Stapled peptide with further functionality

2025/02/06
Literature Seminar
B4 Suzuki

Today's Contents

1. Introduction
   History of stapled peptides

2. Literature
   Ⅰ. Glowing staple
   Ⅱ. Reversible staple
   Ⅲ. Linkers with different functionality

3. Summary

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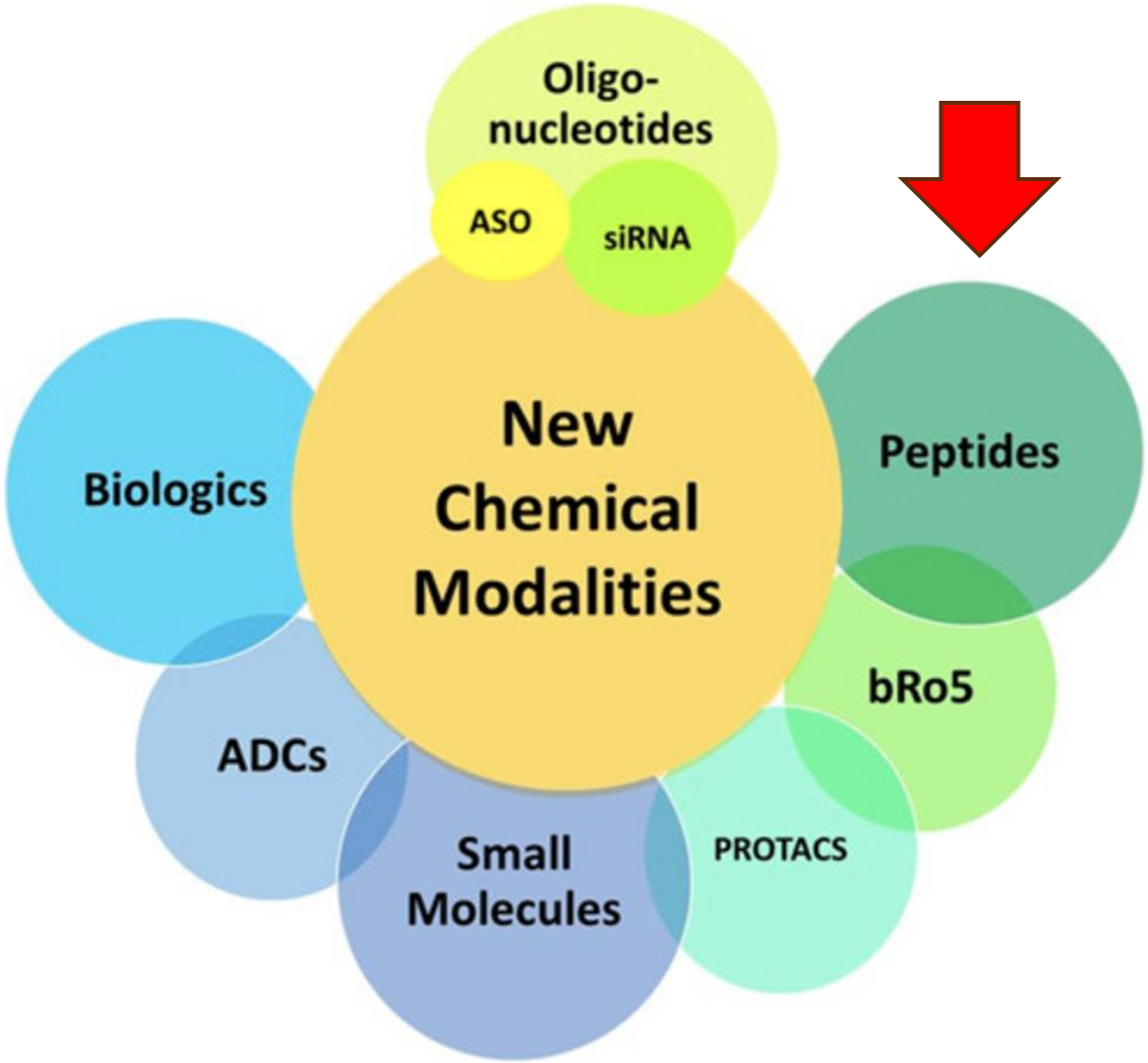
Novel chemical modalities



Drug discovery and development has experienced an incredible paradigm shift

"once" small molecules

→ "now" a variety of modalities

Peptides therapeutics

- the easily designed structure
- the appropriate molecular size
- a good cell penetrability
- a high binding affinity with the PPI interface

Blanco, *et al. ACS Med. Chem. Lett.* **2022**, *13*, 1691–1698.

The critical problem with peptide drugs

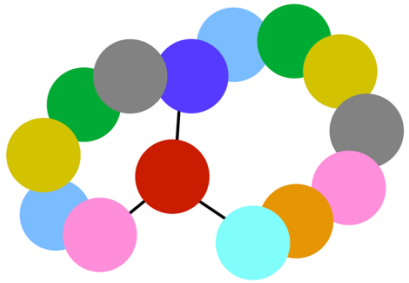
Linear peptide

low helicity
poor stability
uncontrollable hydrophobicity

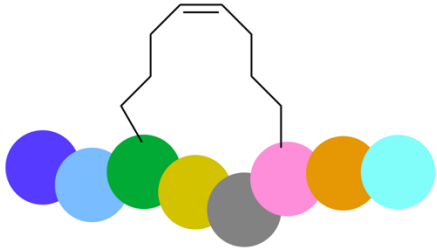
✗ cannot maintain structure and function due to lacking the structural support available from the other parts of protein

