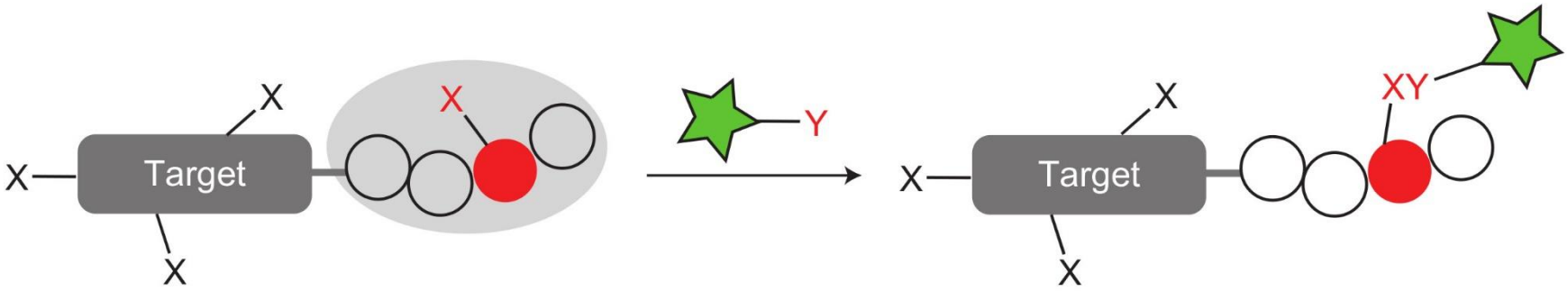


peptide	length (residues)	sequence	yield with enzyme (%)	yield without enzyme(%)
2a	14	NH_2-γ-EC(S-tBu)G-$(GLKAG)_2$-C*-$CONH_2$	93	4
3a	19	NH_2-γ-EC(S-tBu)G-$(GLKAG)_3$-C*-$CONH_2$	96	5
4a	24	NH_2-γ-EC(S-tBu)G-$(GLKAG)_4$-C*-$CONH_2$	96	8

