Chemical Lysine Modification at a single site

2018/12/20

M1 Murata

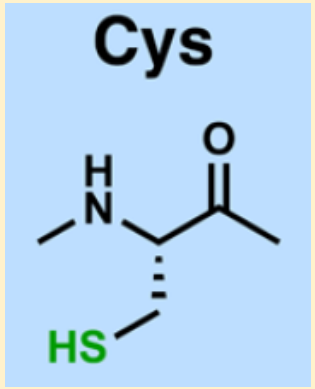
Contents

- Amino acid target for bioconjugation
- Chemical Lysine modification
  - *N*-hydroxysuccinimide (NHS)-ester
  - α, β-unsaturated sulfonamide
  - Sulfotetrafluorophenyl esters
  - Stilbene
  - β-lactam
  - Sulfonyl acrylate（Lysine activated)
- Summary

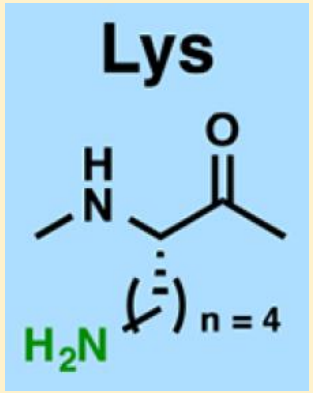
Contents

Amino acids for bioconjugation

Conventional



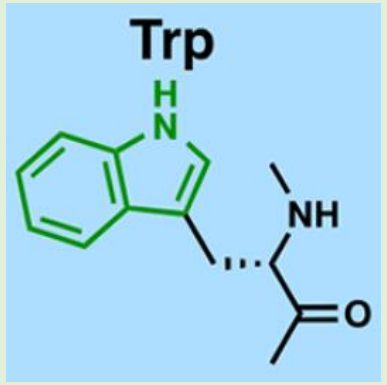
(p*K*a=8.7)

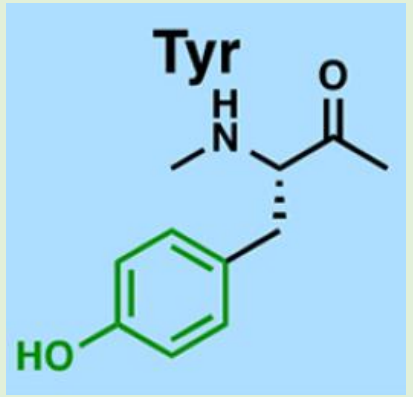


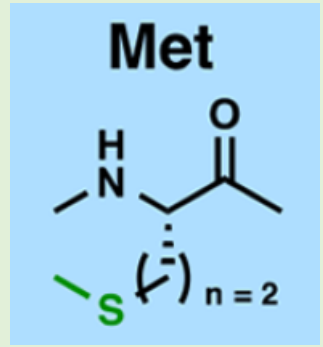
(p*K*a=10.5)

- ✓ Reactive because of nucleophilicity
- ✓ Abundant (Lys)

Recent







(pKa=10.46)

- ✓ Less-reactive
- ✓ Rare

Trp… keto-ABNO

Tyr… reactivity can be efficiently tuned by pH control

Met…only native residue that can be alkylated under acidic conditions

Biochemistry 2017, *56*, 3863−3873

Cys and Lys residues for bioconjugation

The advantage of targeting Cys or Lys residues

① abundant (Lys)
② Easy to taeget because of high reactivity

	Ratio in human protein(%)	The position of residue	Comments
Cys	1.9	within the folded proteins	Not accessible
Lys	5.9	On the surface of proteins	accessible

Native lysine residues are more convenient targets for protein modification than cysteine residues,

Targeting naturally occurring side chains in a chemo- and region-selective fashion remains a great and unexplored challenge.

J. Am. Chem. Soc. **2018**, *140*, 4004–4017

Problems in Lys-bioconjugation

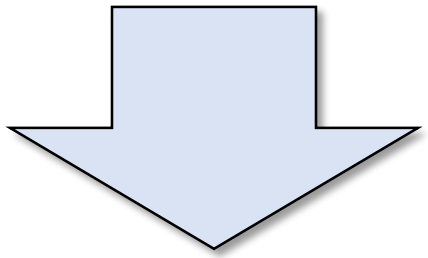
Problem

- Conventional method –

✓ Difficulties to target specific single Lys residue

✓ Require a large excess of often-valuable reagents. (e.g. 10 eq)

✓ Require metals(e.g. Ru, Pd…) or other additives that must later be removed from the reaction mixture.



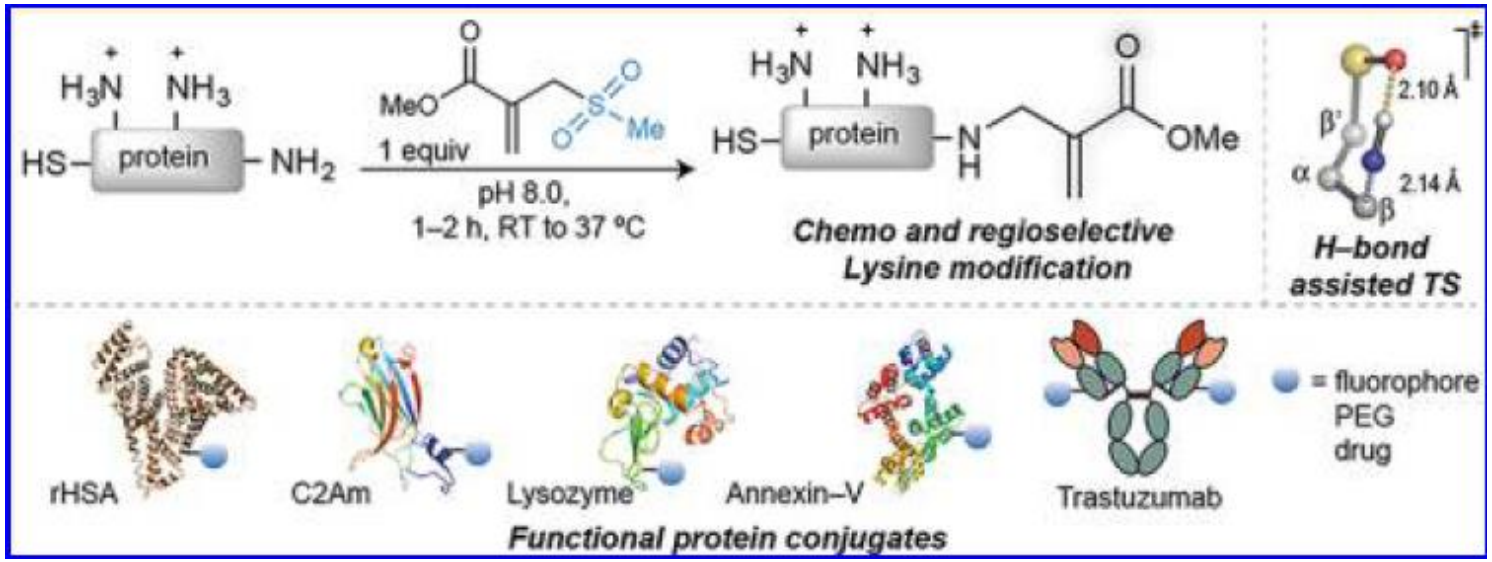
"A general way to selectively target single Lysine is lacking"

J. Am. Chem. Soc. 2018, *140*, 4004–4017

A main literature discussed in this seminar

- Sulfonyl acrylate（Lysine activated）-

Hydrogen bond assisted chemo- and regioselective modification of lysine on native proteins



- ✓ Stoichiometric amount of sulfonyl acrylate reagent
- ✓ Proceed to completion rapidly (1~2h)
- ✓ Under mild conditions (pH=8.0, rt-37℃)
- ✓ Applicable to a range of native protein types
- ✓ The products are stable
- ✓ The method is compatible with Cys bioconjugation method

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

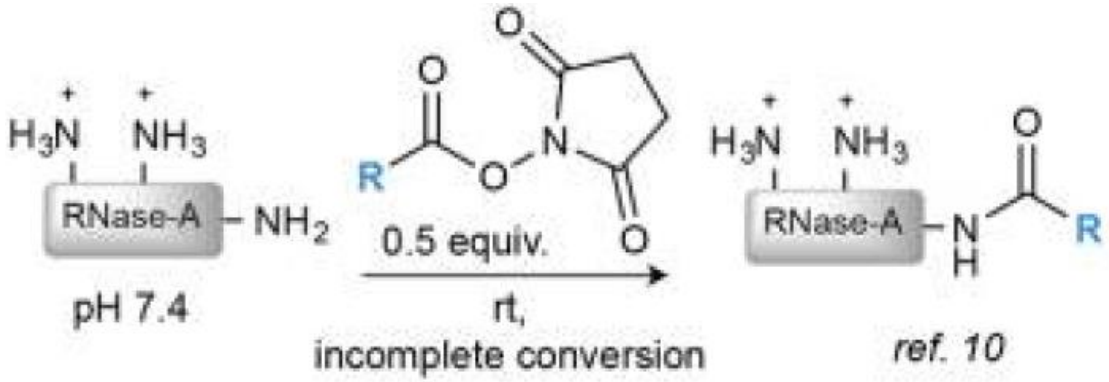
Contents

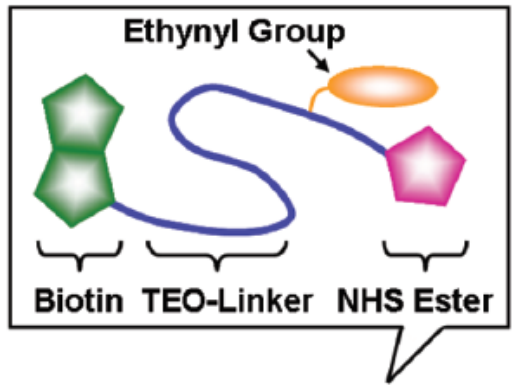
Chemical Lysine modification

- *N*-hydroxysuccinimide (NHS)-ester
- α,β-unsaturated sulfonamide
- Sulfotetrafluorophenyl esters
- Stilbene
- β-lactam

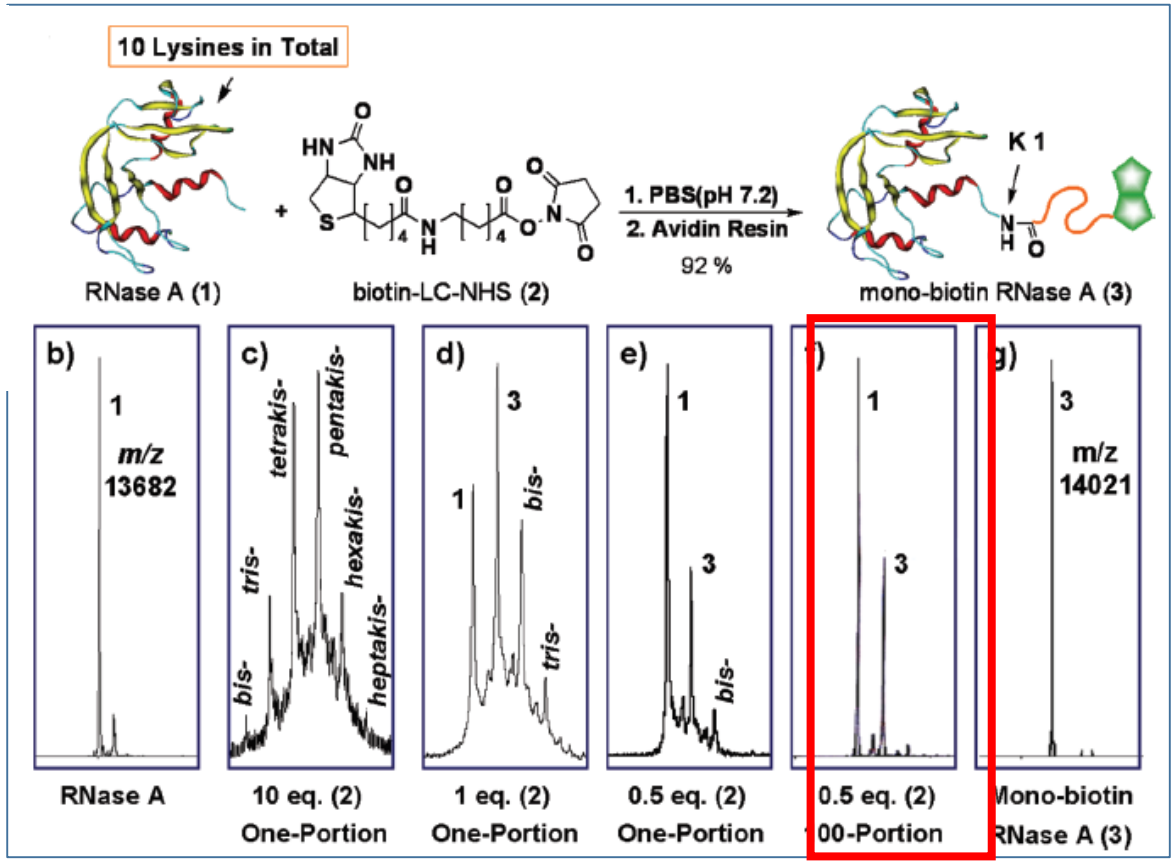
Chemical Lysine modification 1

- *N*-hydroxysuccinimide (NHS)-ester type -





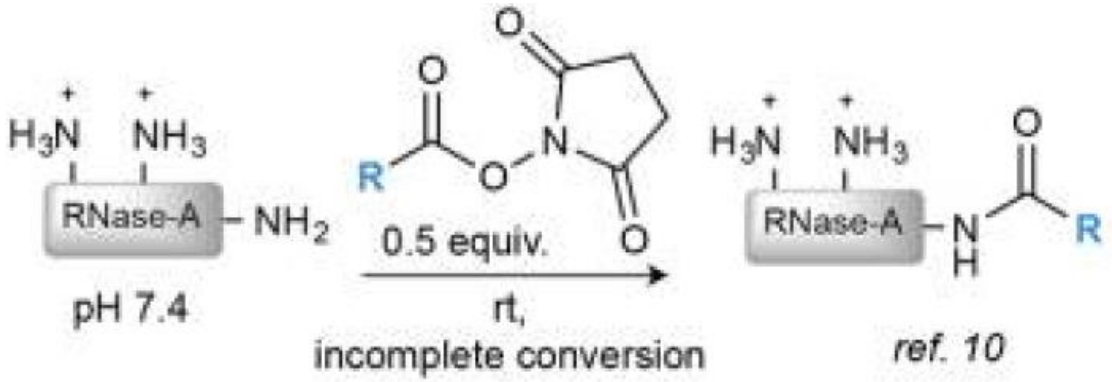
Biotin-TEO-Ethynyl-NHS (18)

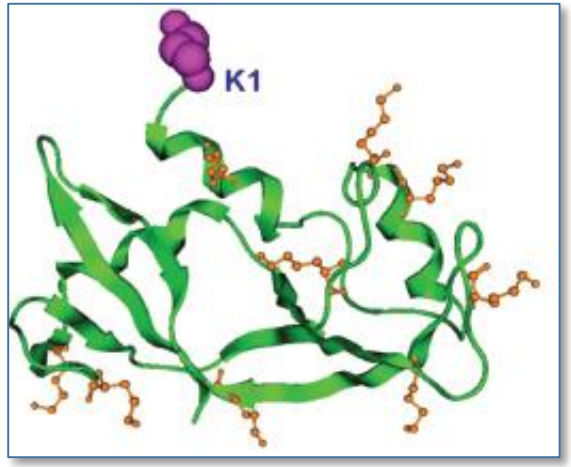


Chen *et al.*, *Bioconjugate Chem.* **2012**, *23*, 500−508

Chemical Lysine modification 1

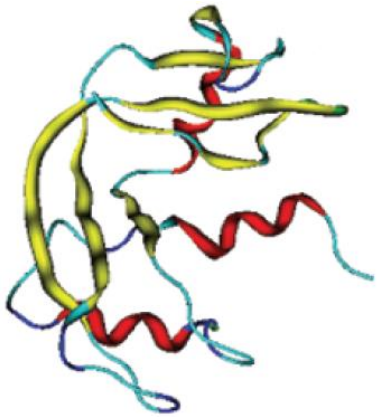
- *N*-hydroxysuccinimide (NHS)-ester type -



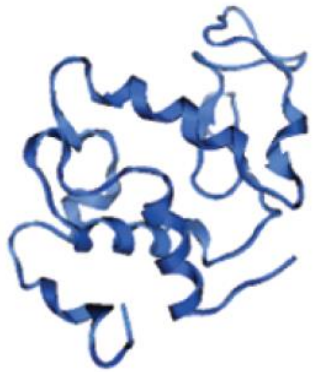


Rnase A

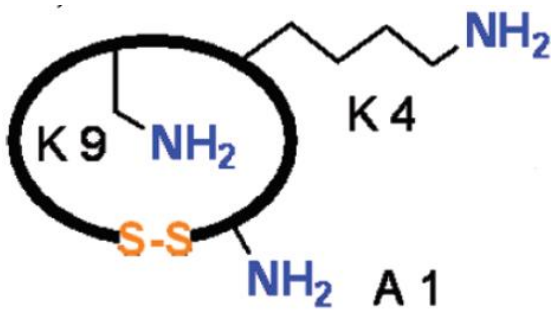
✓ the highest solvent accessibility.