Strategies of peptide stabilization

Cyclic peptide



Bicyclic peptide

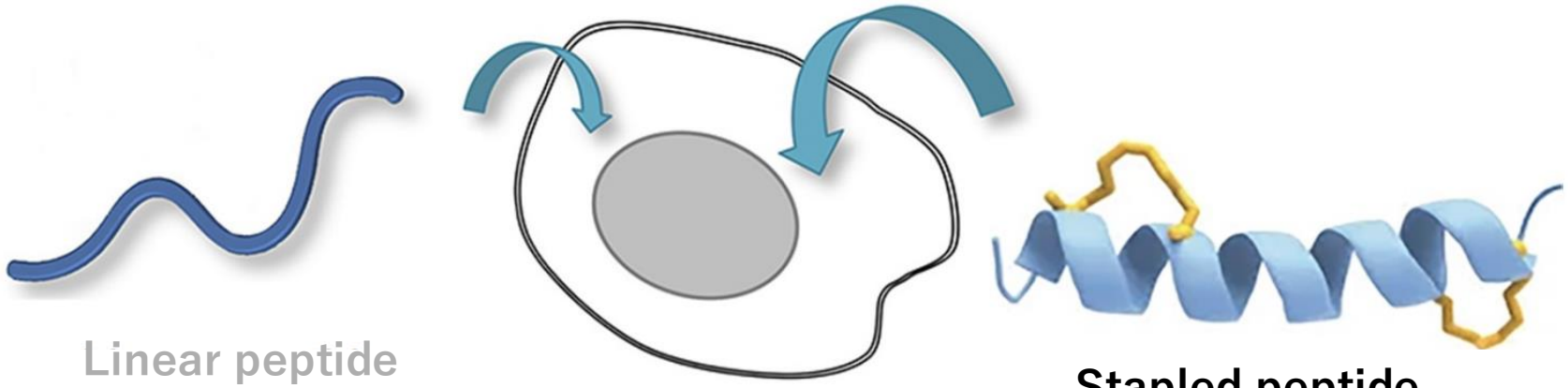


Staple peptide

- ✓ **more stable structure**
- ✓ **unique bioactive properties**
- ✓ **more resistance to proteolysis**

Zhang, *et al. Explor Drug Sci*. **2024**, *2*, 154–89.

Advantages of stapled peptides



Linear peptide

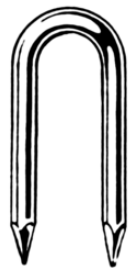
low helicity
poor stability
uncontrollable hydrophobicity

cell

Stapled peptide

high helicity
enhanced stability
programmable hydrophobicity

- ✔ **longer serum half-time**
- ✔ **higher levels of cell permeability**
- ✔ **stabilize the α-helix conformation → restore binding affinity**



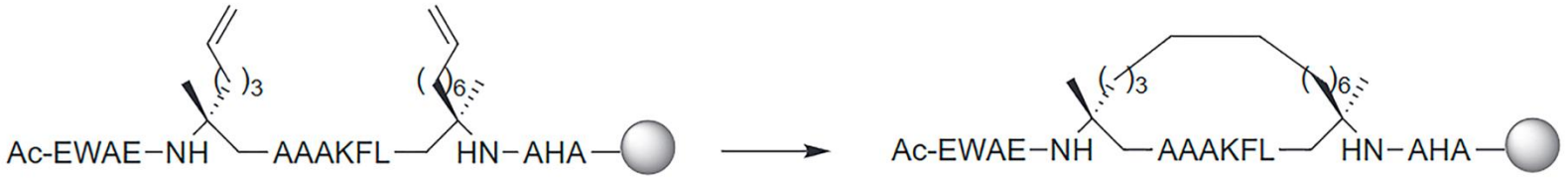
←Staple

Zhang, *et al. Explor Drug Sci*. **2024**, *2*, 154–89.