GST = Glutathione *S*-transferase

S_NAr reaction is very sluggish in water without an enzyme

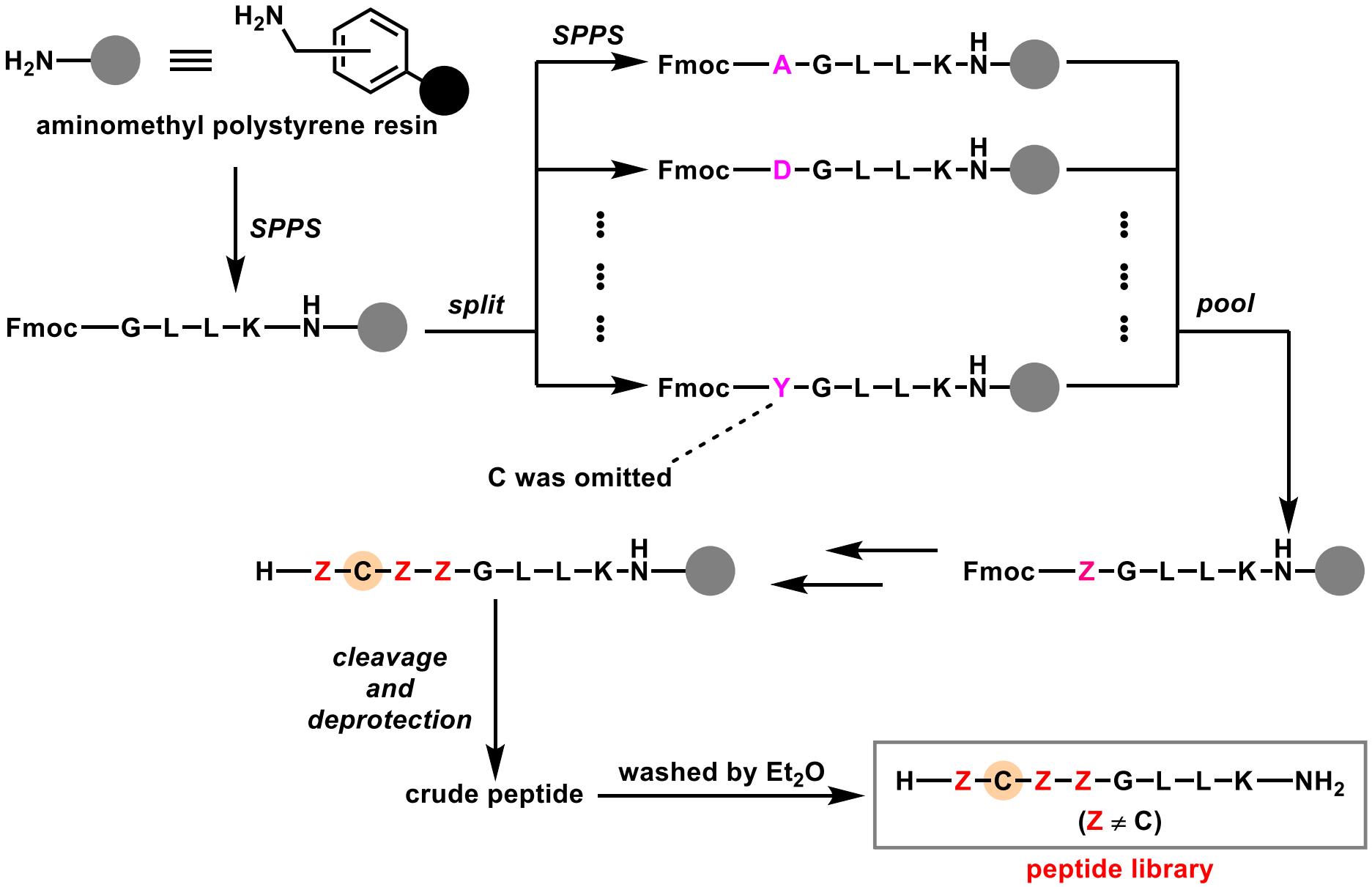
3-3. Design of the study



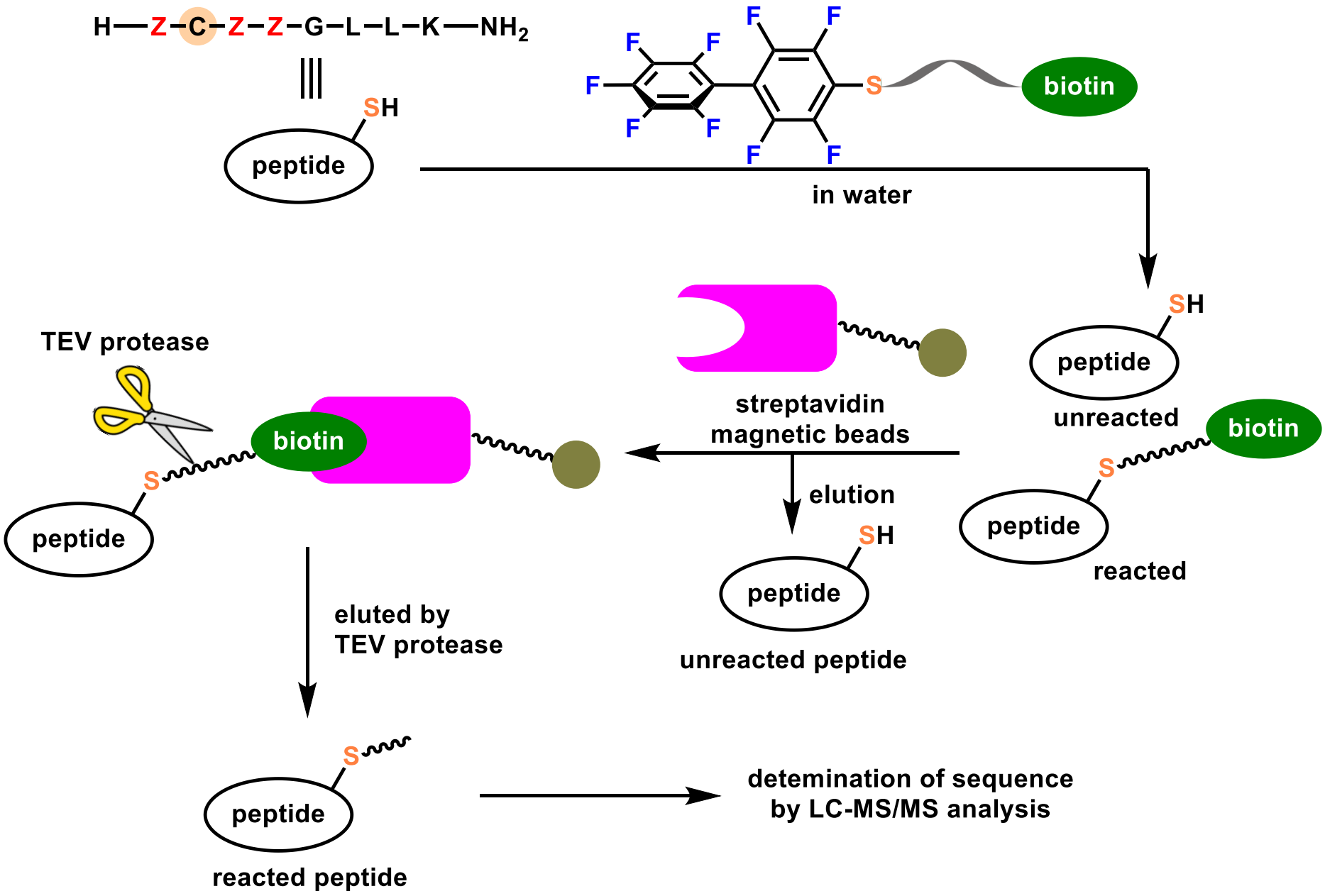
solvent	with an enzyme	without an enzyme
DMF	–	○
water	○	×

In water, the local environment around cysteine seems to be inappropriate for perfluoroarylation. The enzyme might change the local environment. Can the local environment be modified with the sequence around cysteine, without the enzyme?

3-4. Synthesis of peptide library



3-5. Identification of reaction products



3-6. Reacted sequences and hypothesis

H—F–C–I—I–G–L–L–K—NH_2

H—F–C–I—L–G–L–L–K—NH_2

H—F–C–I—M–G–L–L–K—NH_2

H—F–C–L—I–G–L–L–K—NH_2

H—F–C–L—L–G–L–L–K—NH_2

H—F–C–L—M–G–L–L–K—NH_2

H—F–C–P–W–G–L–L–K—NH_2

7 possible hits

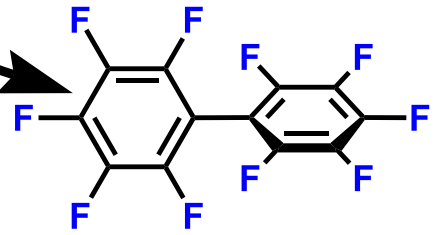
resynthesized and reactivity was tested

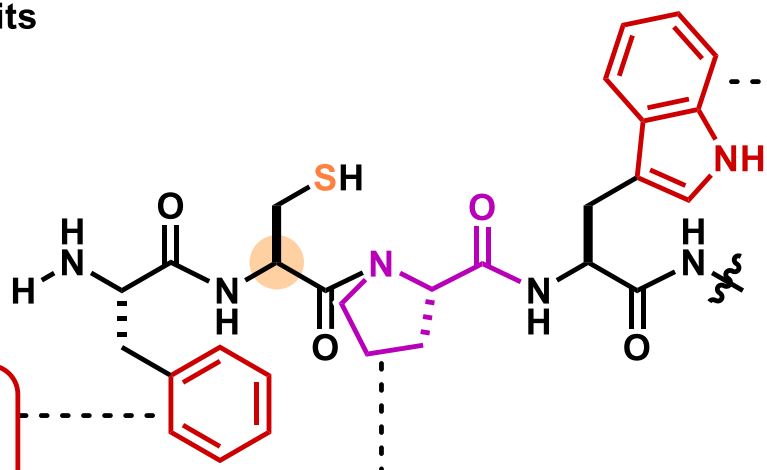
1 positive hit

H—F–C–P–W–G–L–L–K—NH_2

$\ominus$S

S_NAr in water





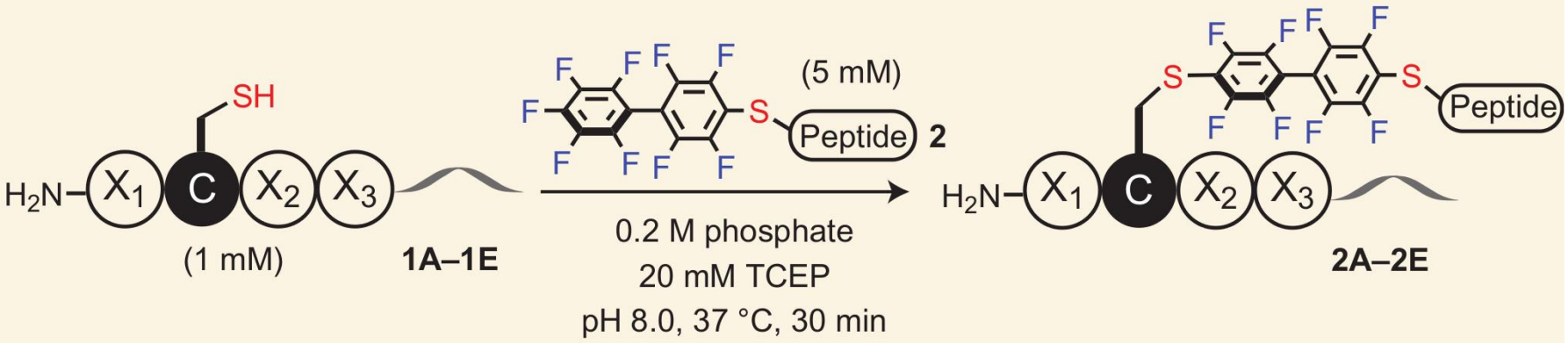
W→G: no reaction interacts with perfluoroaryl group?

F→G: low yield interacts with perfluoroaryl group?

F C P W

P→D-P: low yield positions C, F and W?