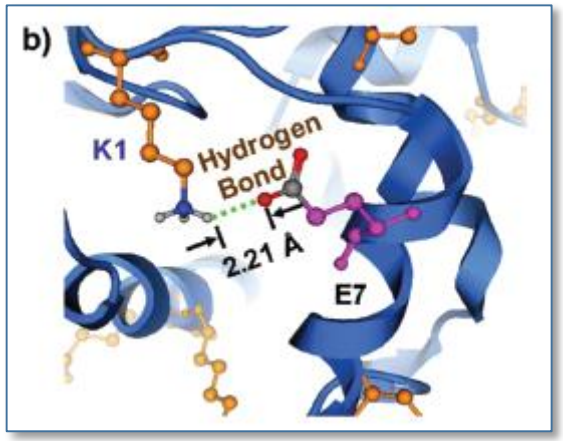
RNase A (1)



Lysozyme C



SST-14



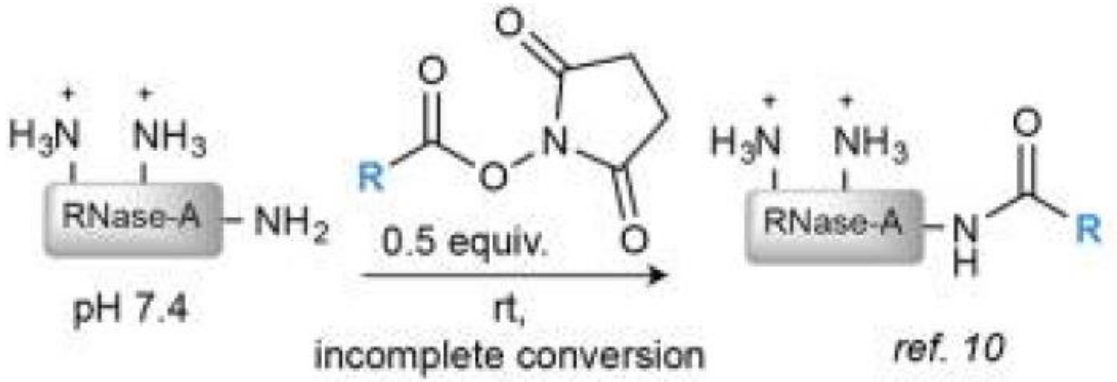
Lysozyme C

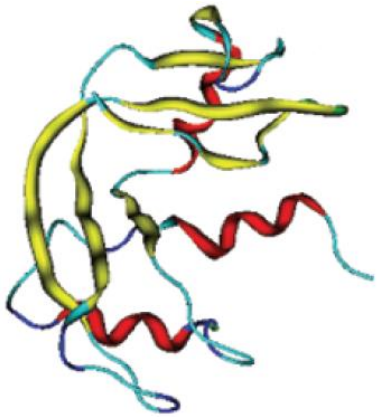
✓ hydrogen bond interaction between K1 and E7

Chen et al., *Bioconjugate Chem*. **2012**, *23*, 500−508

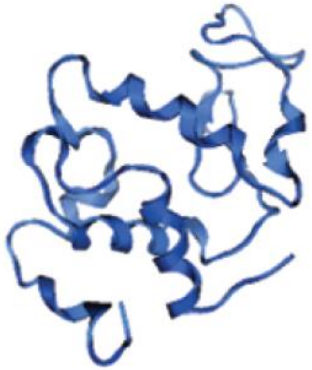
Chemical Lysine modification 1

- *N*-hydroxysuccinimide (NHS)-ester type -

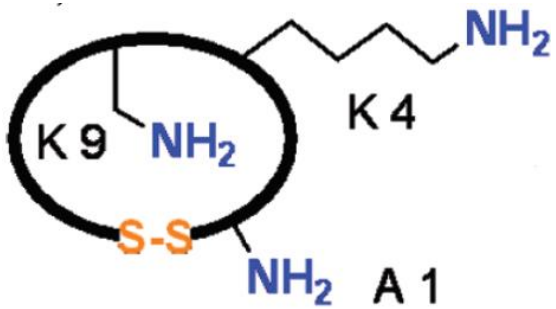




RNase A (1)



Lysozyme C



SST-14

Advantage

- ✓ Fast (2h)
- ✓ Low cost
- ✓ The risk of misfolding is low

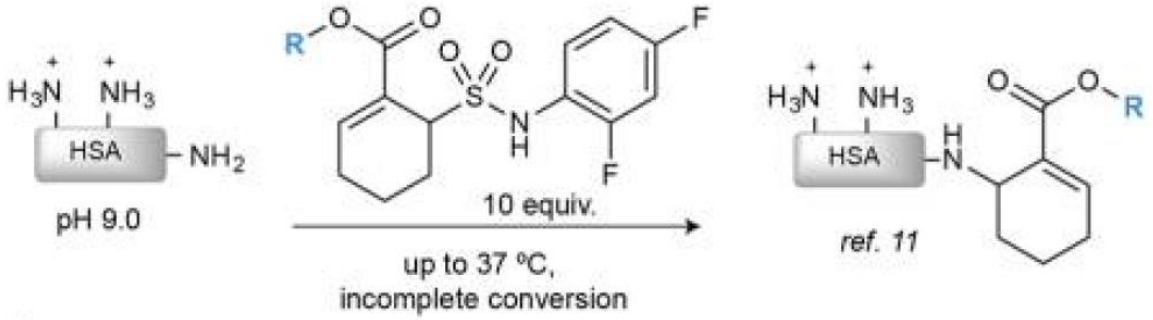
Disadvantage

- ✓ the requirement of substoichiometric amounts of reagent
  - ➡ imcomplete conversion

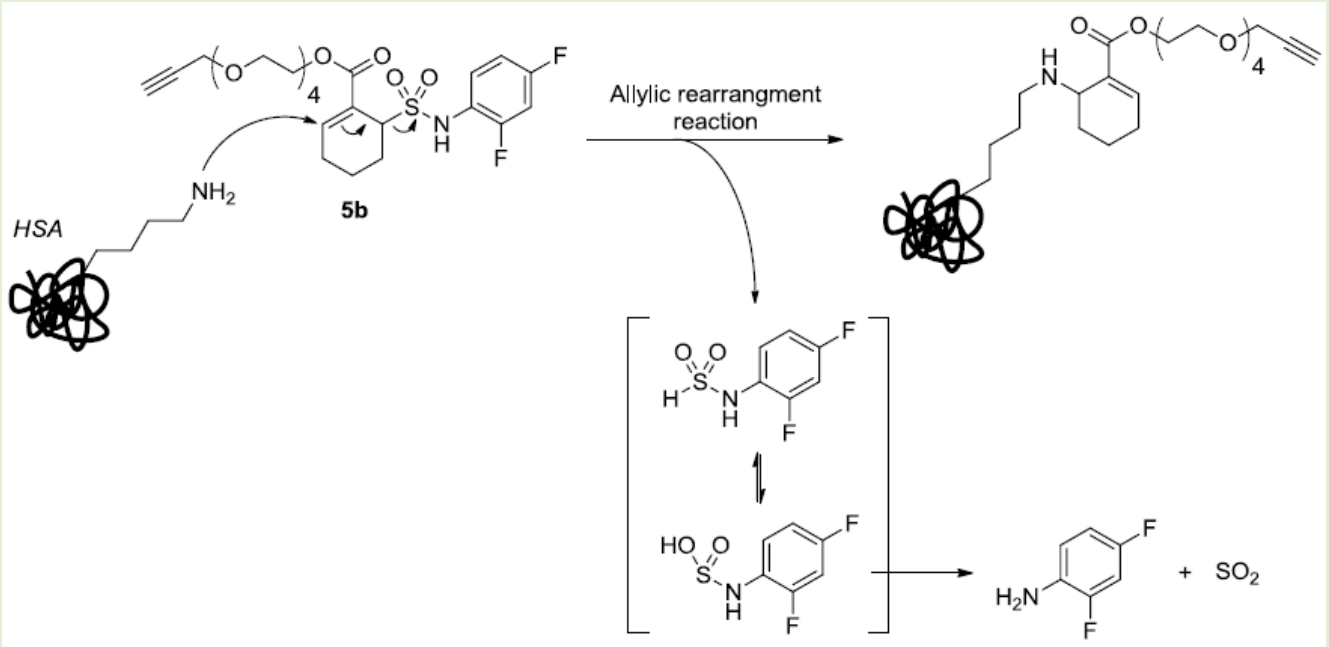
Chen *et al*., *Bioconjugate Chem*. **2012**, *23*, 500−508

Chemical Lysine modification 2

- α,β-unsaturated sulfonamide type -



Proposed Mechanism of the Conjugation Reaction with HSA



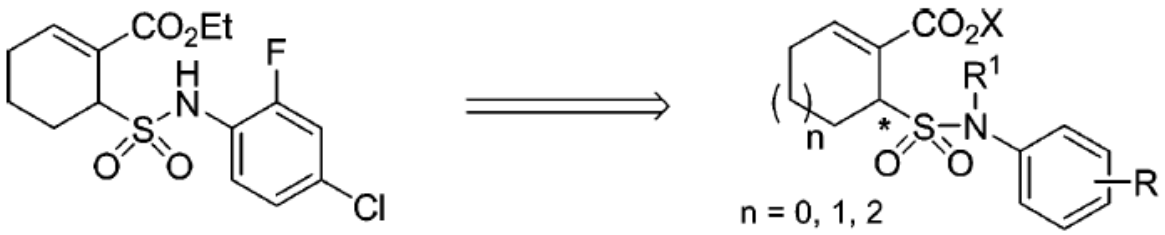
Angew. Chem. Int. Ed. 2014, 53, 11783 –11786

Bioconjugate Chem. 2016, 27, 2271−2275

Chemical Lysine modification 2

- α,β-unsaturated sulfonamide type -

Screening of the Takeda chemical library was carried out
To develop a new therapeutic agent for sepsis.



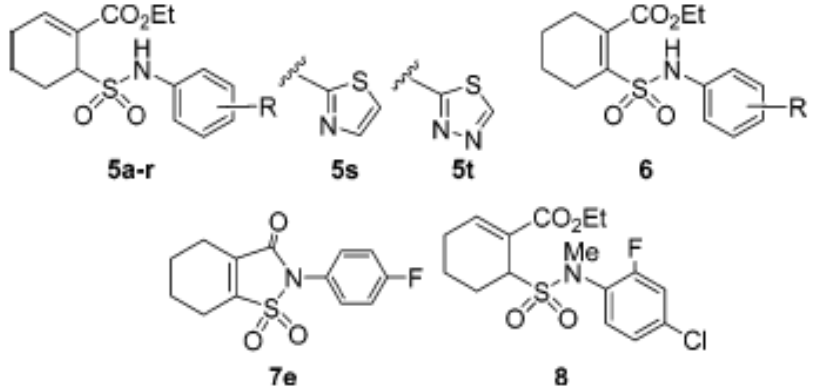
Lead compound

Yamada *et al., J. Med. Chem.* **2005,** *48,* 7457-7467

Barbas *et al., Angew. Chem. Int. Ed.* **2014**, *53*, 11783 –11786

Barbas *et al., Bioconjugate Chem*. **2016**, *27*, 2271−2275

Table 1. Inhibitory Activities of *N*-Arylsufamoyl Derivatives **5a−t, 6a,b,e, 7e,** and **8** on NO Production

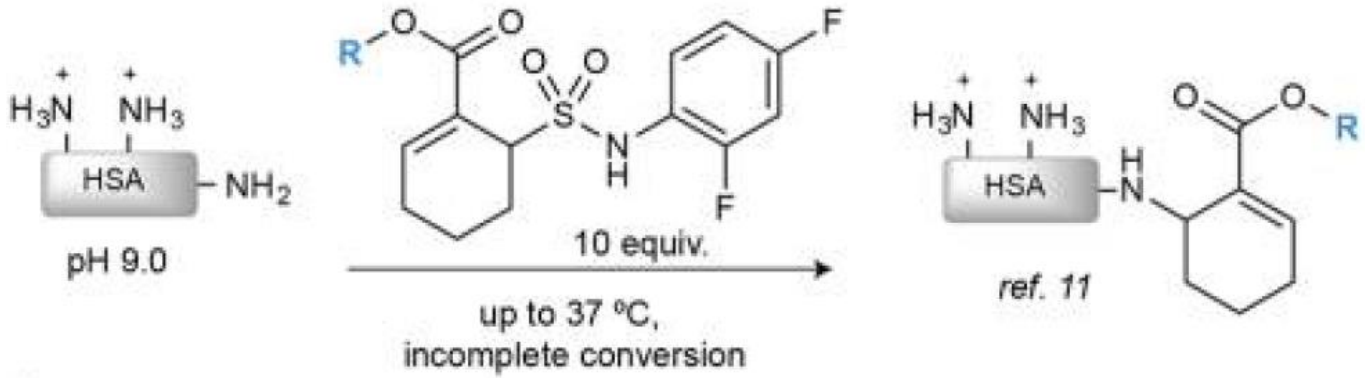


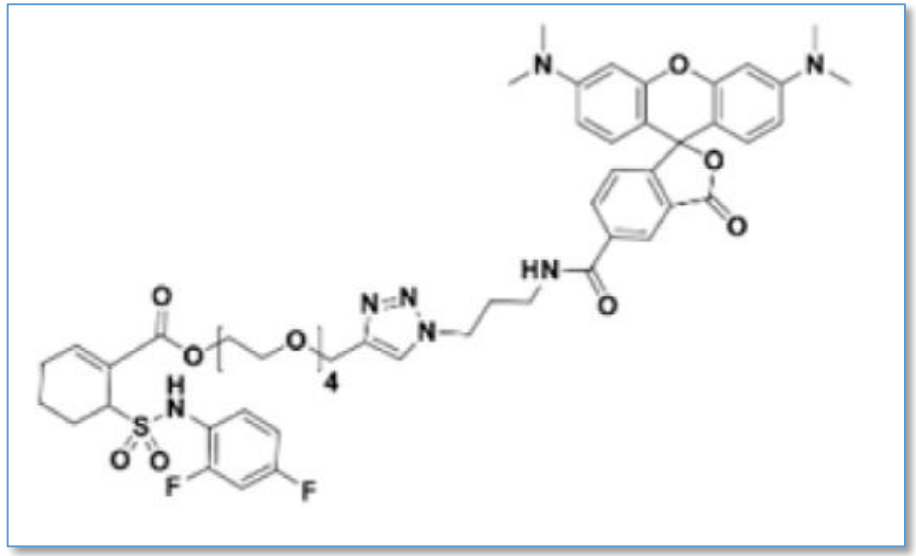
compd	R	IC_{50}[a] (nM)	compd	R	IC_{50}[a] (nM)
5a	2-F, 4-Cl	160 ± 65	**5n**	2-Cl, 4-F	3.2 ± 0.89
5b	H	260 ± 113	**5o**	2-Cl, 4-Me	41 ± 14
5c	2-F	75 ± 45	**5p**	2-Cl, 4-CN	1600 ± 172
5d	3-F	150 ± 14	**5q**	2-Et	130 ± 16
5e	4-F	110 ± 11	**5r**	2-CO_2Me	1100 ± 194
5f	2-Cl	12 ± 1.2	**5s**	–	>10000
5g	3-Cl	66 ± 19	**5t**	–	>10000
5h	4-Cl	400 ± 279	**6a**	2-F, 4-Cl	1700 ± 495
5i	2,3-F_2	140 ± 49	**6b**	H	>8200
5j	2,6-F_2	160 ± 4.9	**6e**	4-F	1400 ± 363
5k	2,4-F_2	16 ± 1.4	**7e**	–	4100 ± 1742
5l	2,4,5-F_3	30 ± 1.8	**8**	–	230 ± 45
5m	2,4-Cl_2	20 ± 2.0			

[a] The inhibitory activity is shown as an IC_{50} value, which is the concentration of test compound required to suppress the production of NO by 50% of control. Values are the mean ± SD of two or three experiments.