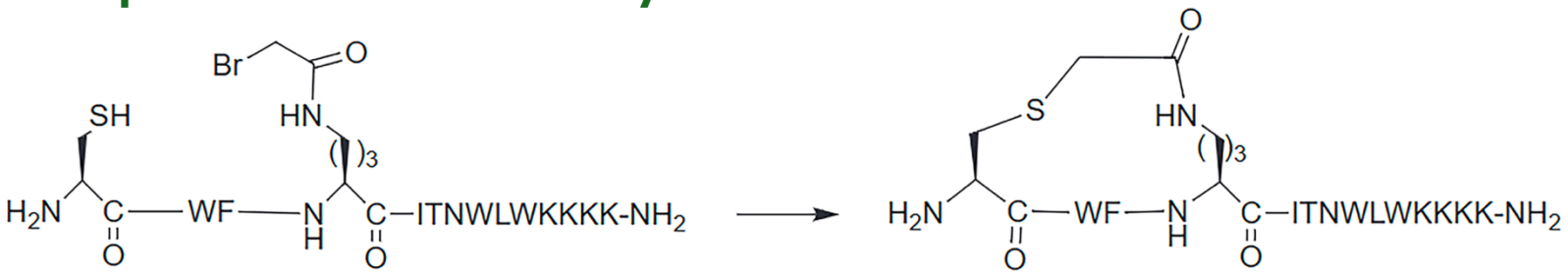
Reaction type of the staple

The recent advances of the stapled peptides

Olefin RCM

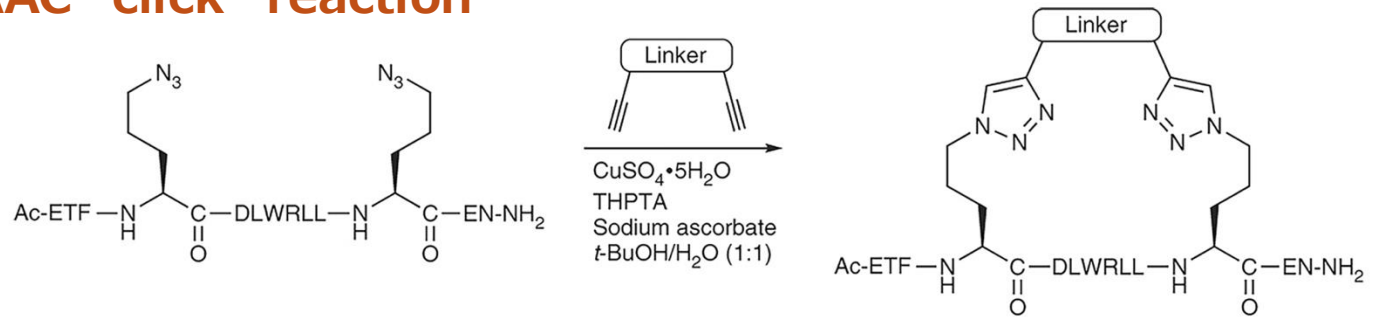


Nucleophilic substation of cysteine



What kind of reaction ?

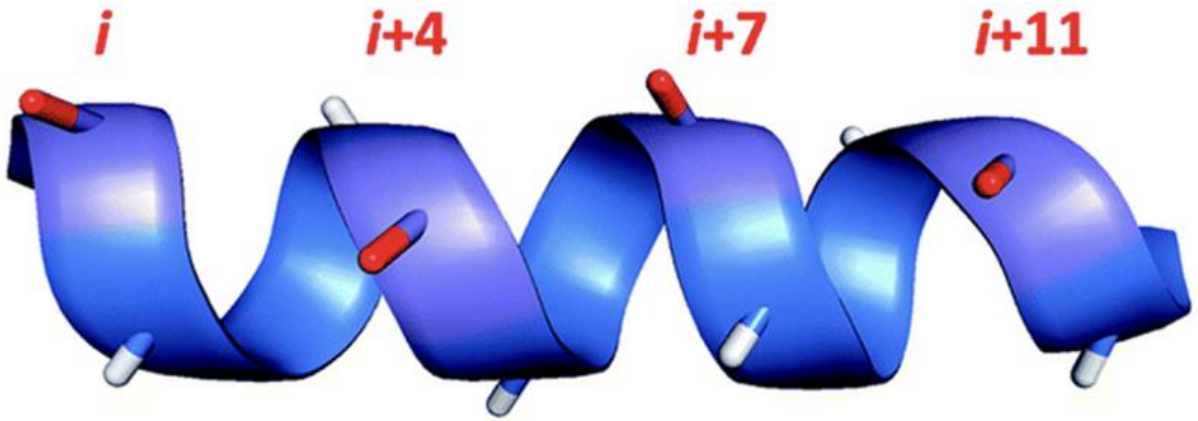
CuAAC “click” reaction



Zhang, *et al. Explor Drug Sci*. **2024**, *2*, 154–89.

Position of the staple

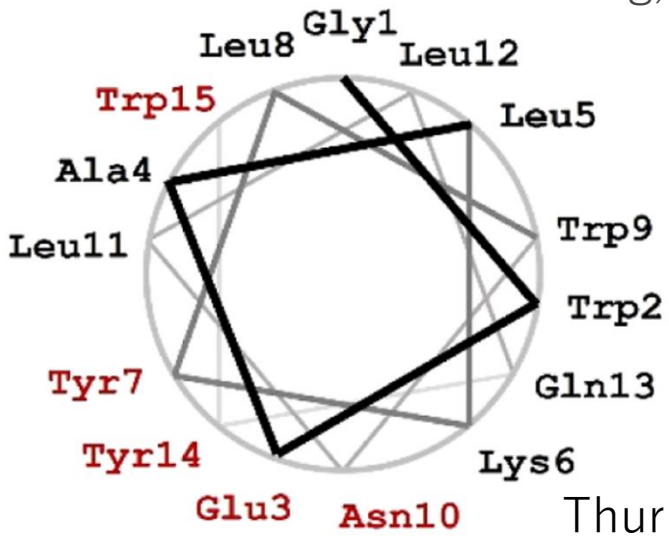
The recent advances of the stapled peptides



The spatial position of i, i+4/7/11 locating amino acids on a helical peptide

Zhang, *et al. Explor Drug Sci*. **2024**, *2*, 154–89.

Which position ?