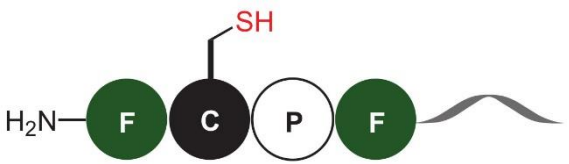
3-7. Mutation studies



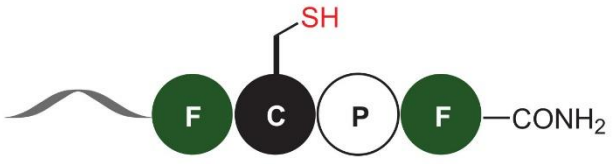
Entry	Peptide	X_1	X_2	X_3	k_2 (M^{-1} s^{-1})	Yield (%)
1	1A	Gly	Pro	Gly	N/A	<1
2	1B	Phe	Pro	Gly	N/A	<1
3	1C	Gly	Pro	Phe	0.09	50
4	1D	Phe	D-Pro	Phe	0.05	30
5	1E	Phe	Pro	Phe	0.73	>99

"π-clamp"

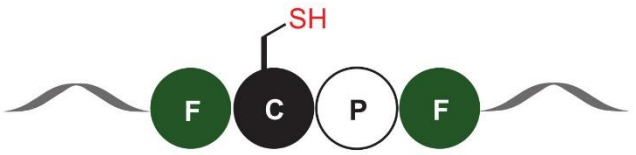
3-8. High reactivity of π-clamp



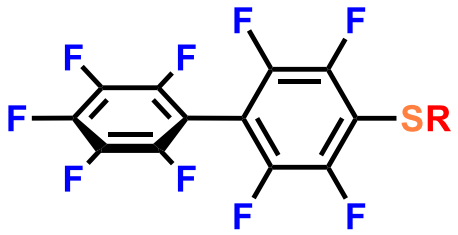
N-terminal π-clamp (**1E**)



C-terminal π-clamp (**1N**)



Middle π-clamp (**1O**)