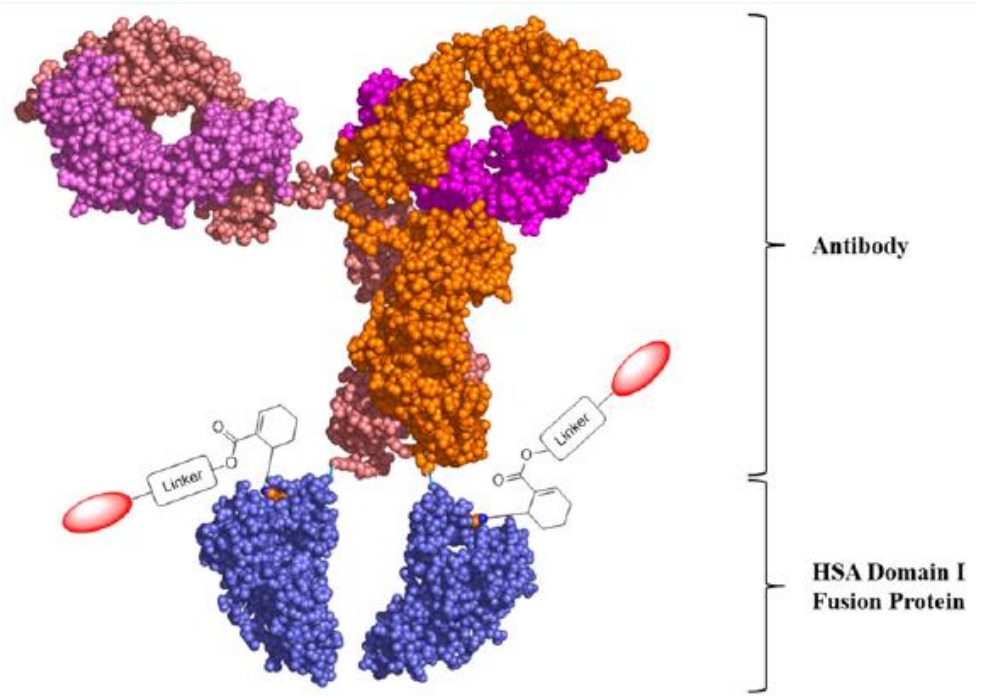
Chemical Lysine modification 2

- α,β-unsaturated sulfonamide type -





Rhodamine-linked cyclohexene sulfonamide compound (cHx-Rho).



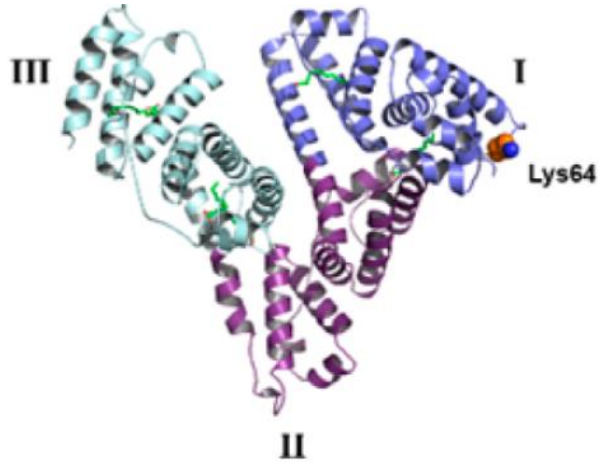
- ✓ rapidly modify HSA
- ✓ excellent serum stability

Barbas *et al., Angew. Chem. Int. Ed.* **2014**, *53*, 11783 –11786

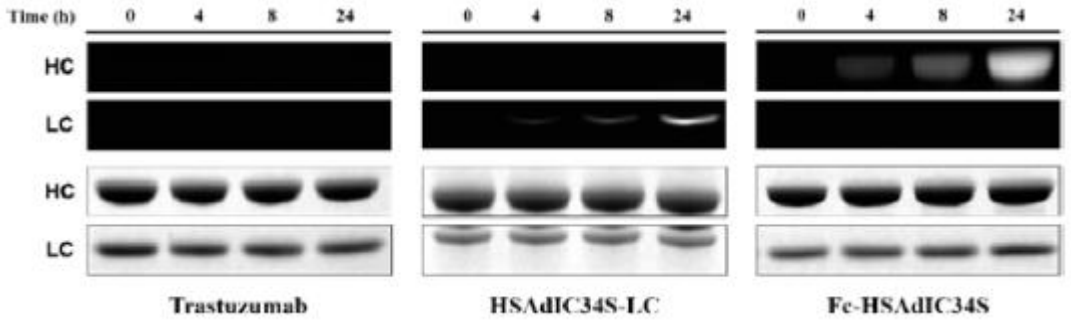
Barbas *et al., Bioconjugate Chem*. **2016**, *27*, 2271–2275

Chemical Lysine modification 2

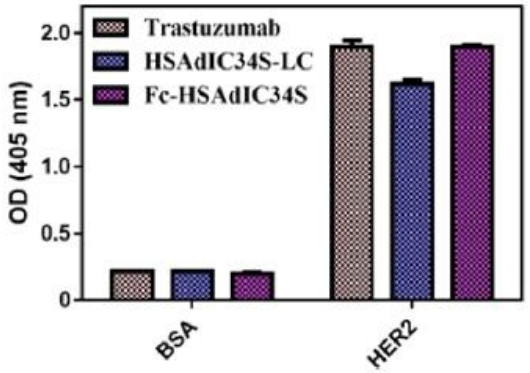
- α, β-unsaturated sulfonamide type -



Time-course study of trastuzumab, HSAdIC34S-LC, and Fc-HSAdIC34S labeling with 10 equiv of cHx-Rho.



HER2 for trastuzumab and fusion conjugates.



Antibody conjugation using HSAdI as a fusion protein should be amenable to therapeutic applications.

<u>Advantage</u>

- ✓ Site-selectivity
- ✓ Antibody conjugates were also stable in human plasma

<u>Disadvantage</u>

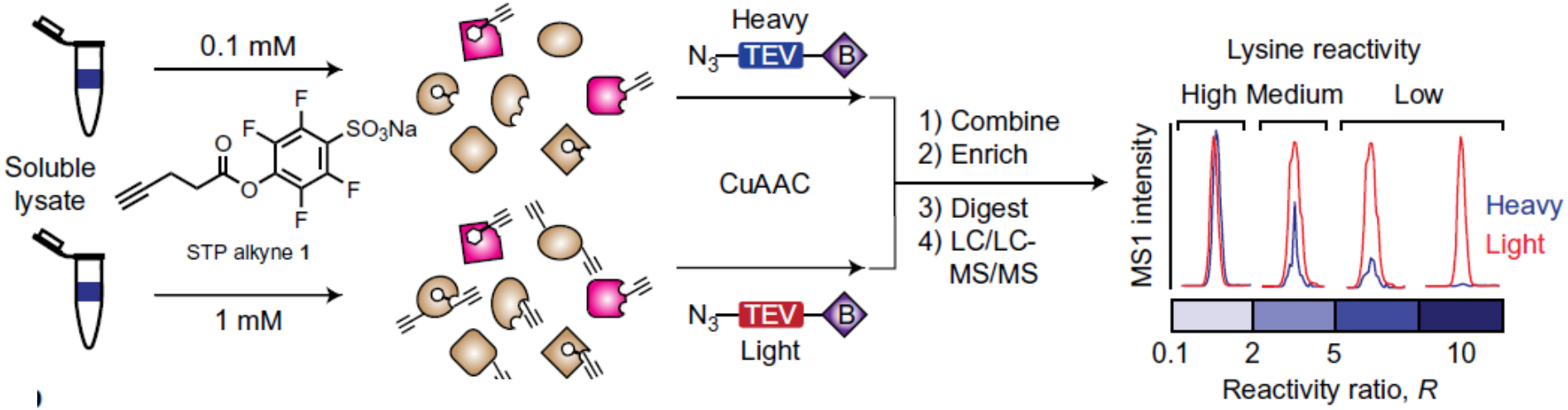
- ✓ Imcomplete conversion
- ✓ Need large amount of reagent (10 eq)
- ✓ Basic condition. (pH=9.0)

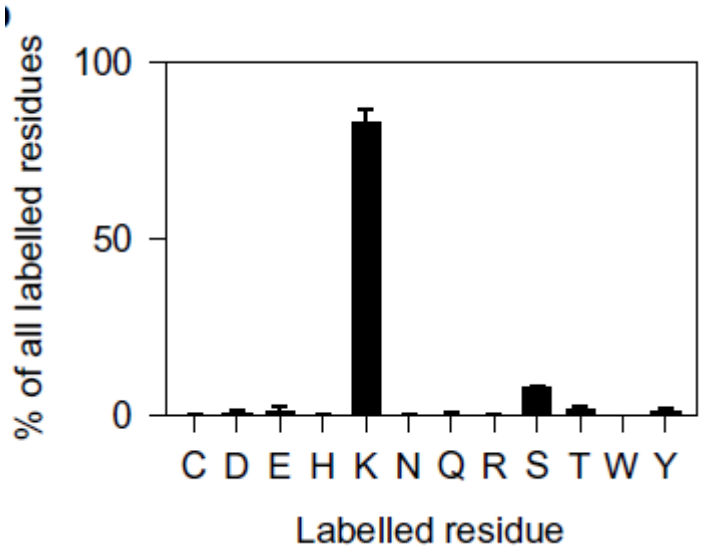
Barbas *et al., Angew. Chem. Int. Ed.* **2014**, *53*, 11783 –11786

Barbas *et al., Bioconjugate Chem*. **2016**, *27*, 2271–2275

Chemical Lysine modification 3

- sulfotetrafluorophenyl esters -



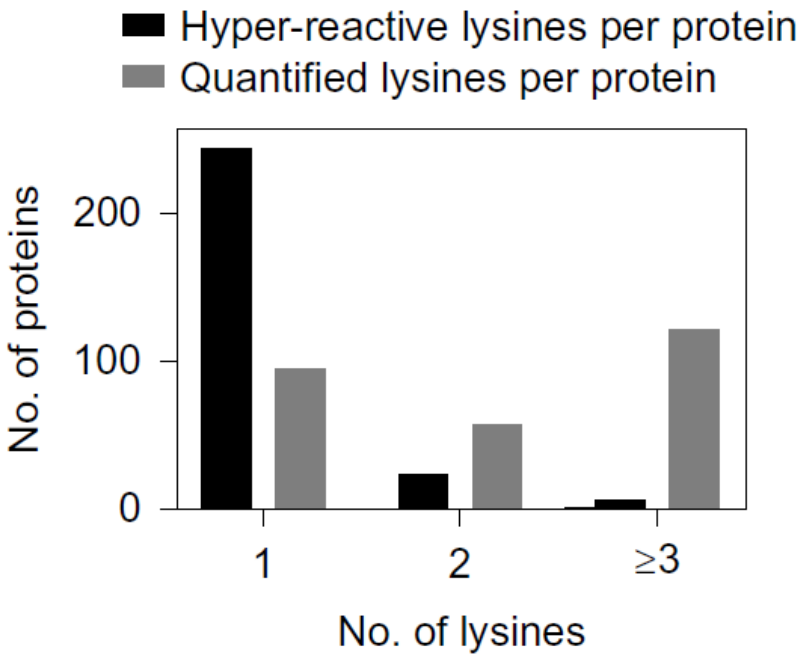


- ✓ broad reactivity
- ✓ good selectivity for lysine residues in the human proteome.

Cravatt *et al.*, *Nat. Chem.*, **2017**, *9*,

Chemical Lysine modification 3

- sulfotetrafluorophenyl esters -



Advantage

✓ more stable in aqueous solution than NHS esters.

Disadvantage

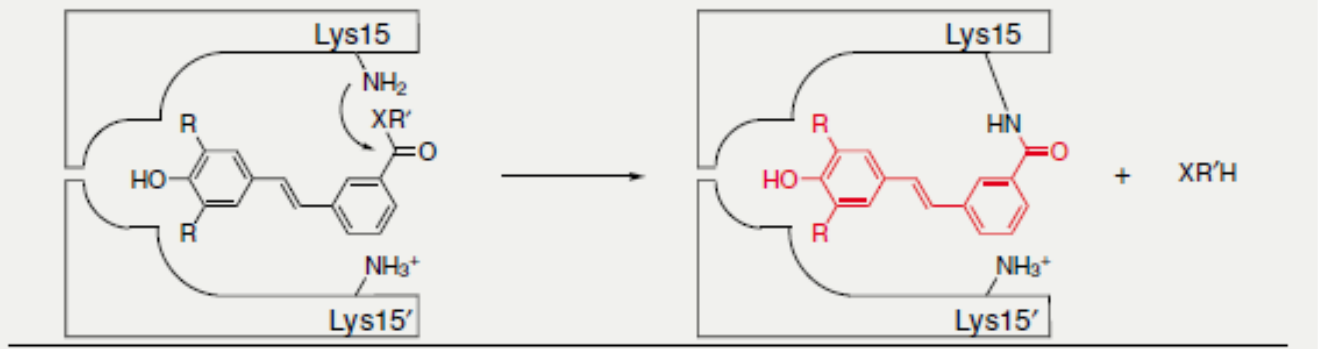
✓ the localization of hyper-reactive lysines to pockets could also restrict their access to post-translational machinery, such as ubiquitylation processes

✓ In order to prevent overreaction it is necessary to well control the equivalence of reagent and the reaction time.

Cravatt *et al.*, *Nat. Chem.*, **2017**, *9*,

Chemical Lysine modification 4

- Stilbene- *designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin*



Transthyretin(TTR) : One of the causative proteins of amyloidosis.

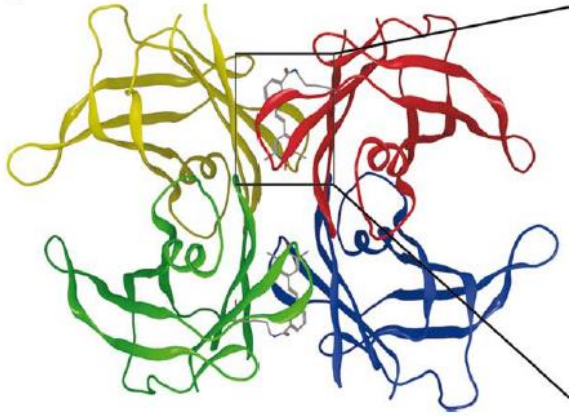


Figure. Crystal structure of the WT TTR

- Amyloidosis caused by transthyretin -

- Senile systemic amyloidosis (SSA)
- Familial amyloid polyneuropathy (FAP)

When transthyretin becomes unstable, it becomes amyloid fiber and aggregates.

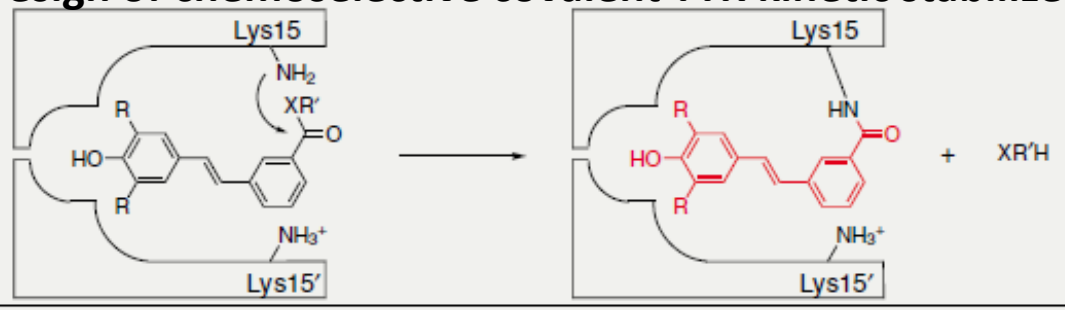
A treatment method to stabilize transthyretin is required.

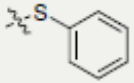
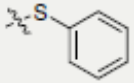
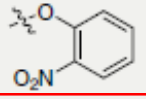
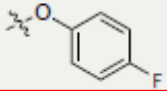
Kelly *et al., Nat. Chem. Biol.*, **2010**, *6*

Chemical Lysine modification 4

- Stilbene- *designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin*

Design of chemoselective covalent TTR kinetic stabilizers



Compound	R	XR′	% fibril formation[a] 7.2 μM	% fibril formation[a] 3.6 μM	IC_{50} (μM)[b]	% modification of TTR subunits in human blood plasma[c]
1	Br		2% (±1%) 3% (±0.3%)	12% (±1%) 17% (±0.2%)	2.00	36% (±0.4%)
2	CH_3		2% (±1%) 3% (±0.2%)	16% (±2%) 34% (±0.5%)	2.00	48% (±1.3%)
3	CH_3		4% (±1%) 3% (±0.2%)	21% (±2%) 33% (±2%)	2.26	49% (±0.7%)
4	CH_3		1% (±1%) 7% (±0.2%)	16% (±1%) 36% (±3%)	1.96	32% (±3.3%)

Most chemoselective

- Percent fibril formation of WT TTR(3.6 μM, black font) and V30M TTR(3.6 μM, blue font)
- Maximum modification percent is 50%.