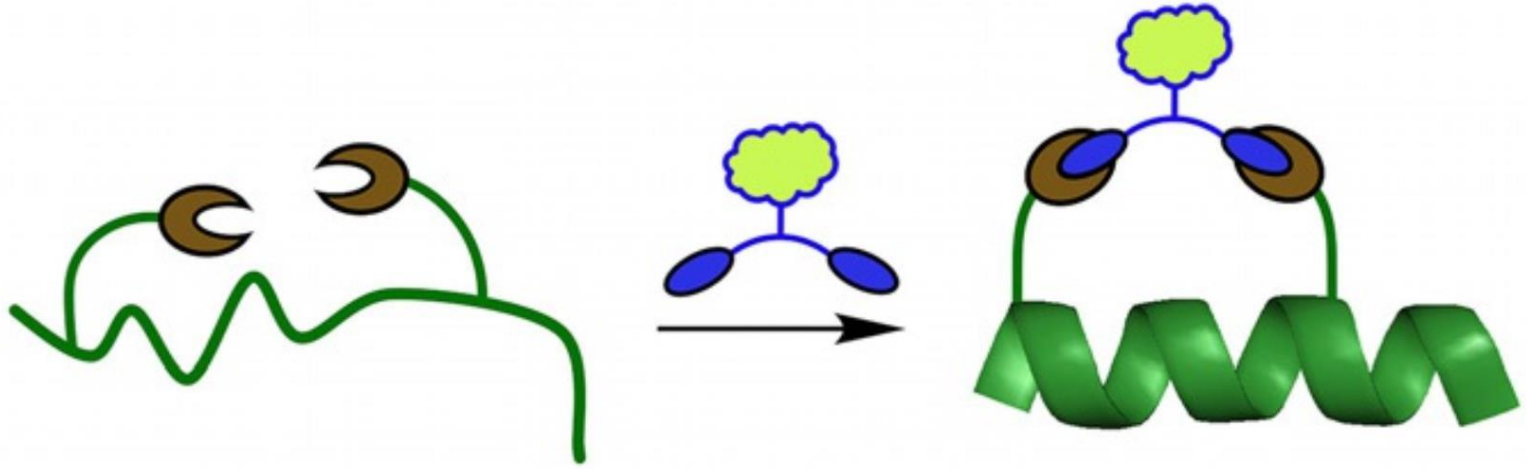


Wheel diagram of the F9170 peptide

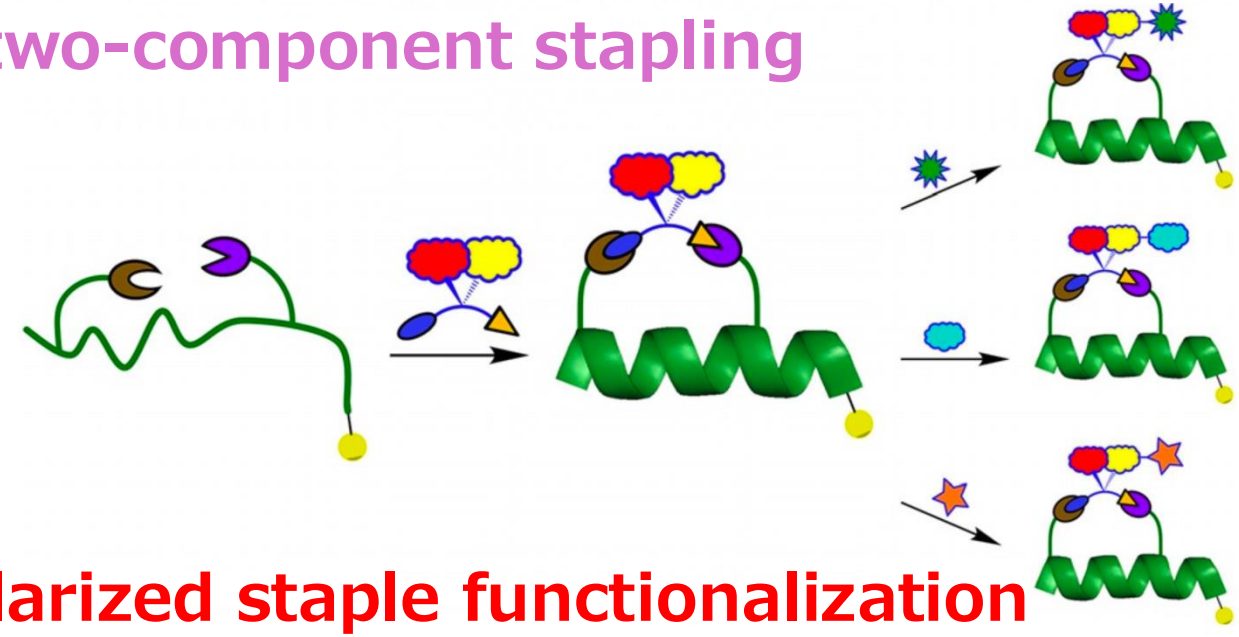
Thurber, *et al. J. Med. Chem.* **2024**, *67*, 9991-10004