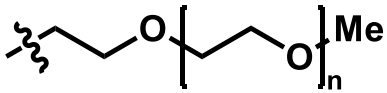
perfluoroaryl probes

2: R = peptide

3: R = biotin

4: R = FITC

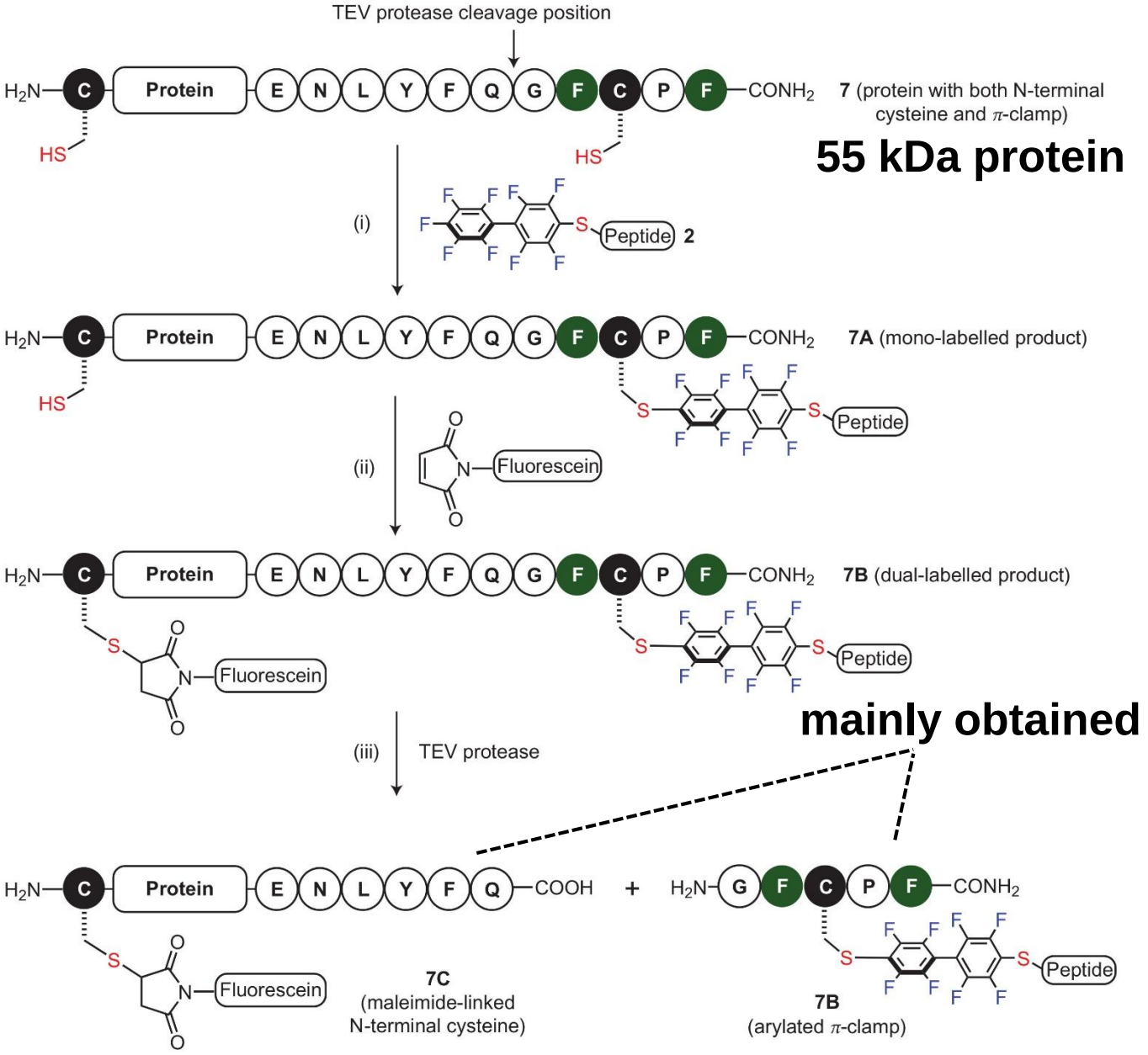
5: R =

6: R = 

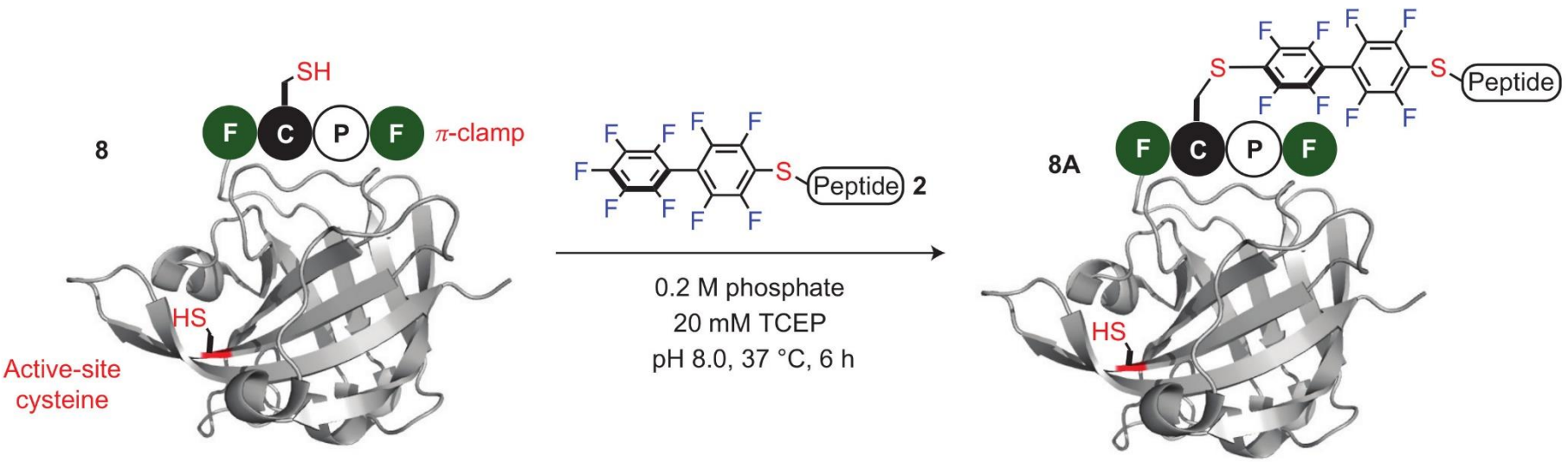
Entry	Peptide	Probe	Yield (%)
1	1E	2	99
2	1E	3	99
3	1E	4	81 (93*)
4	1E	5	99
5	1E	6	99
6	1N	2	92 (99*)
7	1N	3	99
8	1N	4	62 (80*)
9	1N	5	83 (97*)
10	1N	6	99
11	1O	2	99
12	1O	3	99
13	1O	4	86 (94*)
14	1O	5	99
15	1O	6	99

Yields shown are from LC-MS analysis of the crude reactions at 60 min. Lower reaction yields were observed for the FITC-linked probe **4**, potentially because of the low solubility of probe **4** in water. *Yields at 120 min. See Supplementary Figs 3–6 for LC-MS chromatograms.

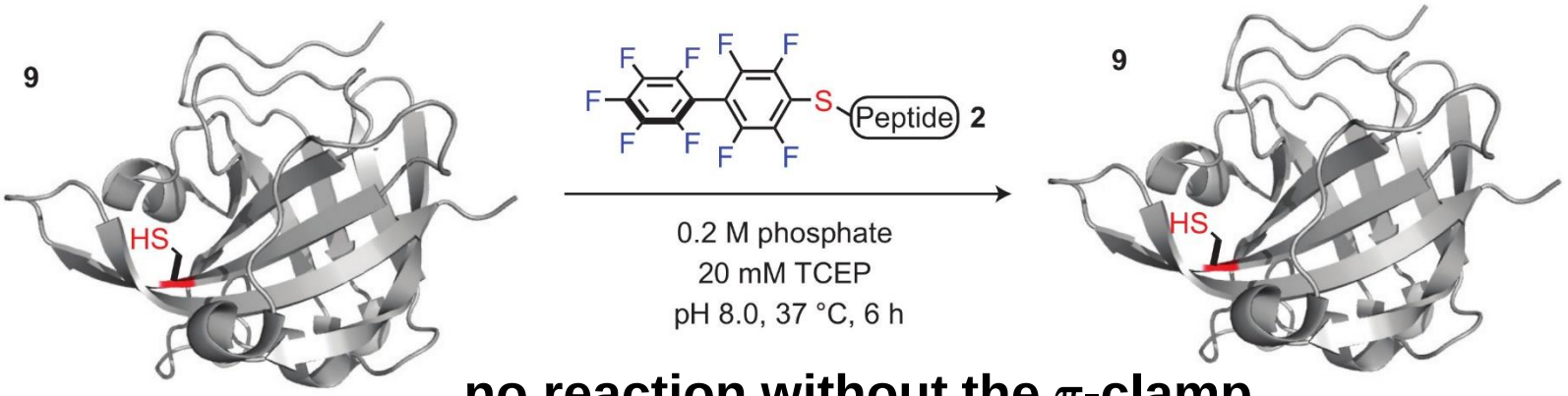
3-9. Siteselectivity in protein (1)



3-10. Siteselectivity in protein (2)