Reduce electrostatic repulsion between lysine residues

↓

Stabilization of transthyretin

✓ Ester or thioester group
→allow amino group of Lys to approach at Burgi-Dunitz angle

Kelly *et al., Nat. Chem. Biol.*, **2010**, *6*

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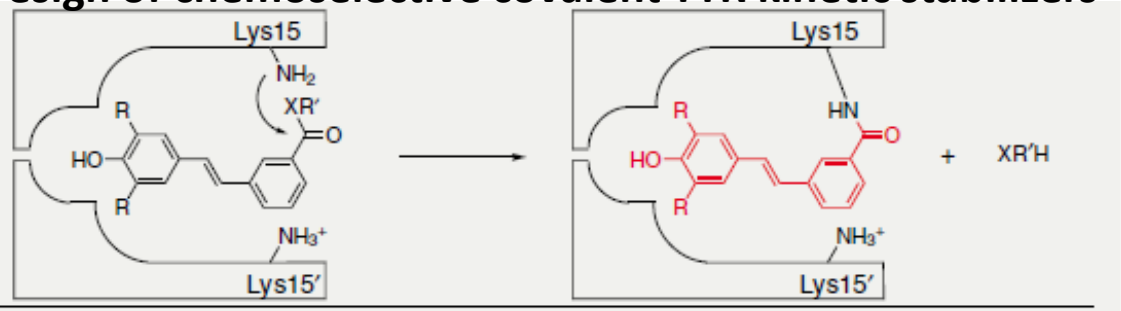
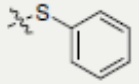
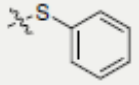
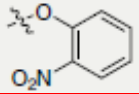
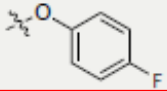
- Stilbene- *designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin*

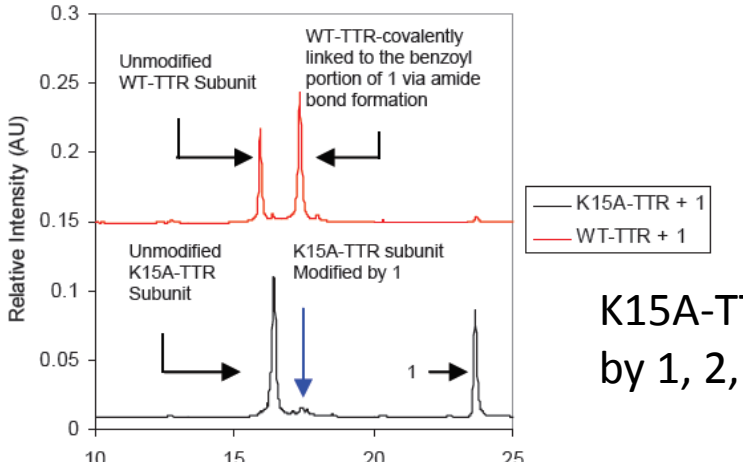
Design of chemoselective covalent TTR kinetic stabilizers



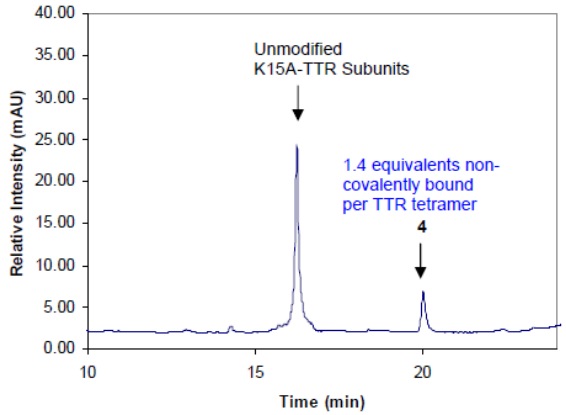
Compound	R	XR'	% fibril formation[a] 7.2 μM	% fibril formation[a] 3.6 μM	IC_{50} (μM)[b]	% modification of TTR subunits in human blood plasma[c]
1	Br		2% (±1%) 3% (±0.3%)	12% (±1%) 17% (±0.2%)	2.00	36% (±0.4%)
2	CH_3		2% (±1%) 3% (±0.2%)	16% (±2%) 34% (±0.5%)	2.00	48% (±1.3%)
3	CH_3		4% (±1%) 3% (±0.2%)	21% (±2%) 33% (±2%)	2.26	49% (±0.7%)
4	CH_3		1% (±1%) 7% (±0.2%)	16% (±1%) 36% (±3%)	1.96	32% (±3.3%)

Most chemoselective

- Percent fibril formation of WT TTR(3.6 μM, black font) and V30M TTR(3.6 μM, blue font)
- Maximum modification percent is 50%.



K15A-TTR subunit modified by 1, 2, 3 was observed.



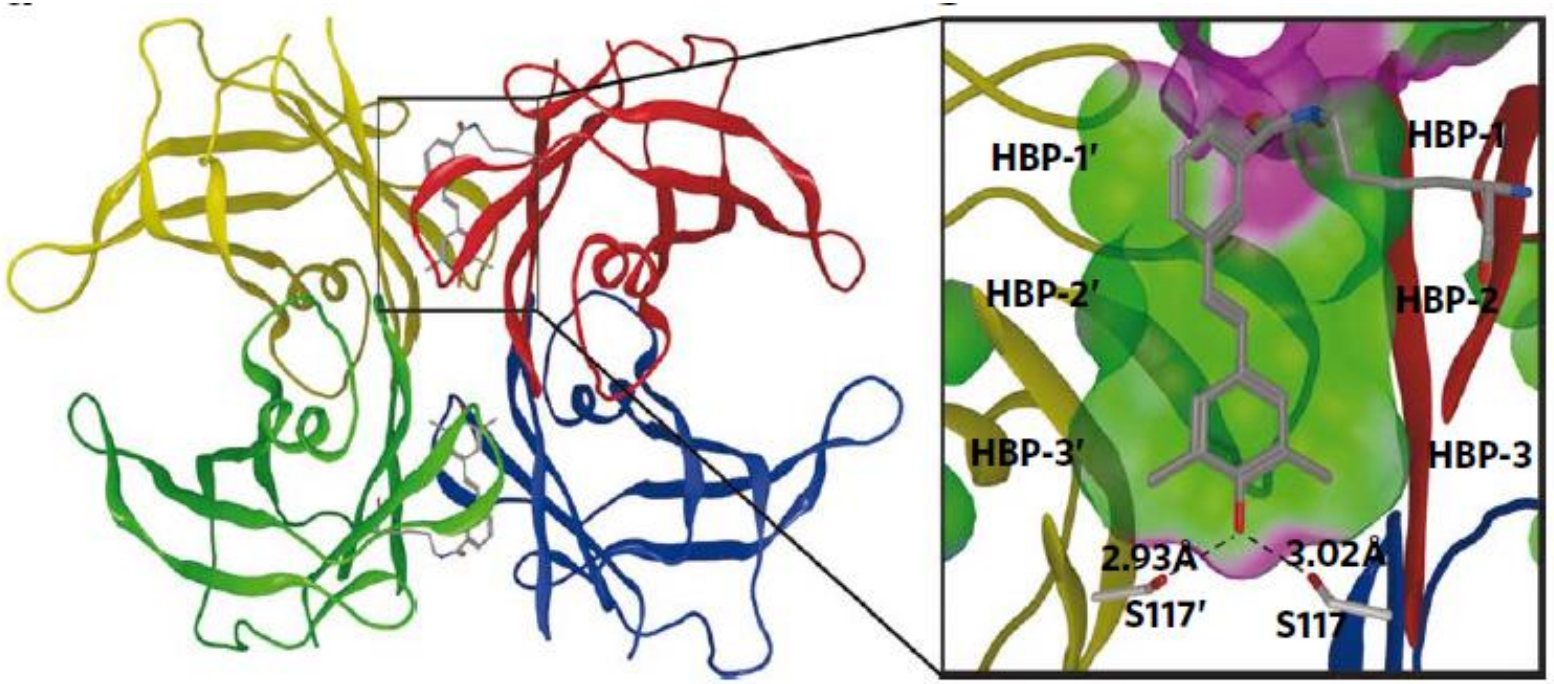
Not observed
→High chemoselectivity

Kelly *et al., Nat. Chem. Biol.*, **2010**, *6*

Chemical Lysine modification 4

- Stilbene- *designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin*

Transthyretin(TTR) : One of the causative proteins of amyloidosis.



Green: hydrophobic
Purple: polar
Blue: exposed

Figure. Crystal structure of the WT TTR

- ✓ Bridging hydrogen bonds are formed between the 4-OH of the benzoyl substructure and the Ser117 and Ser117′ hydroxyls from adjacent TTR monomers.
- ✓ One Lys15 ε-amine group and one Lys15′ ε-ammonium group at pH 7.

Kelly *et al., Nat. Chem. Biol.*, **2010**, *6*

Chemical Lysine modification 4

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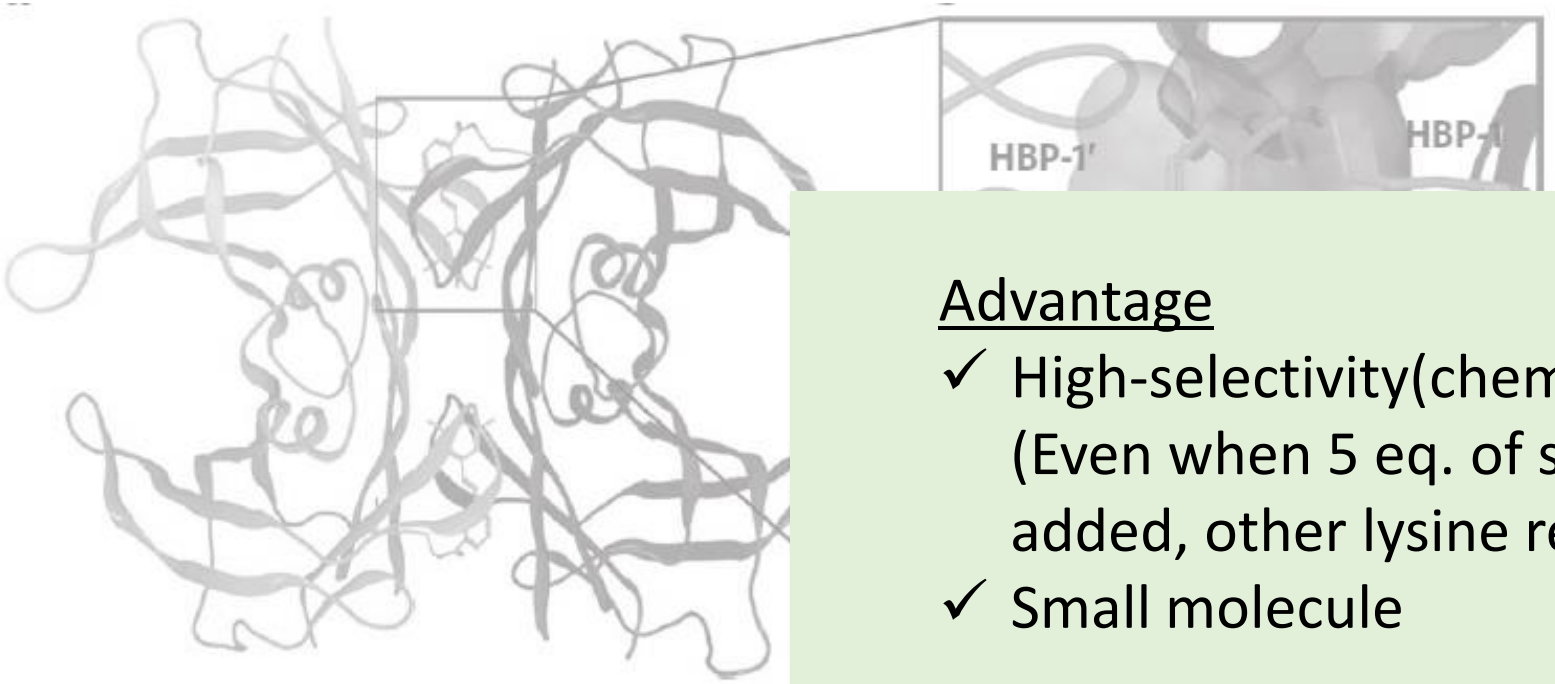
Transthyretin(TTR) : One of the causative proteins of amyloidosis.



- ✓ Bridging hydrogen bonds are forme
  Ser117′ hydroxyls from adjacent TTR
- ✓ One Lys15 ε-amine group and one L

Advantage

- ✓ High-selectivity(chemo-, site-)
  (Even when 5 eq. of stilbene derivative were added, other lysine residues did not react)
- ✓ Small molecule

Disadvantage

- ✓ Applicable range is limited to TTR

Kelly *et al., Nat. Chem. Biol.*, **2010**, *6*

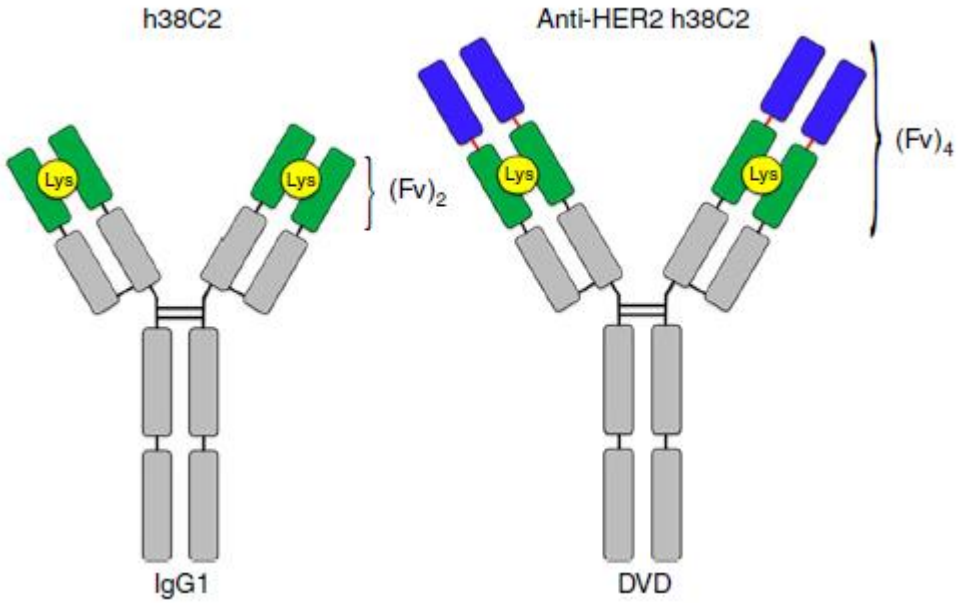
Chemical Lysine modification 5

- β-lactam- *The first site-specific ADC to be generated using a natural Lys for conjugation.*

ADC : useful for cancer treatment.

Conventional Site-Selective reaction

- ✓ unnatural amino acid by a genetic engineering technique
- ✓ introduce a sequence suitable for an enzymatic reaction



h38C2 :(humanized anti-hapten monoclonal antibody)

- ✓ Having a nucleophilic Lys residue (p*K*a=~6) in the hydrophobic pocket

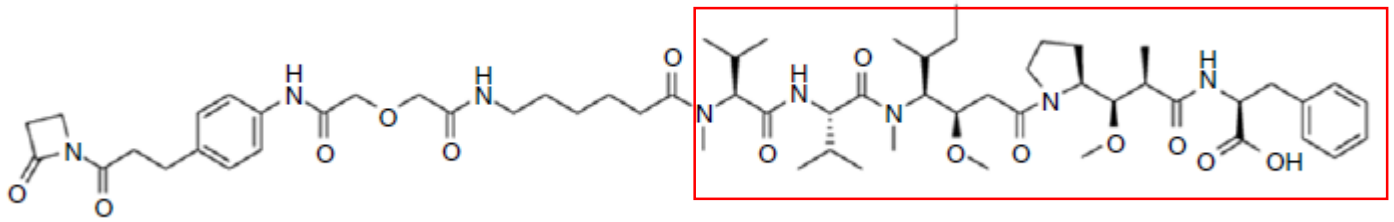
The DVD is composed of
- variable domains of trastuzumab (blue),
- h38C2 (green) with reactive Lys (yellow circle),
- constant domains (gray).

It becomes a selective modified target at in physiological pH.