Composition of the staple

The recent advances of the stapled peptides



two-component stapling

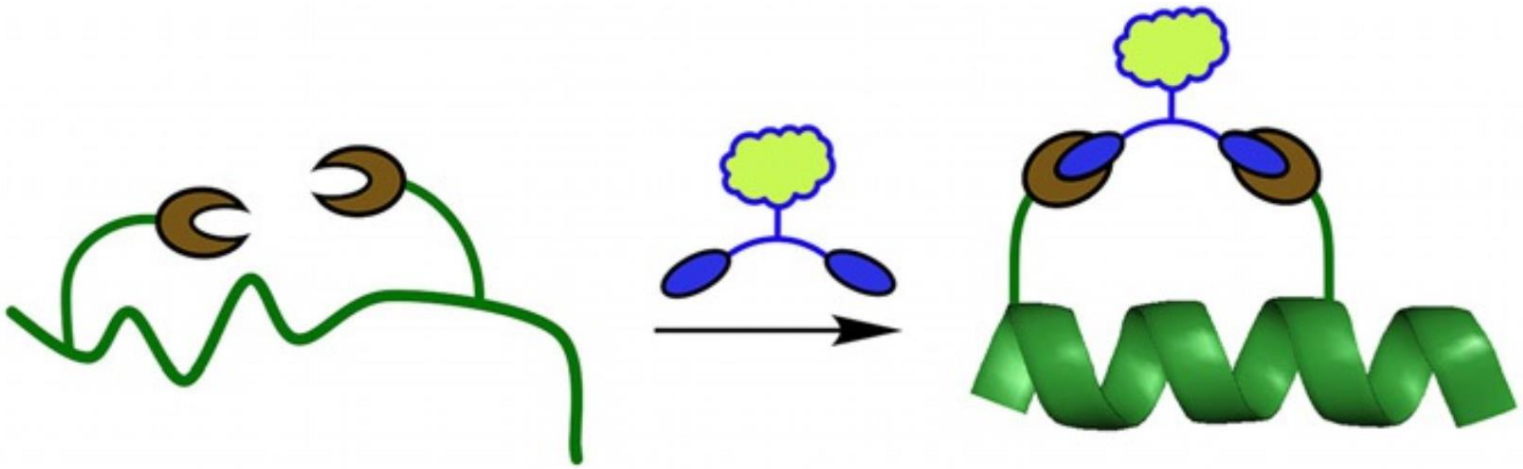


modularized staple functionalization

What kind of staples ?

Sipthorp, *et al. ChemBioChem* **2017**, *18*, 1066-1071.

Introduce additional functionality



What kind of staples ?

Sipthorp, *et al. ChemBioChem* **2017**, *18*, 1066-1071.

Stability of structures

Conventional Purpose

+

Additional functionality

New Purpose

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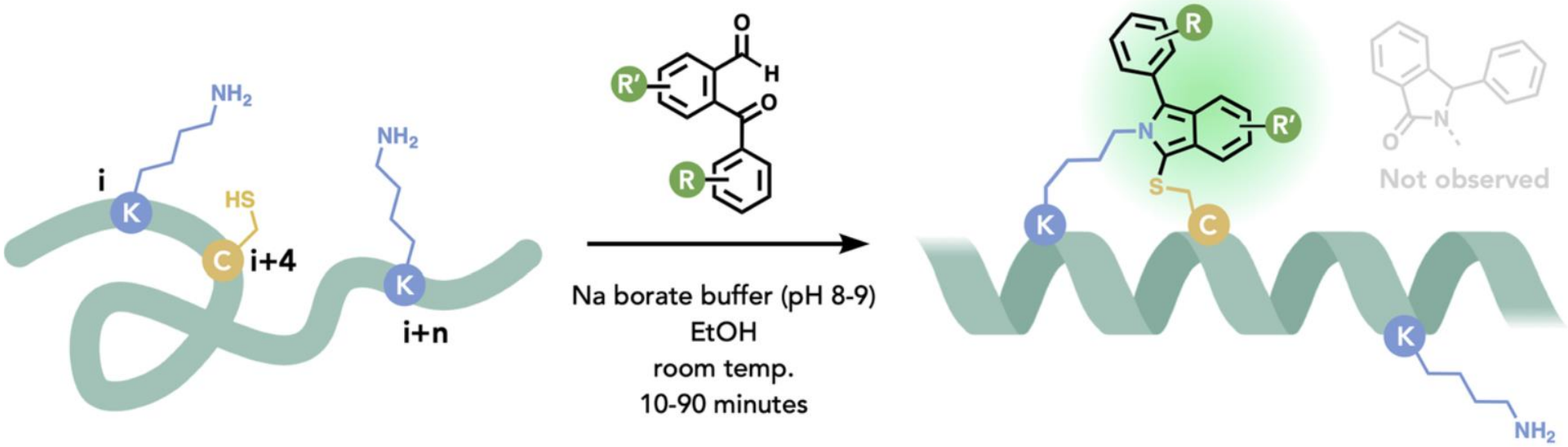
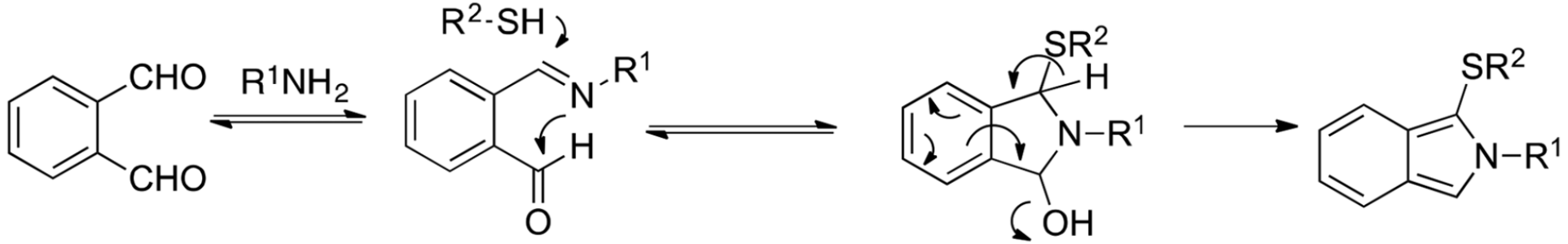
I -(1) Fluorogenic stapling

Fluorescent Isoindole Crosslink (FIICk)