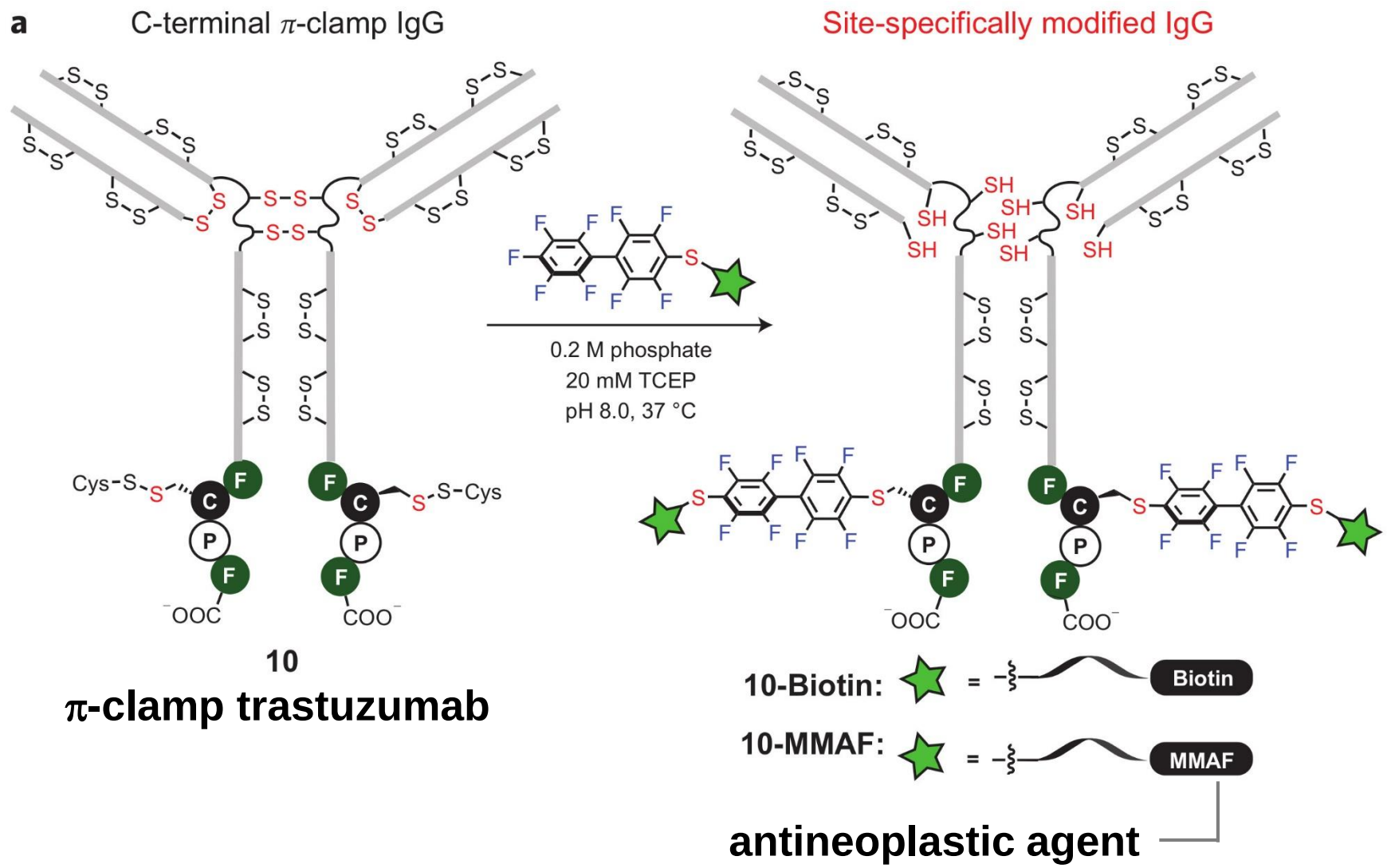


selective conjugation to the π-clamp



no reaction without the π-clamp

3-11. Antibody-drug conjugate using π-clamp



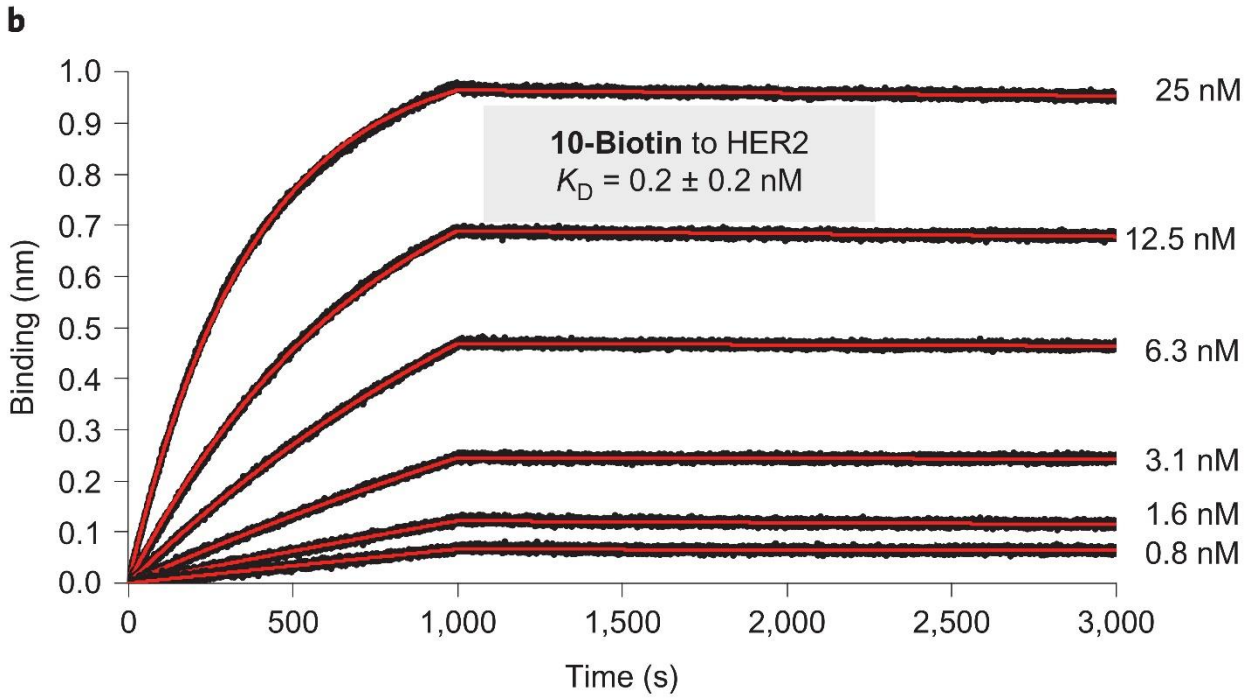
π-clamp trastuzumab

antineoplastic agent

trastuzumab ⋯ an antibody that binds to HER2

3-12. Affinity of biotin-conjugated trastuzumab

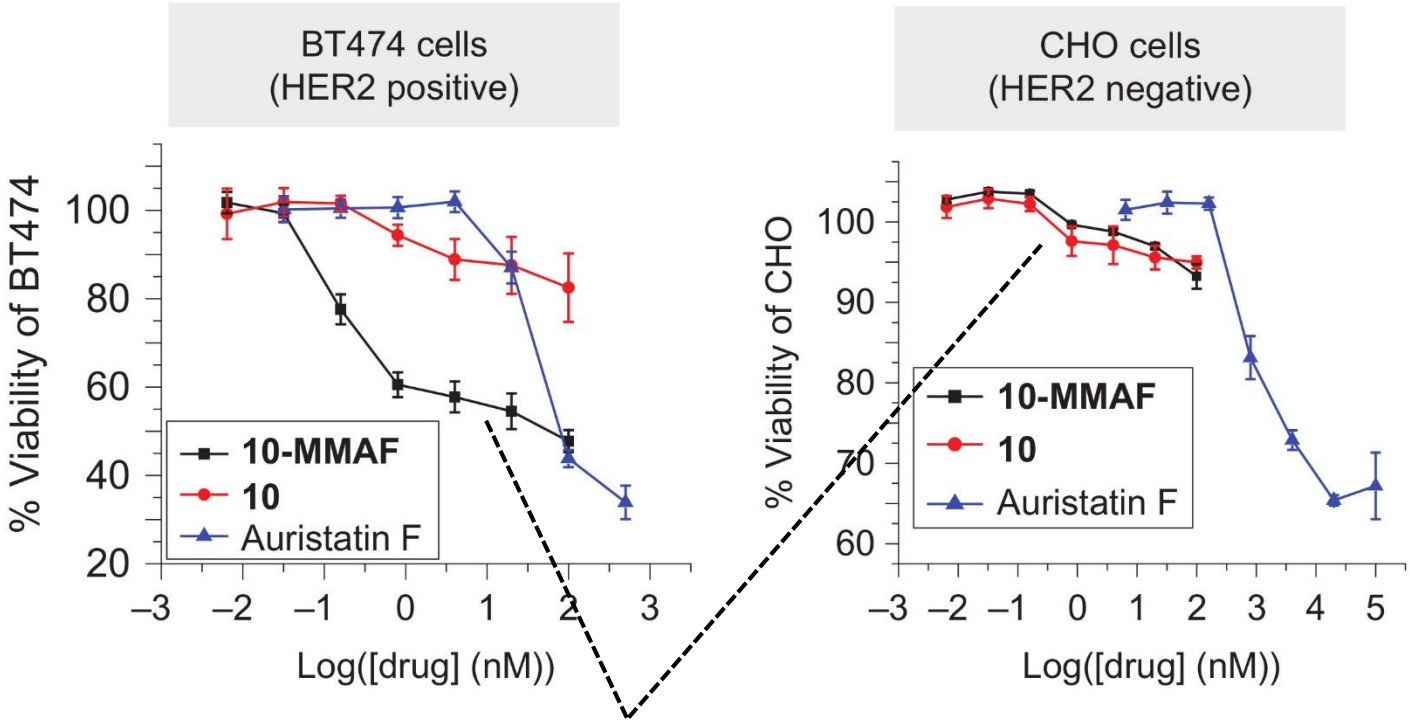
- binding assay to HER2



	K_D (nM)
trastuzumab[1)]	0.1
biotin-labeled π-clamp trastuzumab	0.2 ± 0.2