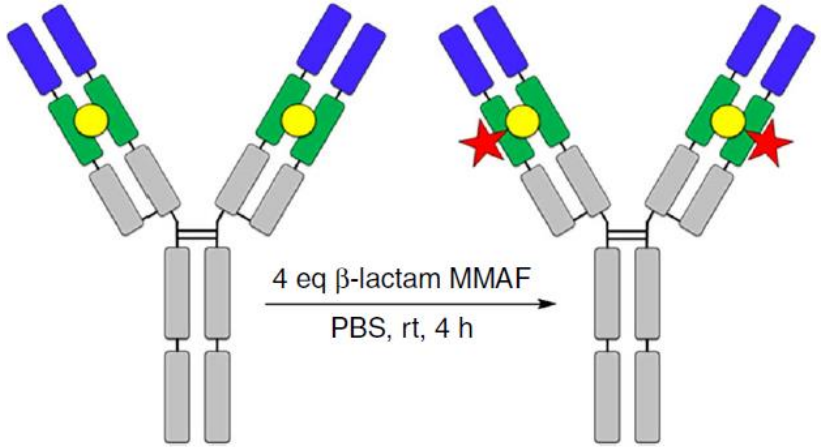
Rader, Roush et al., Nat. Com, **2017**, *.8,* 1112

Chemical Lysine modification 5

- β-lactam- *The first site-specific ADC to be generated using a natural Lys for conjugation.*



Monomethyl auristatin F (MMAF)
…To reduce cytotoxicity



Advantage

- ✓ Mutation free
- ✓ React irreversibly with Lys.
  →prevent premature drug release
- ✓ ADCs to be highly homogeneous
- ✓ conjugation does not eliminate any positive charges on the antibody
  →preserve electrostatic properties of the antibody

Disadvantage

- ✓ The possibility that other Lys react with reagent when the reaction time is longer.

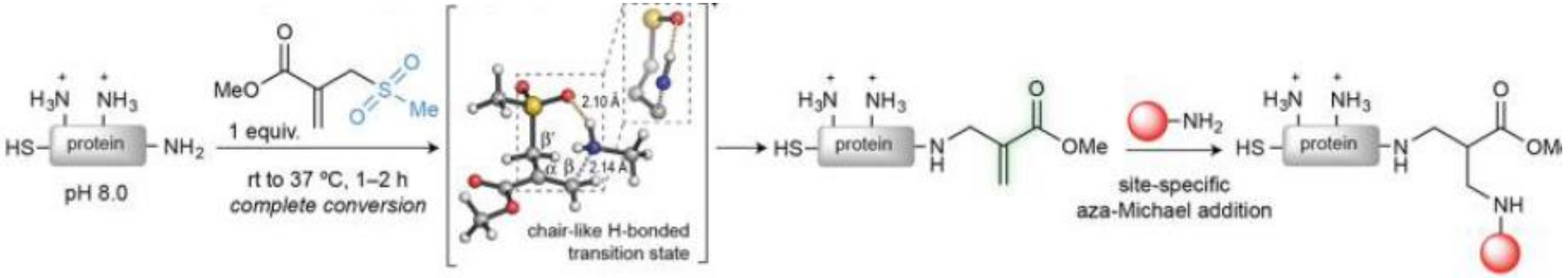
Rader, Roush *et al., Nat. Com*, **2017**, *.8,* 1112

Contents

Sulfonyl acrylate-mediated lysine modification

- Sulfonyl acrylate（Lysine activated）-

Hydrogen bond assisted chemo- and regioselective modification of lysine on native proteins

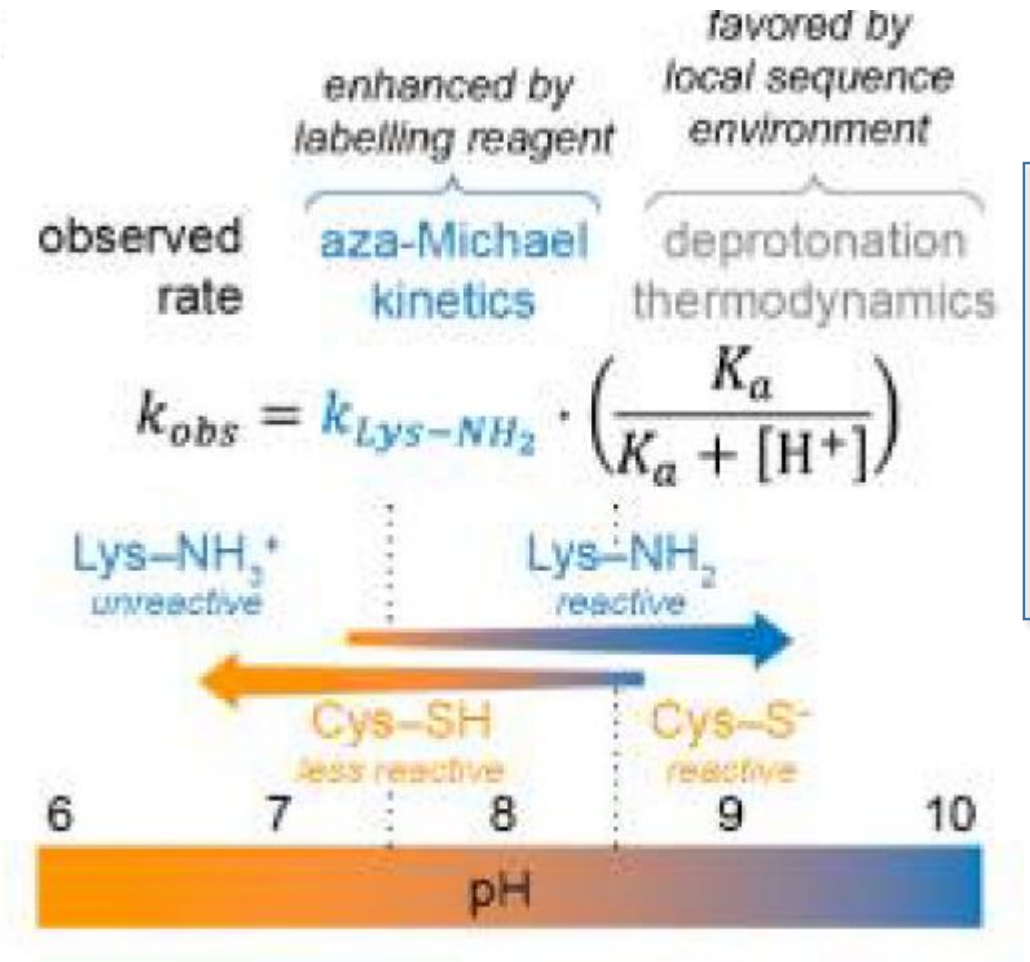


- ✓ Stoichiometric amount of sulfonyl acrylate reagent
- ✓ Proceed to completion rapidly (1~2h)
- ✓ Under mild conditions (pH=8.0, rt-37℃)
- ✓ Applicable to a range of native protein types
- ✓ The products are stable.
- ✓ The method is compatible with Cys bioconjugation.

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

p*K*a & reactivity of amino acids

- The reactivity of Lys and Cys residue at various pH conditions -

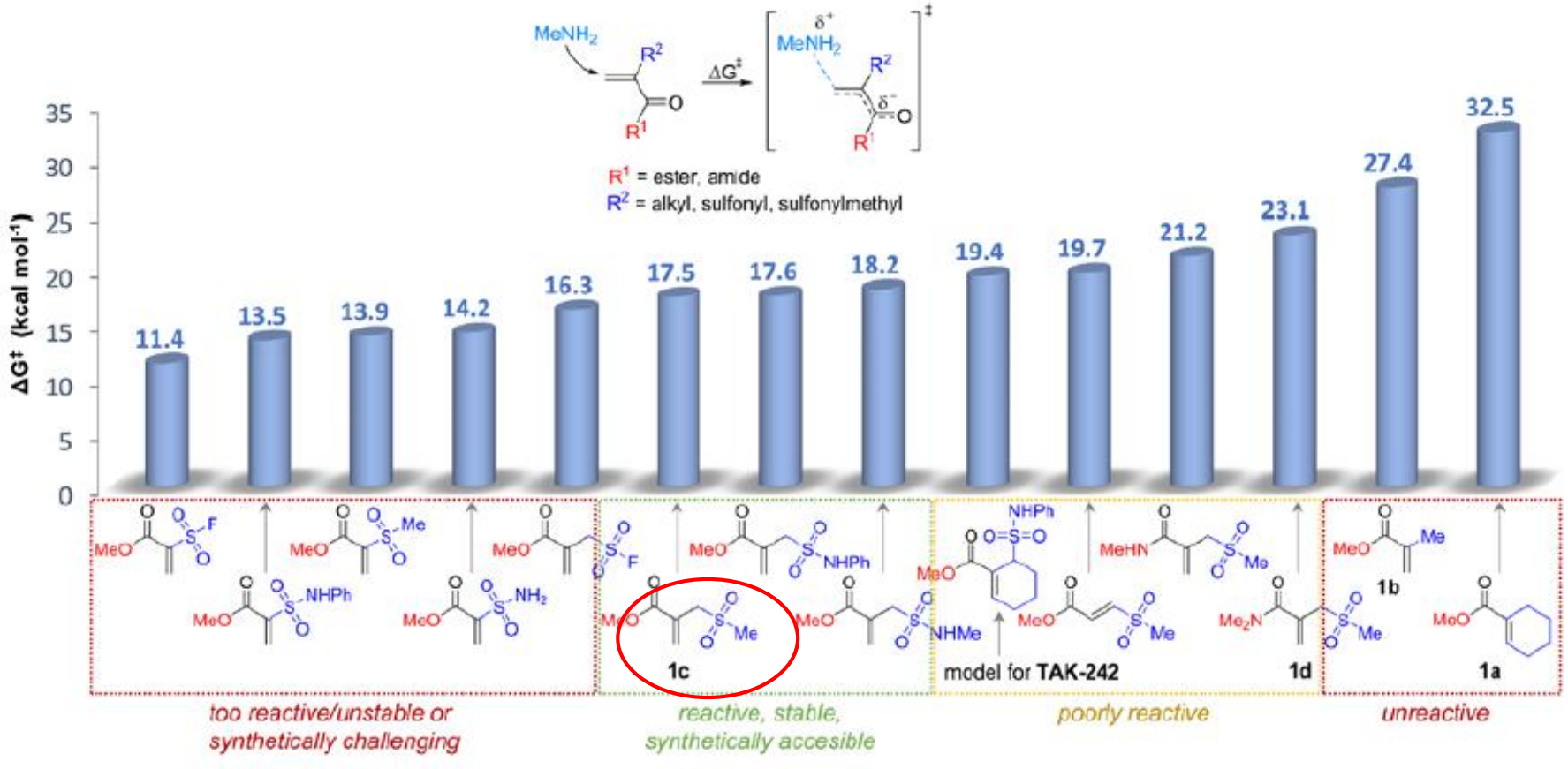


pH 5.5-6.0	Lysine residues are mostly protonated and unreactive.
pH 7.5-.8.0	Lysine residues compete with cysteine as Michael donors.
pH 8.5-	The more nucleophilic thiolate of cysteine residues usually dominate

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Screening of Michael acceptors

- Computational screening of acrylic acid derivatives-



Activation barriers ($\Delta G^{\ddagger}$) were calculated with PCM(H_2O)/M06-2X/6-31+G(d,p)

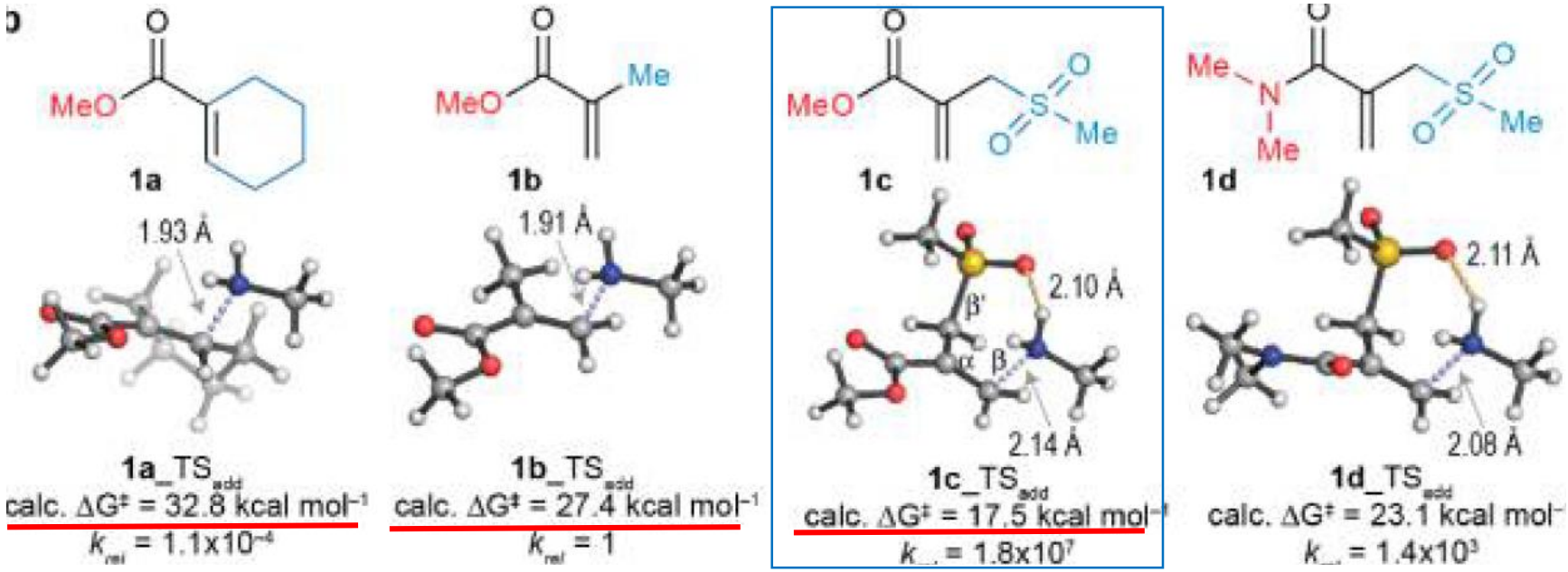
*Sulfonylmethyl acrylate **1c** was predicted to have a superior reactivity compared to its amide analogues*

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Detailed analysis of transition states

- Sulfonyl acrylate（Lysine activated）-

Acrylate electrophile derivatives 1a–d used in this study and transition states

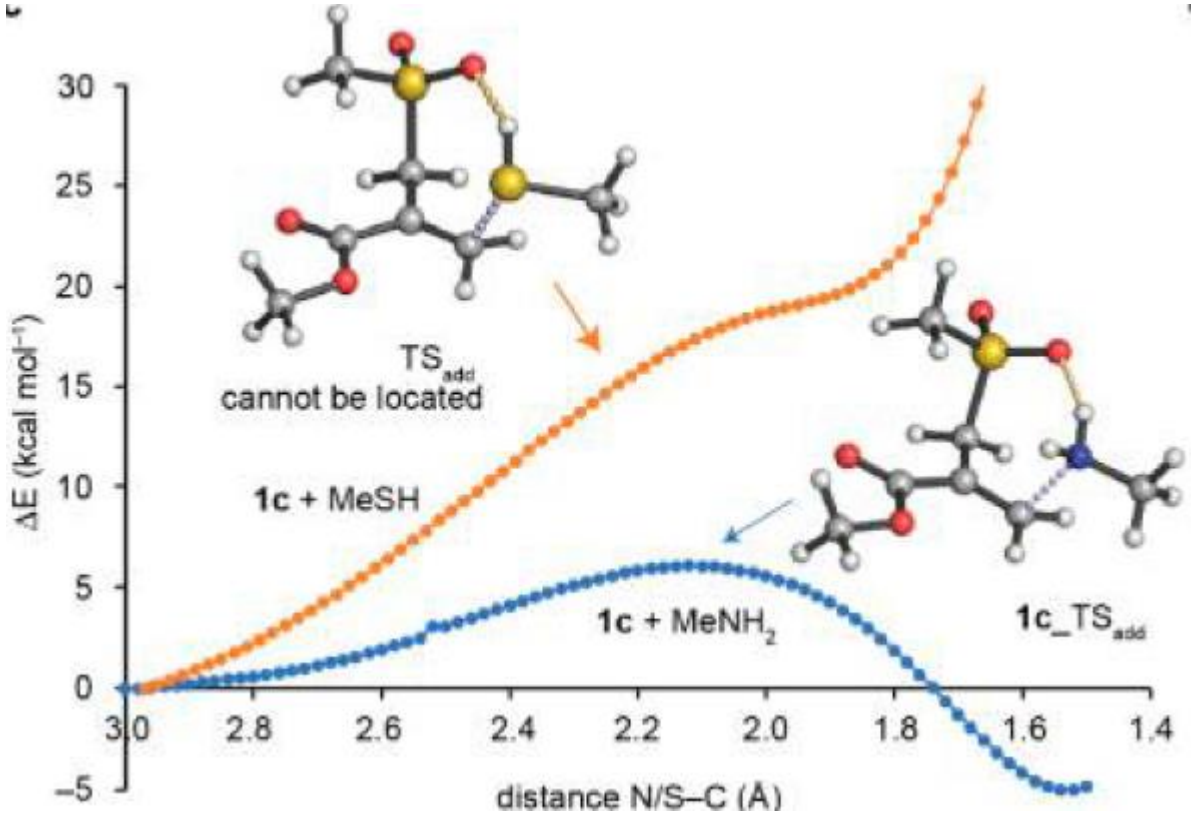


This hydrogen bond interaction between the lysine model and the sulfone lowers the energy barrier by 10–16 kcal mol–1