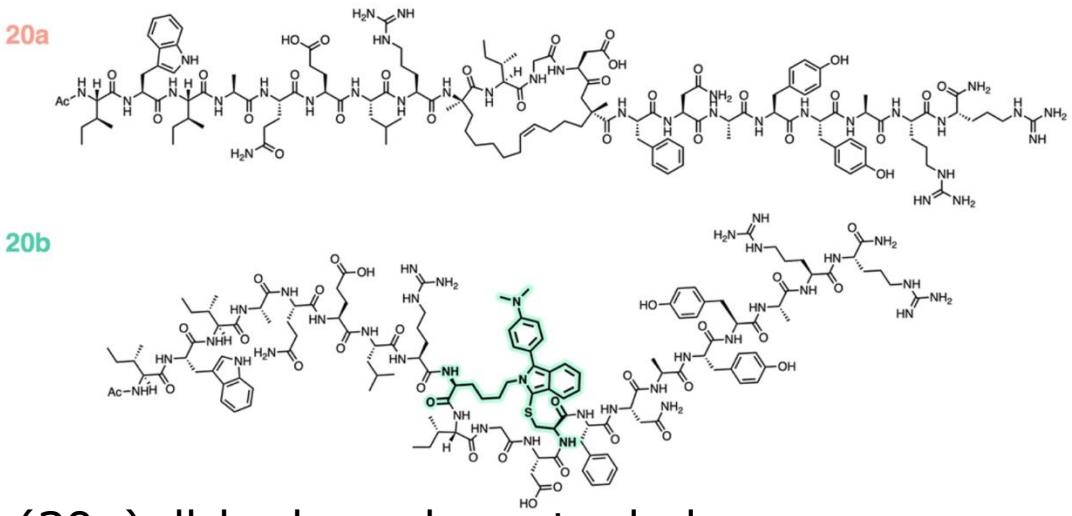
Reaction mechanism



Li, *et al. J. Am. Chem. Soc.* **2019**, *141*, 12274 – 12279

Perrin, *et al. Chem. Sci.* **2025**, *16*, 584–595

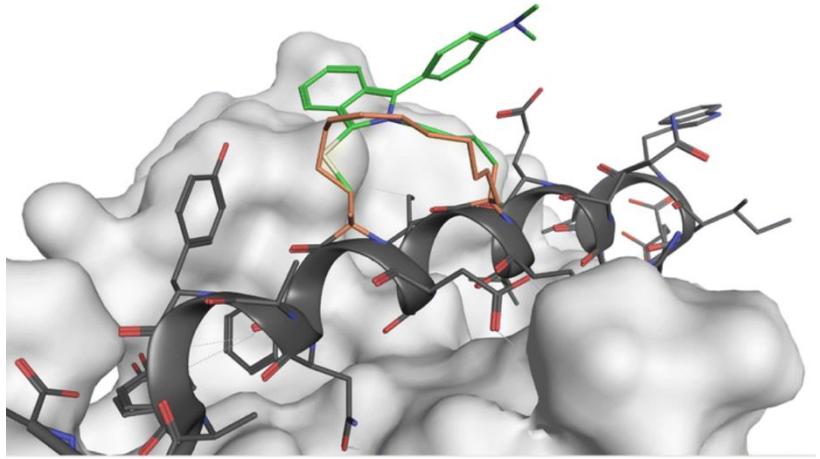
I -(1) Fluorogenic stapling

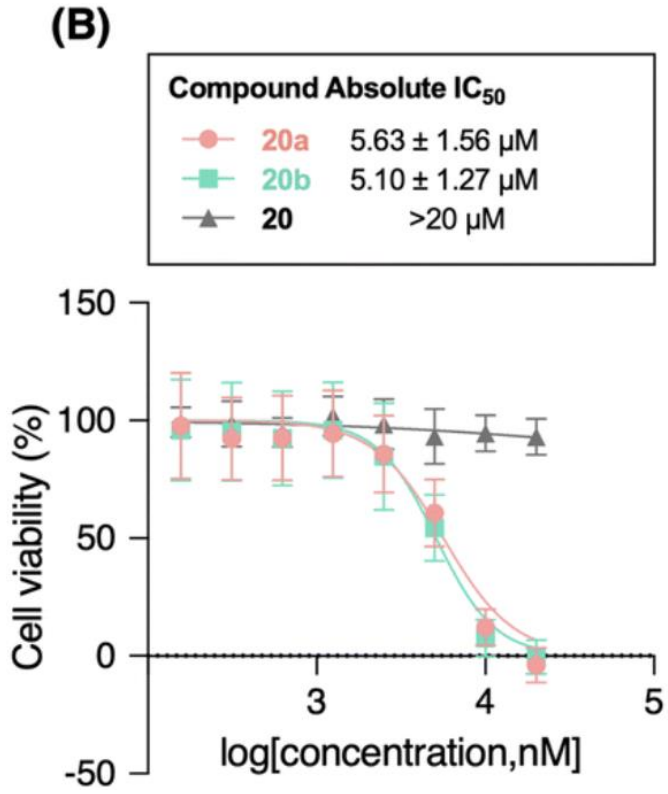


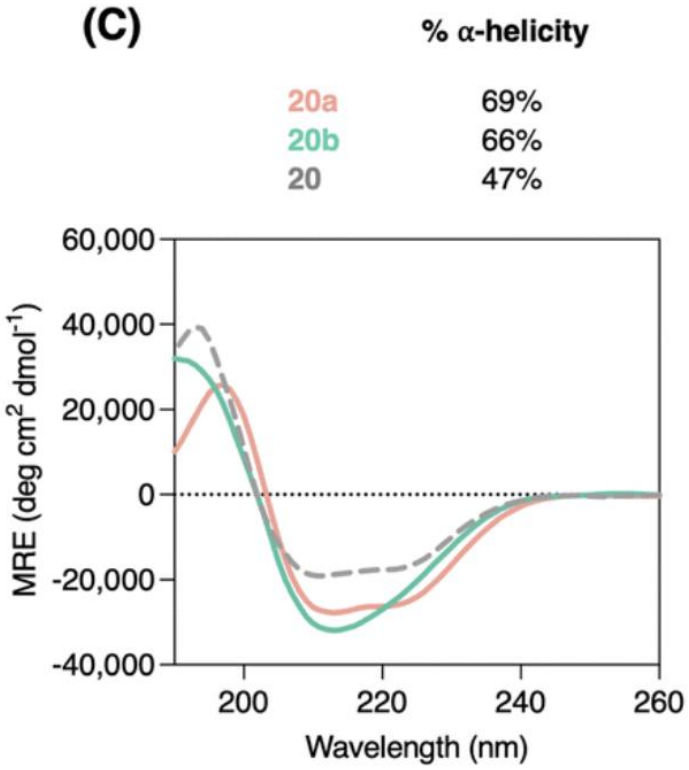
(20a)all-hydrocarbon stapled