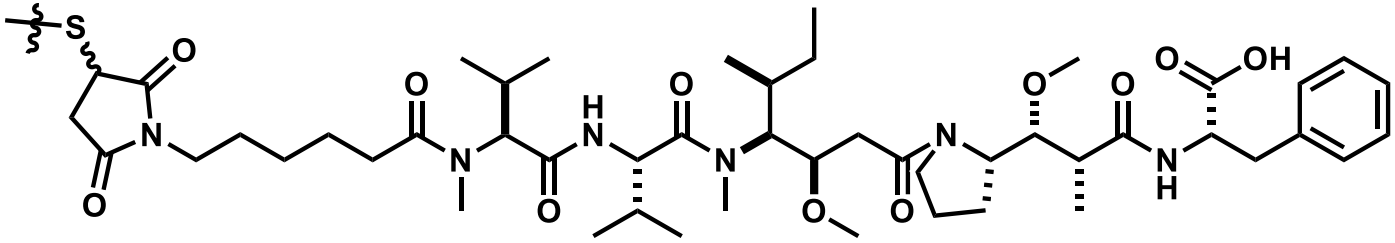
1) Baselga, J. *Ann. Oncol.* **2001**, *12 (suppl 1)*, S49–S55.

3-13. Cytotoxicity of MMAF-conjugated trastuzumab



selective cytotoxicity was observed

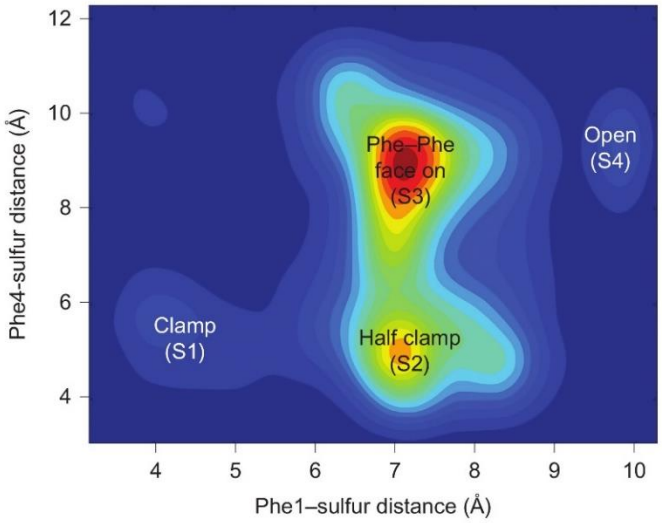
10-MMAF: MMAF-conjugated π-clamp trastuzumab

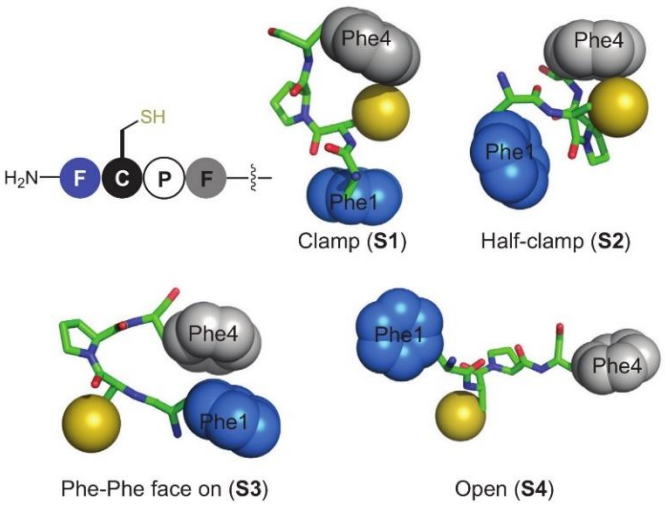


antibody-linked monomethyl auristatin F (MMAF)
tubulin inhibitor

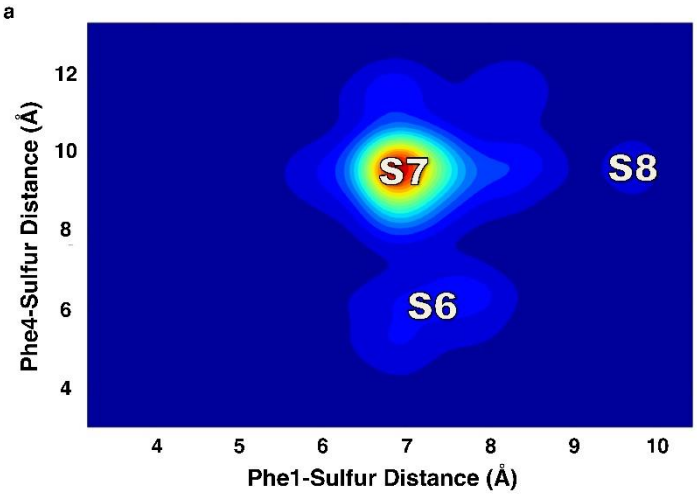
3-14. Mechanistic study (1)