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Comparison between Cys & Lys

- Sulfonylmethyl acrylate（Lysine activated）-



- Hydrogen-bonding

  Cys ＜ Lys

- Cys … less polar character of the S−H bond,

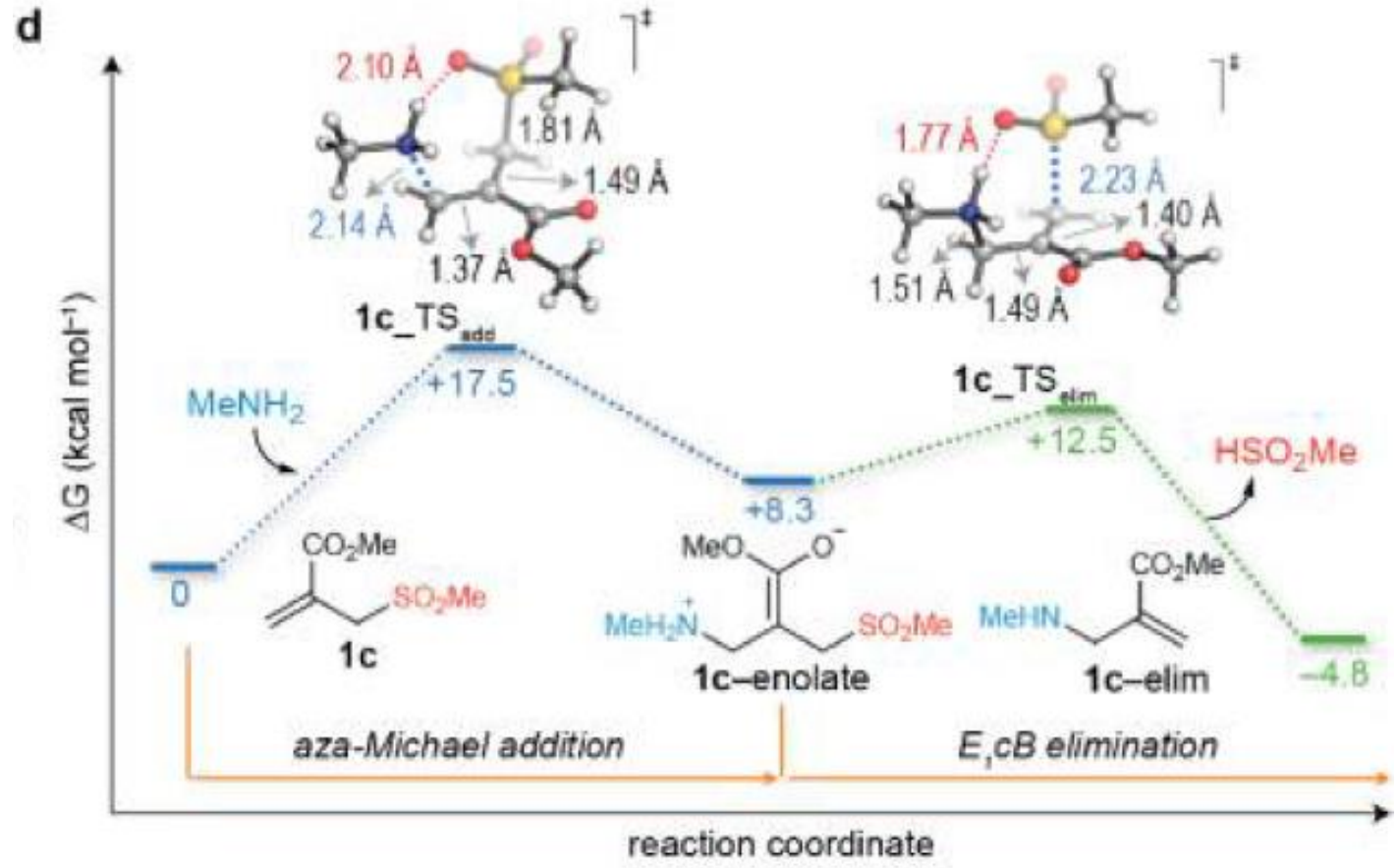
✓ *The positive charge developed at the amino group of the lysine model is efficiently dissipated by the sulfone*

A means to selectively modify lysine even in the presence of free cysteine residues at near neutral pH.

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004−4017

This conjugation is a two step reaction

- Sulfonylmethyl acrylate（Lysine activated）-

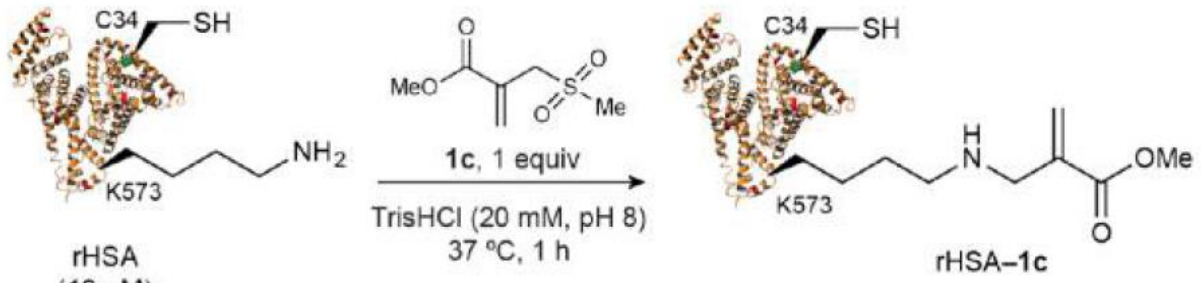


✓ Hydrogen bonding between the nucleophilic amino group and the sulfone moiety promotes both the aza-Michael addition and the elimination of methanesulfinic acid.

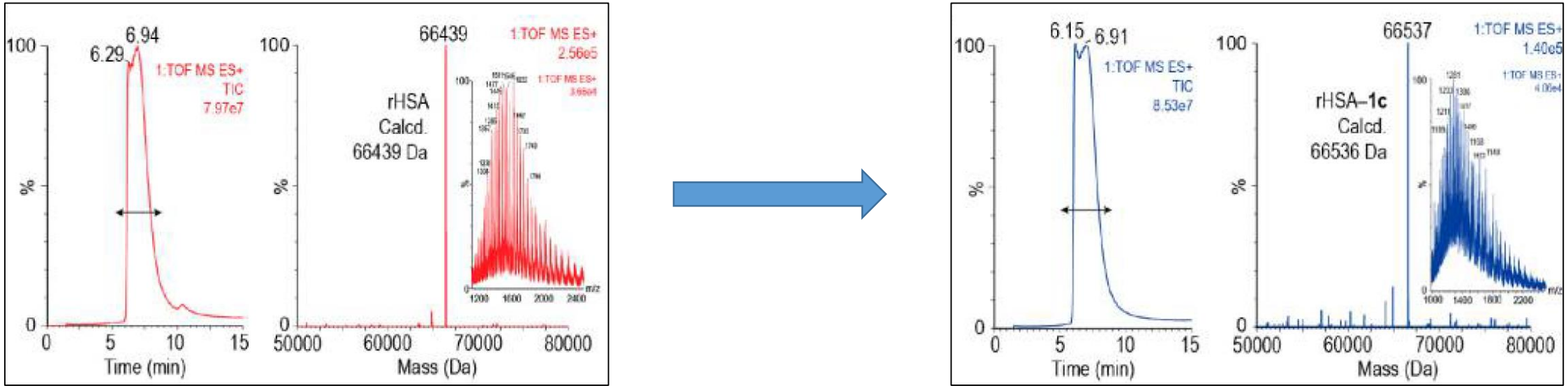
Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

rHSA (MS analysis)

- Sulfonylmethyl acrylate（Lysine activated）-



Total ion chromatogram

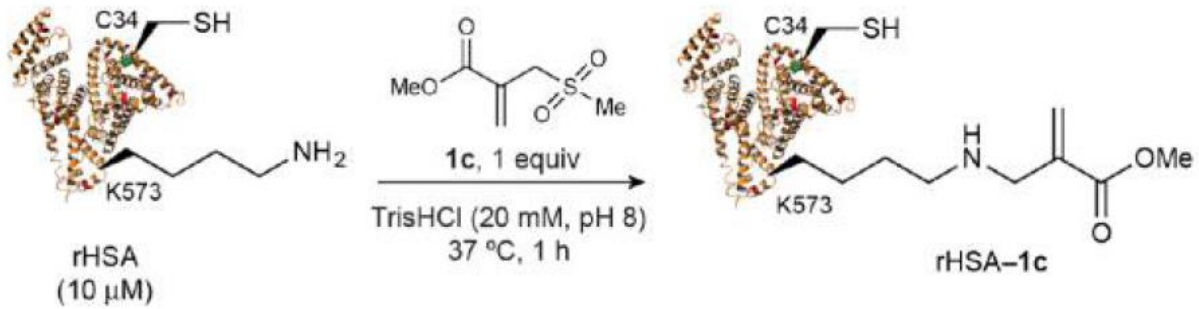


A single modification was produced in >95%

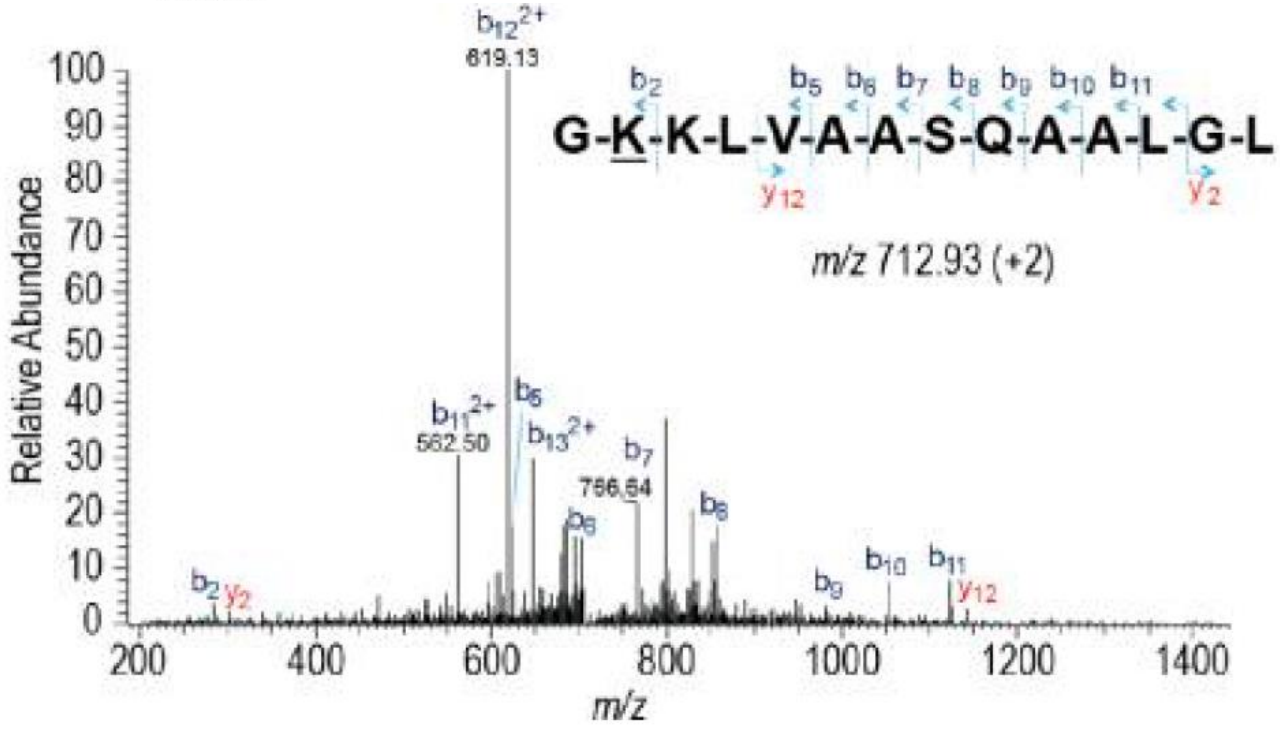
Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

rHSA (LC-MS/MS , CD)

- Sulfonylmethyl acrylate（Lysine activated）-



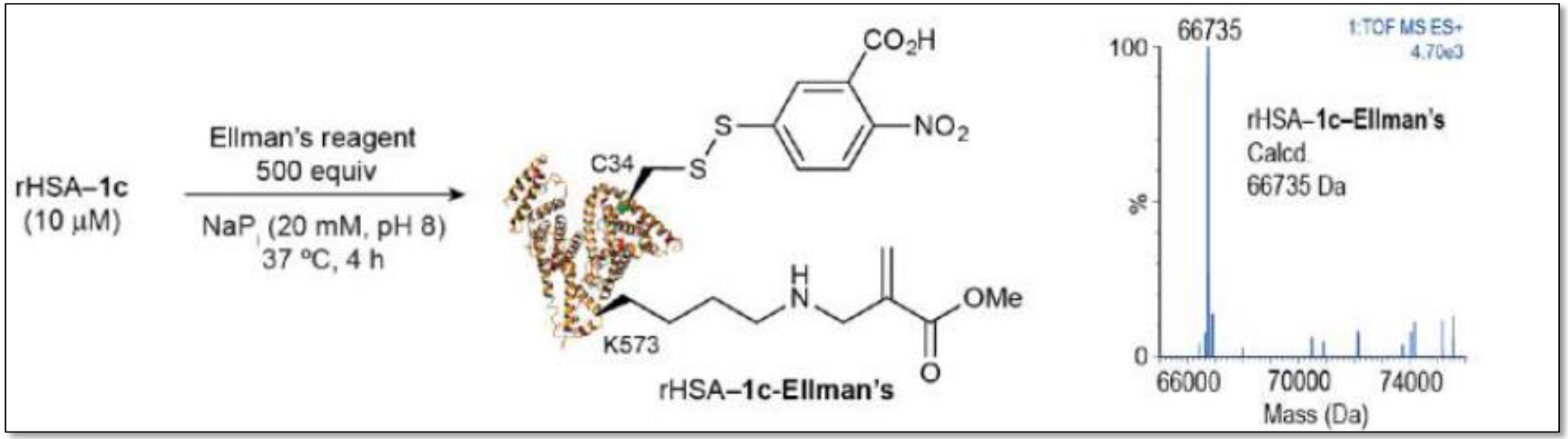
LC-MS/MS analysis



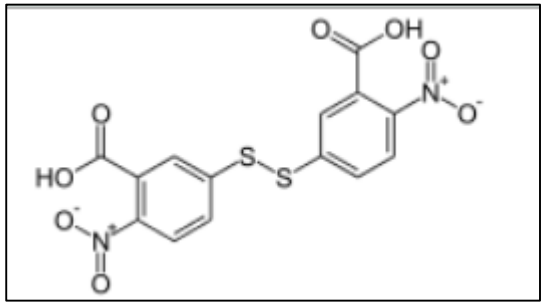
Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Reaction with thiol specific Ellman's reagent

- Sulfonylmethyl acrylate（Lysine activated）-



Ellman's reagent

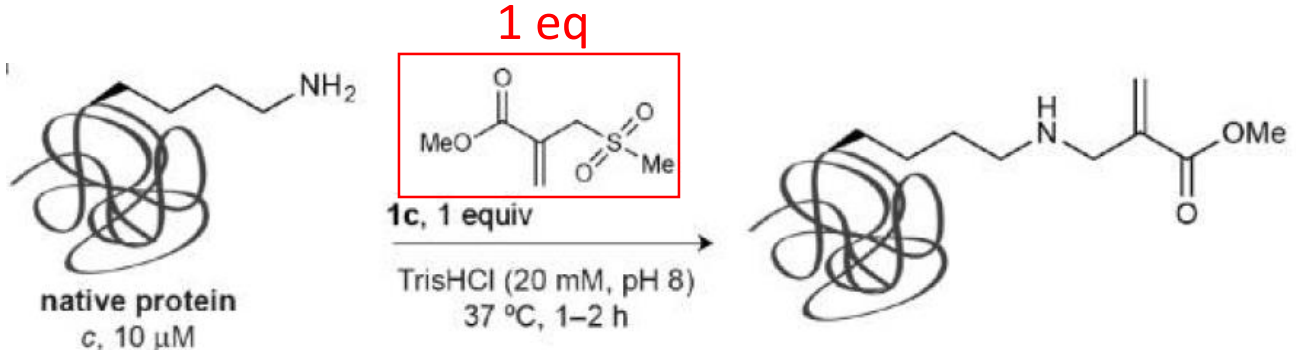


- ✓ Fast
- ✓ Full conversion

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Substrate scope

- Application to various type of native protein-



Conversion (%) native protein–1c (pH8, 37 °C) = 100

Conjugates are stable → Application to ADC is expected(Trastuzumab)