(20b)FlICk-stapled

(A)

20a	Ac-IWIAQELR**X**IGD**X**FNAYYARR-NH_2
20b	Ac-IWIAQELR**K**IGD**C**FNAYYARR-NH_2
20	Ac-IWIAQELRRIGDEFNAYYARR-NH_2





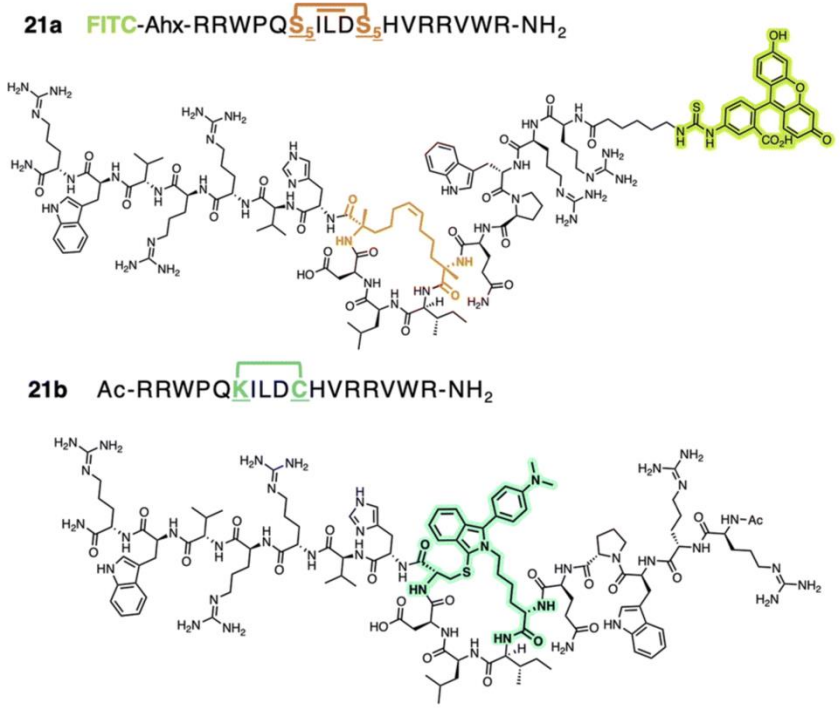


20b FlICk-stapled

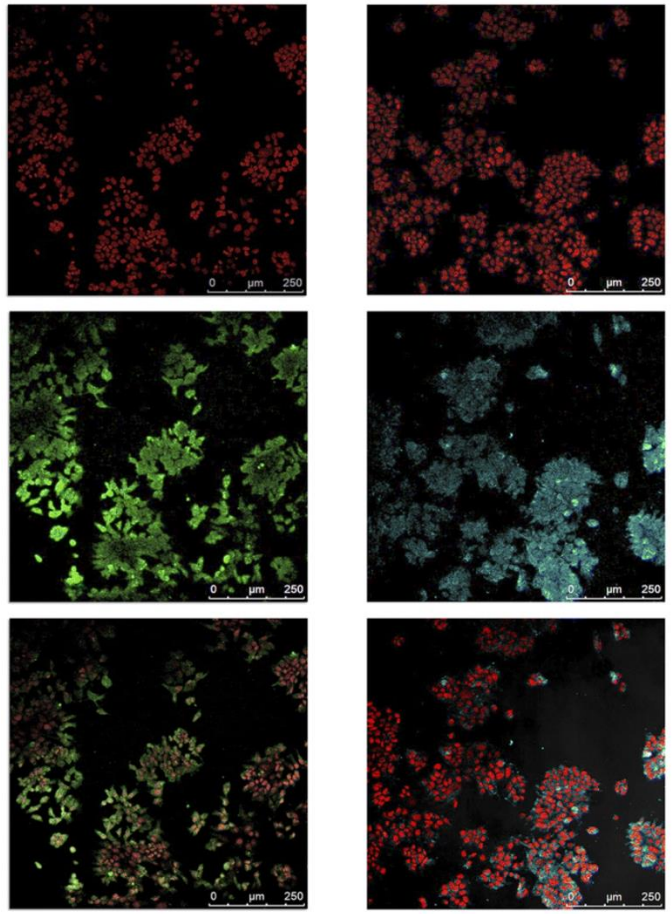
- **very similar stapling scaffold and helicity**
- **equipotent to bioactivity of (20a)**
- **comparable to helicity to (20a)**