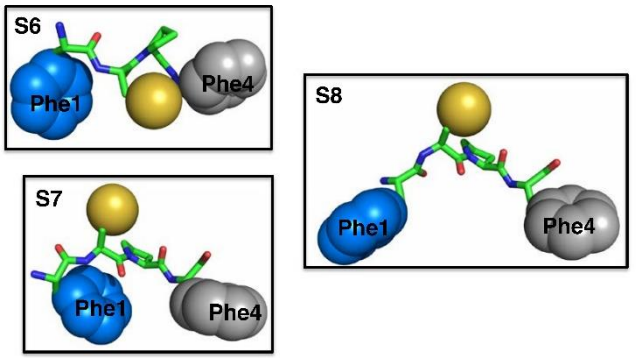
- ***cis*-proline**



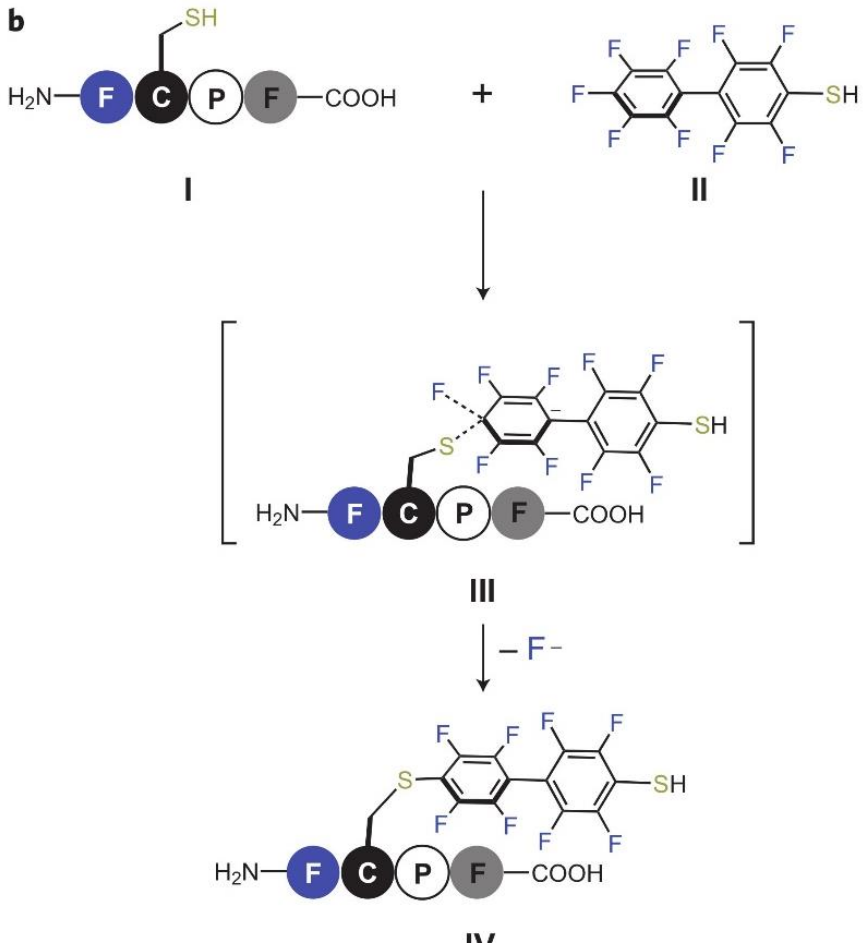


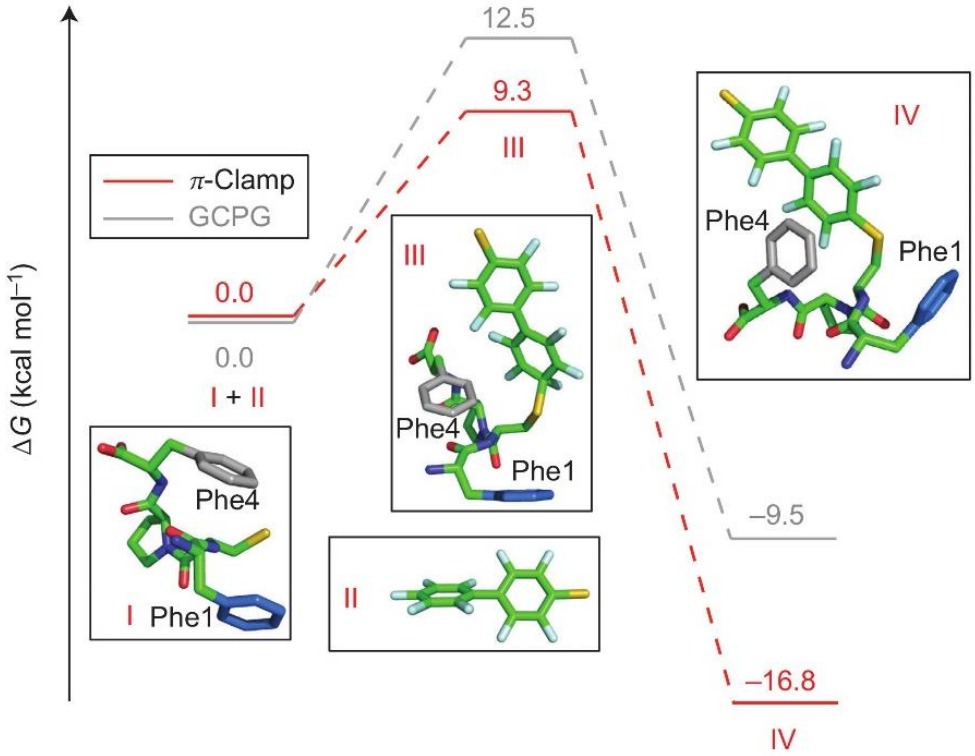
- ***trans*-proline**





3-15. Mechanistic study (2)





3-16. Advantages of π-clamp-mediated bioconjugation

1. **Small size**
   - **minimal structural perturbation to the target protein**

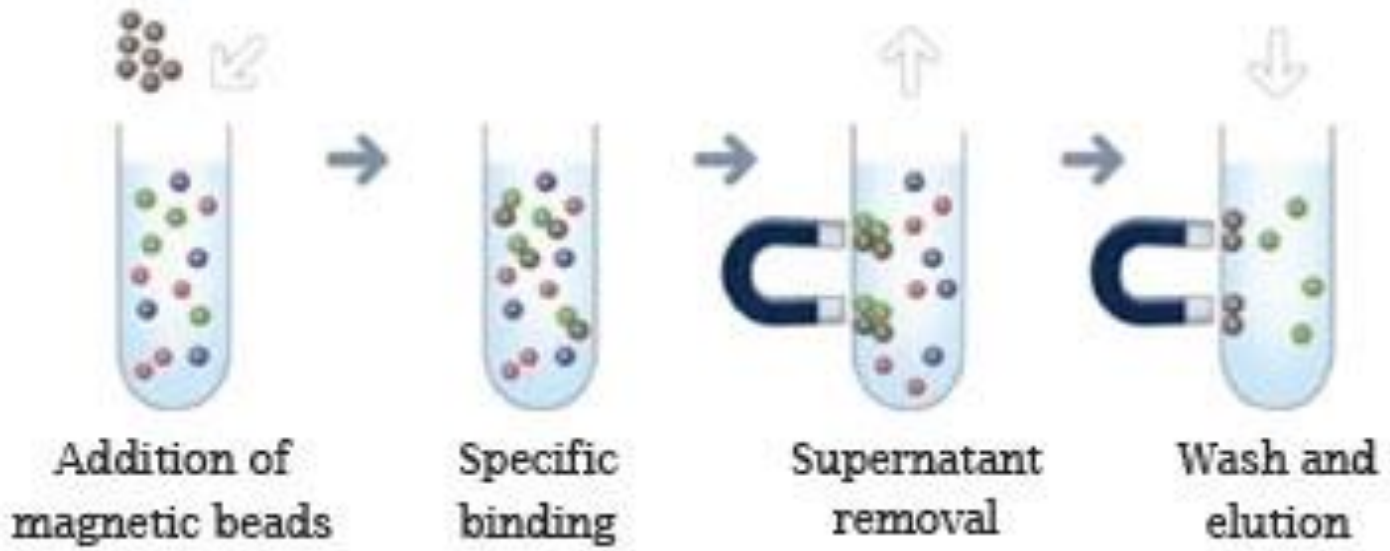
2. **Genetic encodability**
   - **easy preparation**

3. **Selective perfluoroarylation with other cysteines unreacted**
   - **minimal structural perturbation and deactivation**
   - **Ability to perform protecting-group-free dual cysteine modification**

Conclusion

- ✓ **A peptide sequence called "π-clamp" that promoted rapid perfluoroarylation of cysteine in water was discovered by screening library.**
- ✓ **The sequence fine-tuned the local environment and accelerated the reaction regardless of its position in the protein with other cysteines unreacted.**
- ✓ **Addition of the sequence scarcely changed the nature of the target protein.**
- ✓ **The target protein was successfully conjugated with functionalized perfluoroaryl compound.**