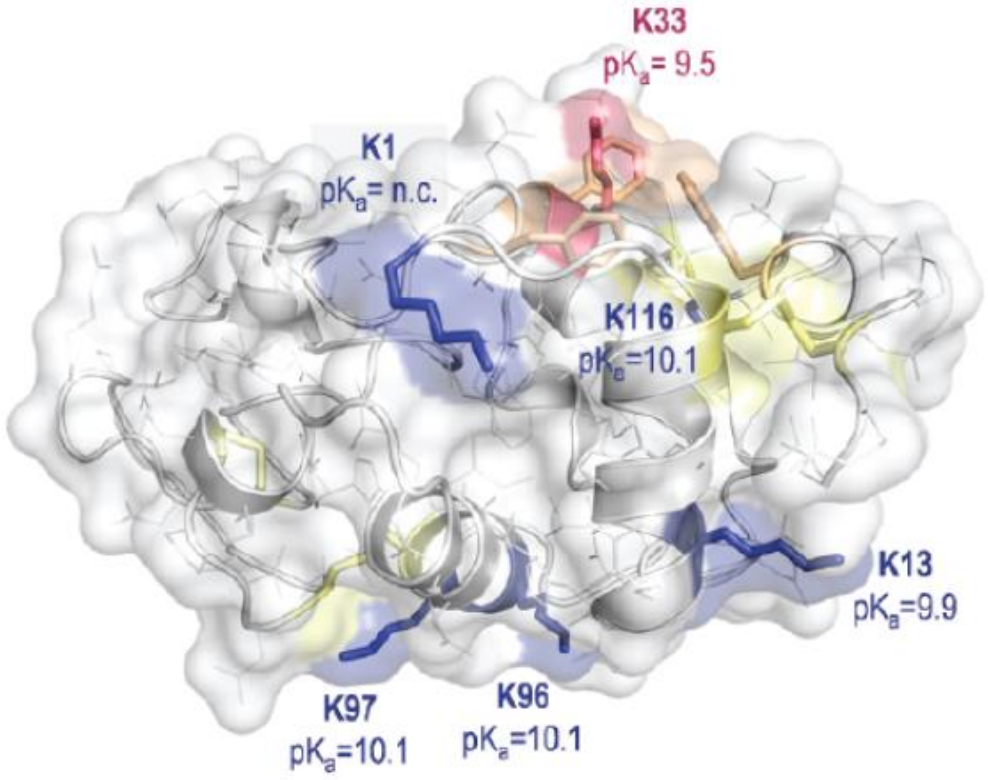
Regioselective lysine modification is applicable to a wide-range of native protein scaffolds.

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Relationships between p*K*a & reactive site

- Sulfonyl acrylate（Lysine activated）-

Theoretical calculation of the most reactive lysine residue

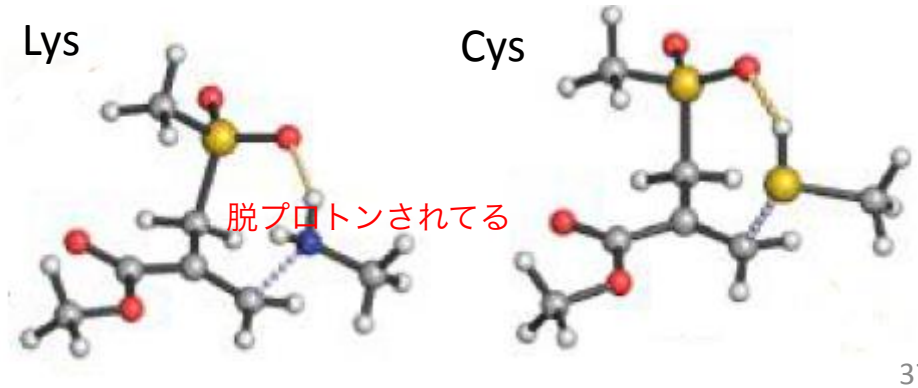


Lysozyme (6 Lys, no free Cys, 5 disulfides)

Predicted site of modification: K100
Observed site of modification: K100

	p*K*a	modification
K100	10.1	observed
C95	10.3	Not observed

The selectivity of Lysine residue was observed because of Hydrogen bond.



Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004−4017

Site-specific labeling

- The ability to precisely conjugate fluorophores and cytotoxic drugs to antibodies -

Conventional antibody labeling

… relied on labeling using disulfide bonds with genetically engineered free lysine residues or non-natural amino acids.

→Produce heterogeneous compounds

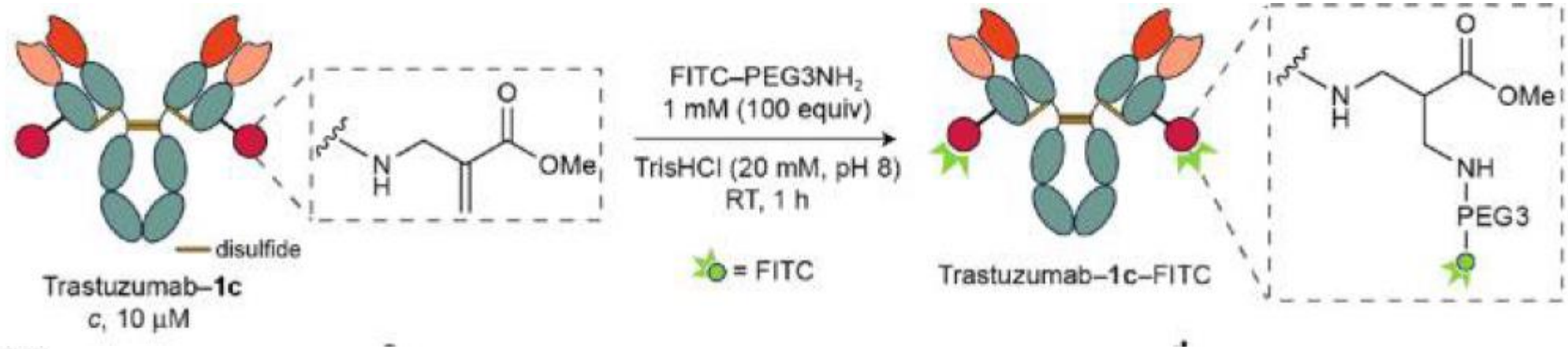
There are possibility that affinity with the target antigen may be compromised.

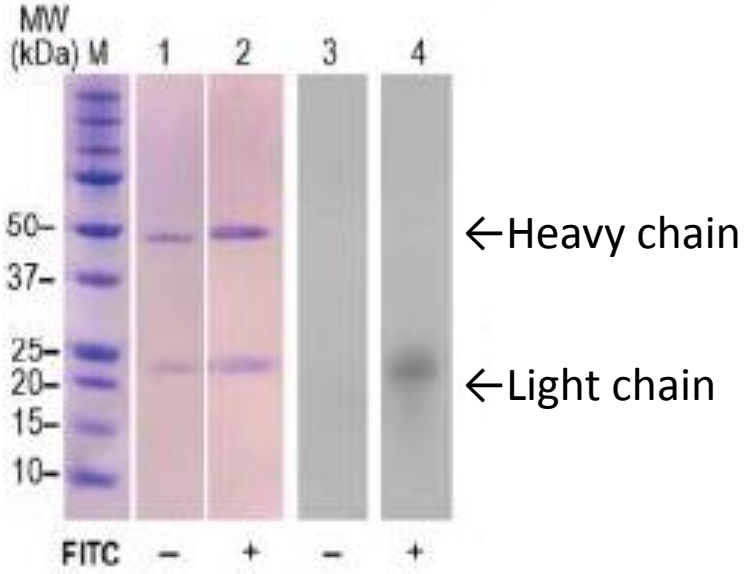
This method can be applied directly to a therapeutic antibody in its native form.

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Site-specific labeling

- The ability to precisely conjugate fluorophores and cytotoxic drugs to antibodies -



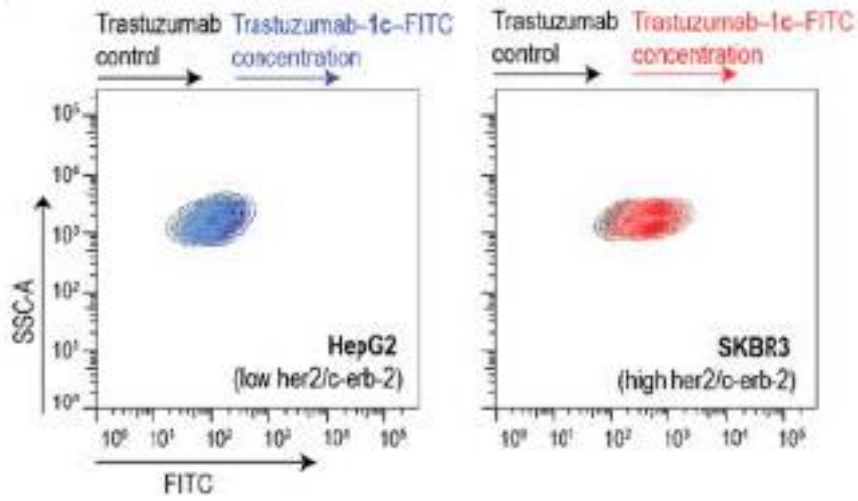


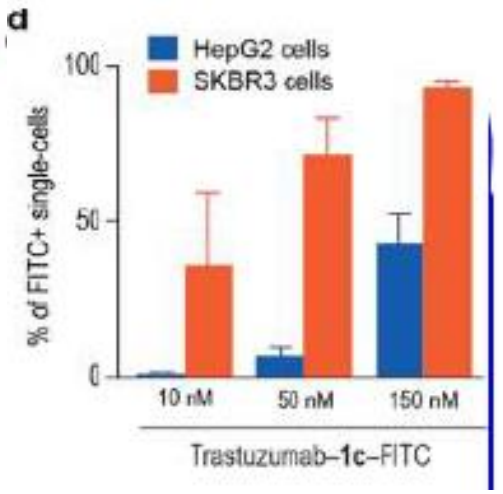
←Heavy chain

←Light chain

SDS-PAGE

Flow cytometry





Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Contents

- Amino acid target for bioconjugation
- Chemical Lysine modification
  - *N*-hydroxysuccinimide (NHS)-ester
  - α, β-unsaturated sulfonamide
  - Sulfotetrafluorophenyl esters
  - β-lactam
  - Sulfonyl acrylate（Lysine activated)
- Summary

Summary

- Lysine residues are abundant and easy to taeget because of their high reactivity.
- A general way to selectively target single Lysine is lacking.
- This method can provide a single lysine one step modification with complete chemo- and region-selectivity.
- Direct applicability to wild-type protein sequence bode well for routinely accessing site-selectively modified proteins for basic biology and therapeutic applications.

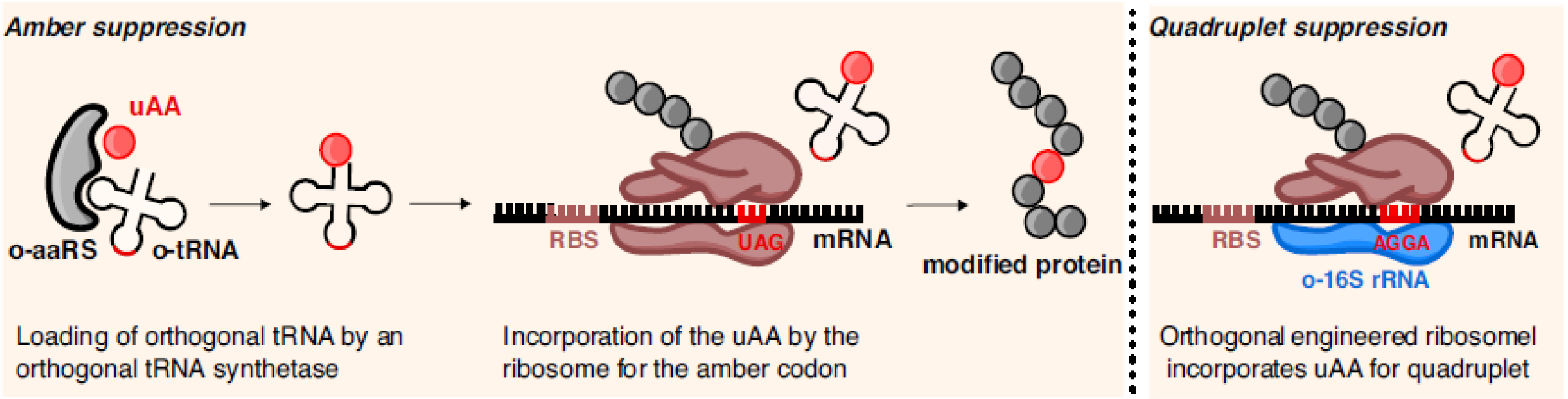
Appendix

Various types of modification

- Ligation
- Genetic Code expansion
- Ligand – directed type
- Chemical conjugation using Dha

Various types of modification

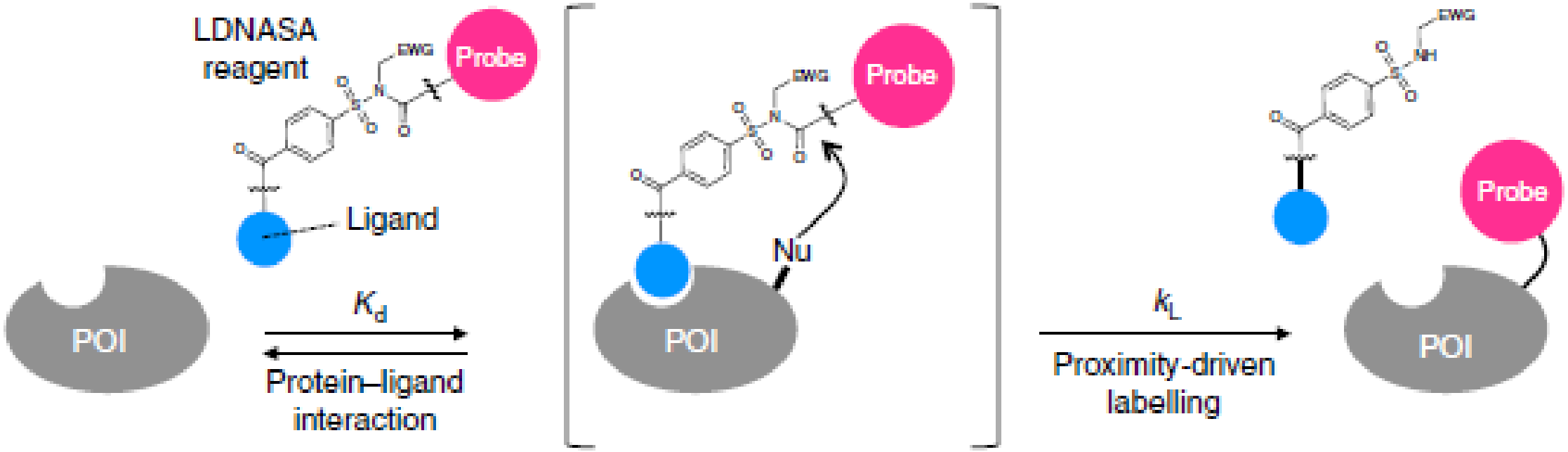
- Genetic code expansion



Current Opinion in Chemical Biology 2018, 45:35–47

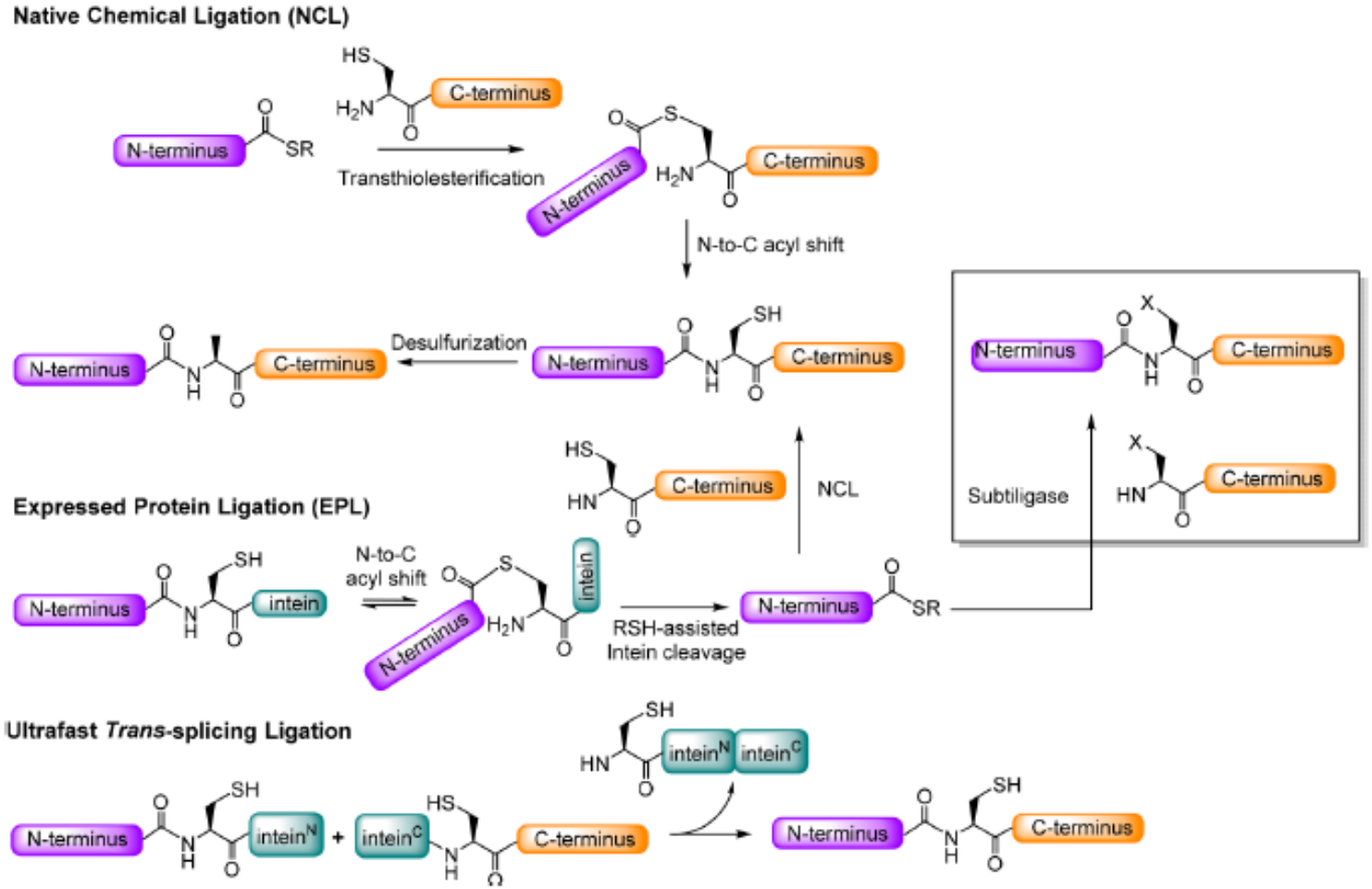
Various types of modification

- Ligand-directed type



Various types of modification

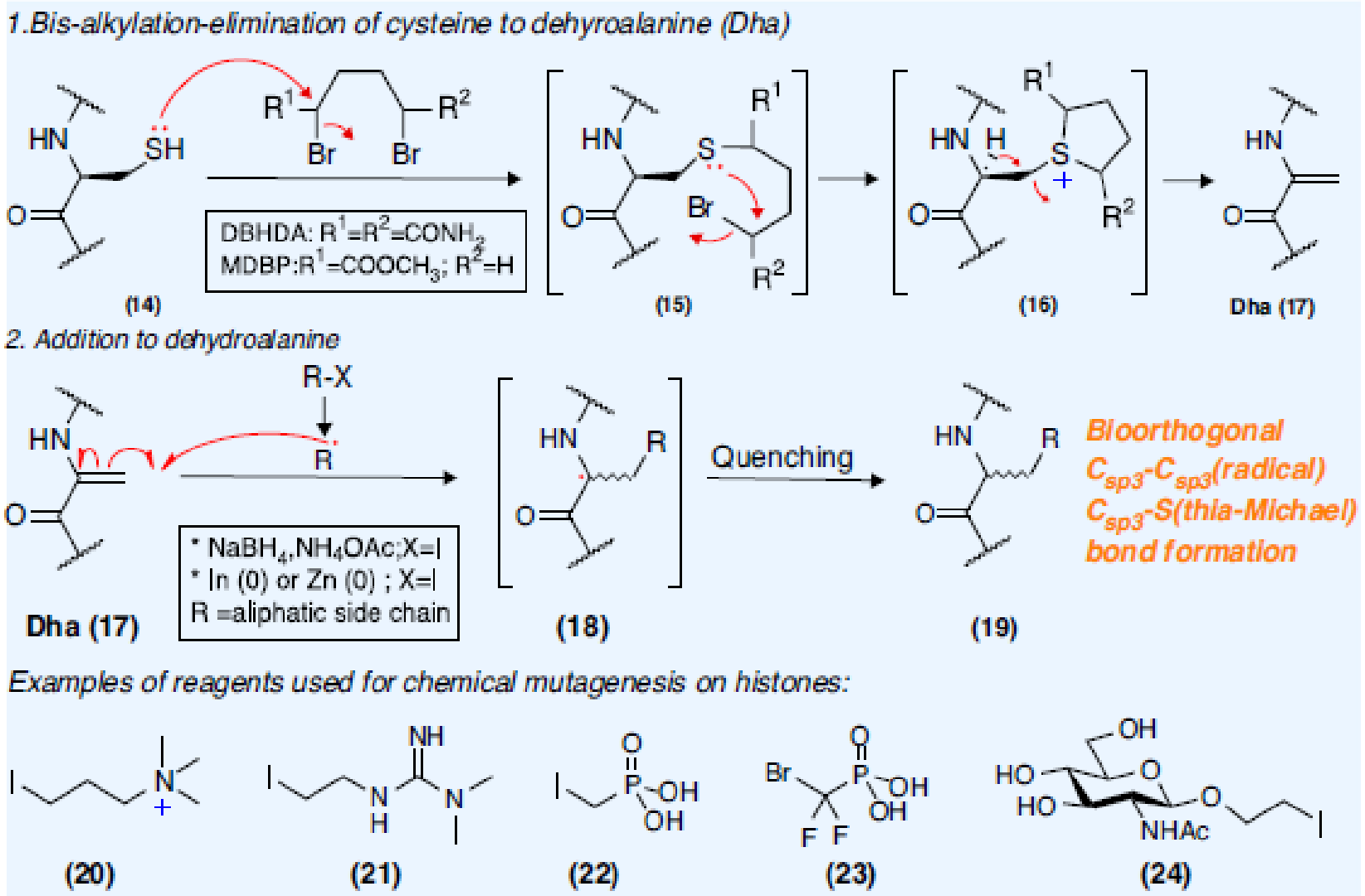
- Ligation



Chem. Rev. 2018, 118, 6656–6705

Various types of modification

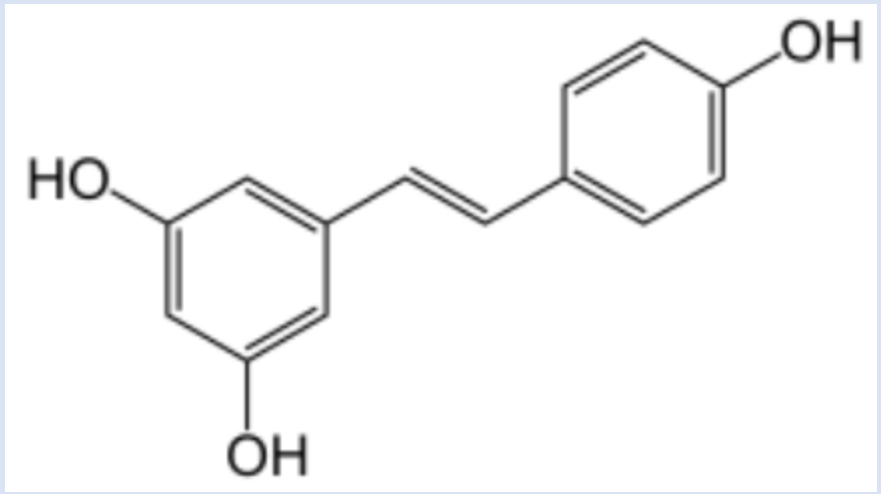
- Chemical conjugation using Dha



Chemical Biology, **2018**, *45*:35–47

Chemical Lysine modification 4

- Stilbene- *designed stilbenes that selectively and covalently modify the prominent plasma protein transthyretin*

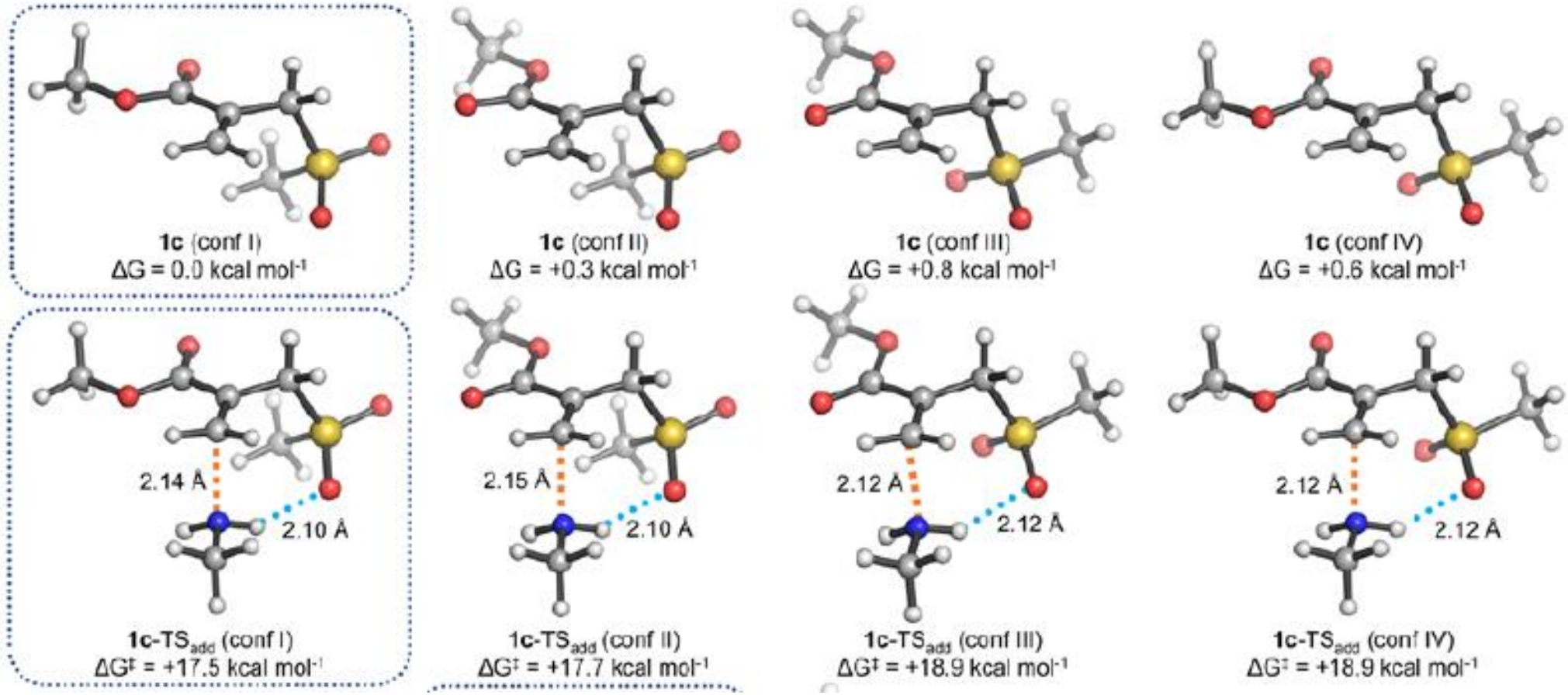


Resveratrol

- ✓ Noncovalent TTR kinetic stabilizers
- ✓ It is known to prevent amyloid formation–associated cytotoxicity, whereas structurally related compounds with poor TTR binding capacity do not inhibit cytotoxicity

Chemical Lysine modification 6

- Sulfonyl acrylate（Lysine activated）-

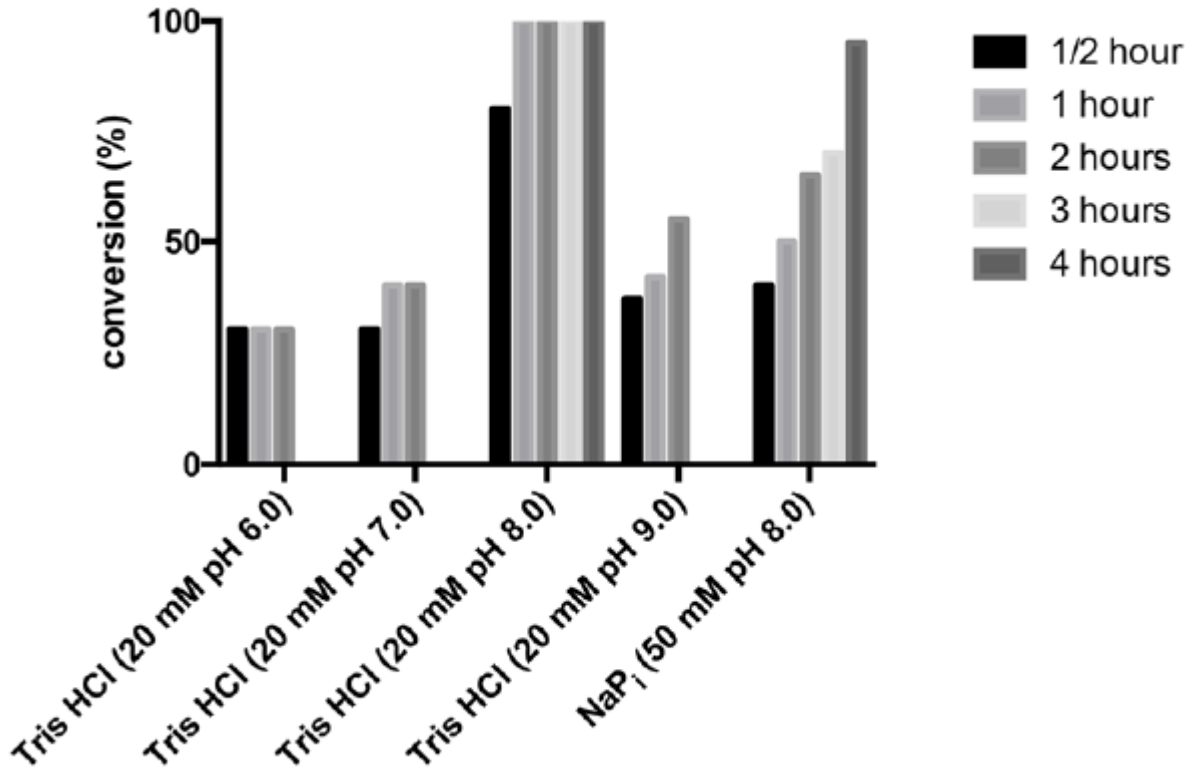


Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Chemical Lysine modification 6

Optimisation of reaction conditions between rHSA and **1c** with respect to pH, buffer and time

Reaction conditions		**Conversion (%) rHSA–1c (10 μM, 37 °C)**				
Buffer	pH	Time (h)				
		½	1	2	3	4
Tris HCl 20	6.0	30	30	30	-	-
mM	7.0	30	40	40	-	-
	8.0	80	**100**	100	100	100
	9.0	37	42	55	-	-
NaPi 50 mM	8.0	40	50	65	70	95



Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017

Chemical Lysine modification 3

Target protein: **Lysozyme**
Production run: **40 ns**
Protonation state change attempted every **5** simulation steps

pH	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0
LYS13	0.999993	0.999947	0.997636	0.992302	0.945720	0.398367	0.188996	0.012162	0.001551	0.000046
LYS33	0.999820	0.999562	0.997475	0.949310	0.775744	0.208294	0.032031	0.004438	0.000303	0.000018
LYS96	0.999989	0.999846	0.998788	0.990135	0.900597	0.547084	0.099162	0.011172	0.001762	0.000174
LYS97	0.999971	0.999822	0.997938	0.978243	0.900403	0.554339	0.091912	0.011426	0.001499	0.000092
LYS116	0.999996	0.999961	0.998962	0.986439	0.898668	0.542344	0.079672	0.010680	0.000642	0.000143

Protonated fraction ($1 - f_d$) for each residue as a function of the pH

Bernardes *et al.*, *J. Am. Chem. Soc.* 2018, *140*, 4004–4017