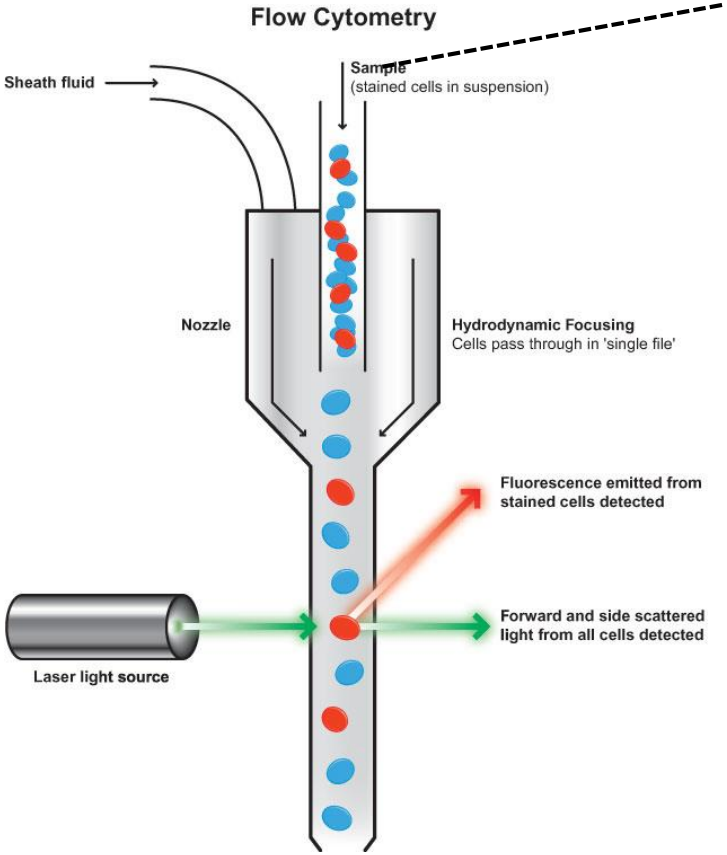
A-1. Magnetic beads separation



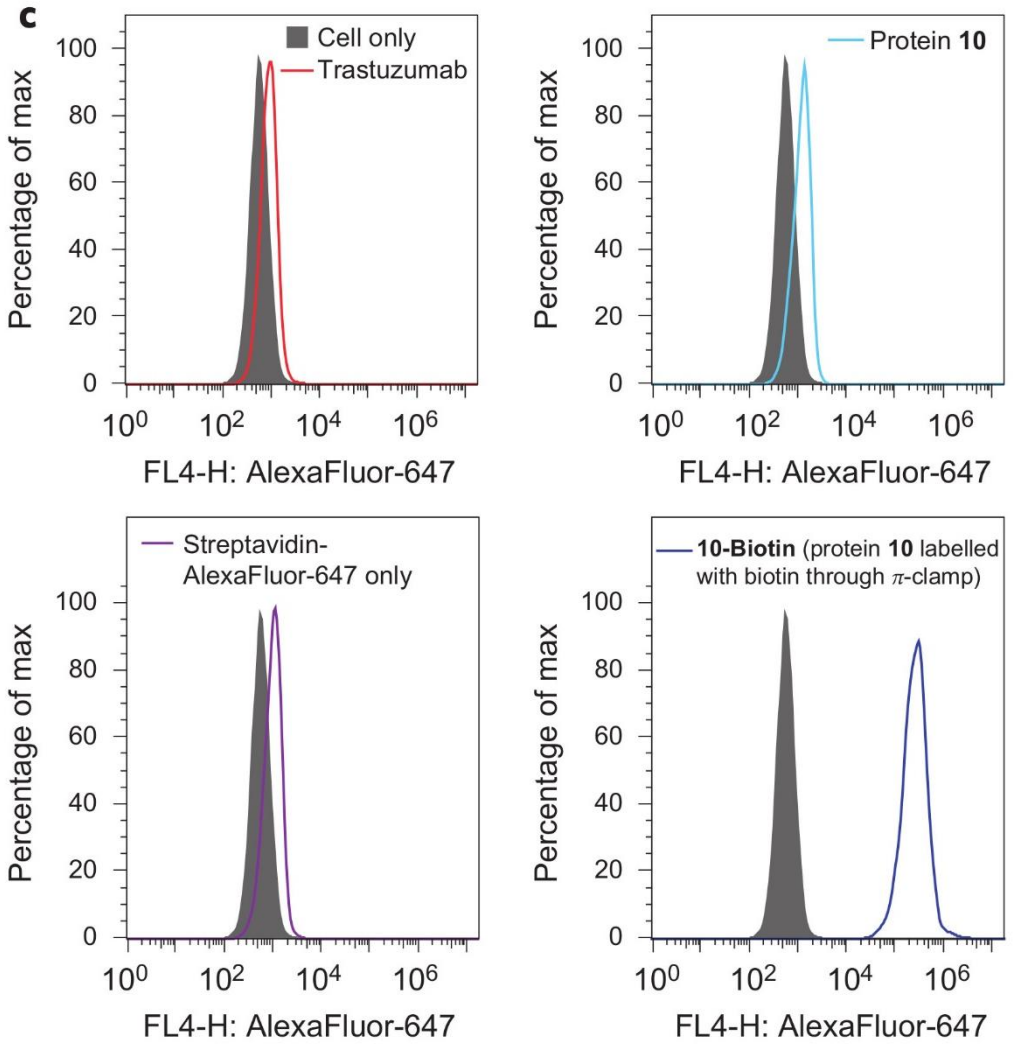
Albertoni, G. A.; Arnoni, C. P.; Barboza Araujo, P. R.; Andrade, S. S.; Carvalho, F. O.; Castello Girão, M. J. B.; Schor, N.; Barreto, J. A. *Brazilian J. Infect. Dis.* **2011**, *15*, 547.

A-2. Flow cytometry

- Flow cytometry



cells treated with biotin-conjugated trastuzumab and fluorescent streptavidin conjugation



A-3. Toxicity of hexafluorobenzene

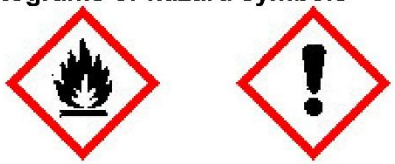
2. HAZARDS IDENTIFICATION

GHS classification

PHYSICAL HAZARDS	
Flammable liquids	Category 2
HEALTH HAZARDS	
Skin corrosion/irritation	Category 2
Serious eye damage/eye irritation	Category 2A
ENVIRONMENTAL HAZARDS	Not classified

GHS label elements, including precautionary statements

Pictograms or hazard symbols



Signal word	Danger
Hazard statements	Highly flammable liquid and vapour Causes skin irritation Causes serious eye irritation

Precautionary statements:

[Prevention]	Keep away from heat/sparks/open flames/hot surfaces. - No smoking. Keep container tightly closed. Use explosion-proof electrical/ventilating/lighting equipment. Take precautionary measures against ignition by the static discharge and the spark. Wash hands thoroughly after handling. Wear protective gloves/eye protection/face protection.
[Response]	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention. IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower. If skin irritation occurs: Get medical advice/attention. Take off contaminated clothing and wash before reuse.
[Storage]	Store in a well-ventilated place. Keep cool.
[Disposal]	Dispose of contents/container through a waste management company authorized by the local government.

http://www.tcichemicals.com/eshop/ja/jp/commodity/H0085/