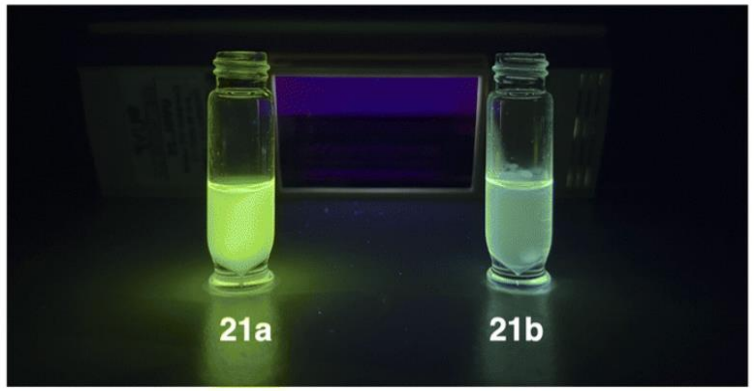
Perrin, *et al. Chem. Sci.* **2025**, *16*, 584–595

I -(1) Fluorogenic stapling



(21a)FITC-labeled peptide
(21b)FlICked-peptide





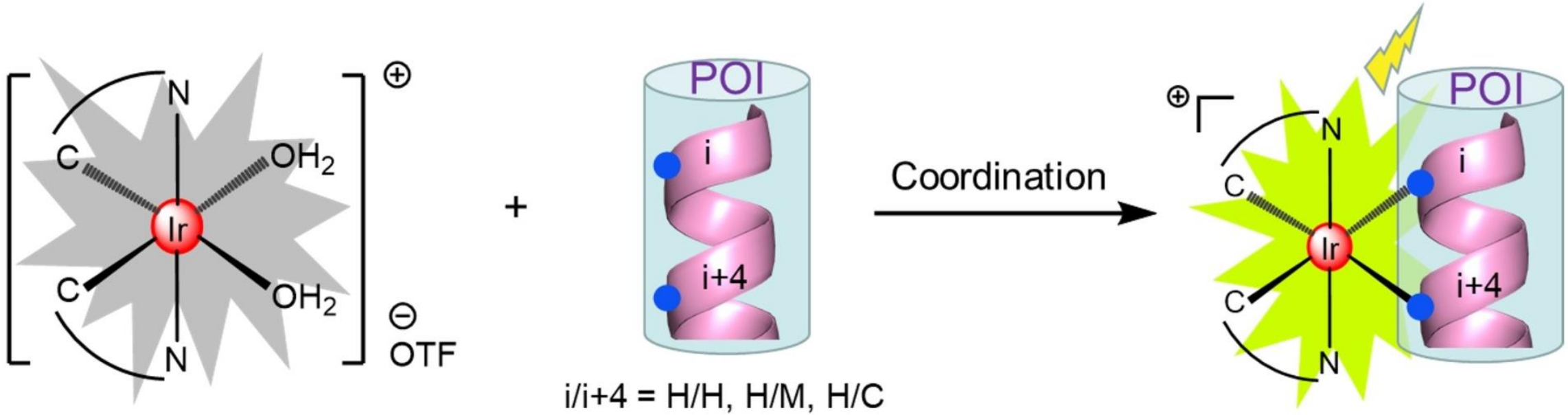
20b FlICk-stapled

- intracellular fluorescence. was observed (405nm).
- good cell permeability
- good stability

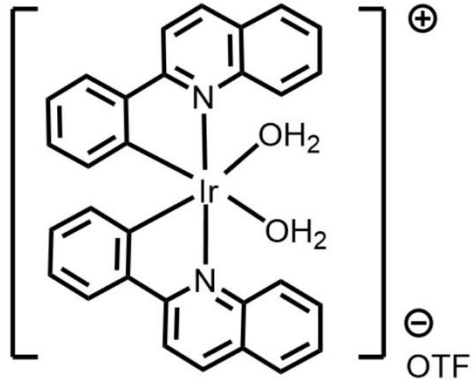
- structuring native a-helices to improve their bioactivity
- positional selectivity favoring i, i+4 helical staples (Lys & Cys)
- obviating the need to append a fluorophore

Perrin, *et al. Chem. Sci.* **2025**, *16*, 584–595

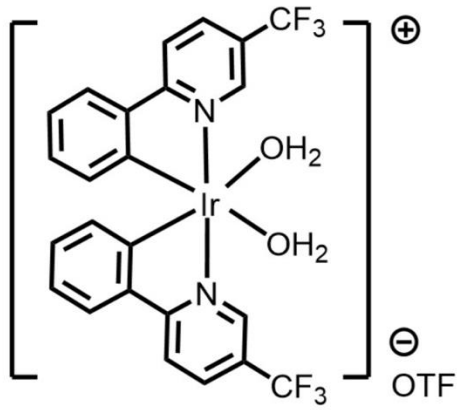
I -(2)Anchoring a luminescent tag in a Protein



i/i+4 = H/H, H/M, H/C



Ir5



Ir6

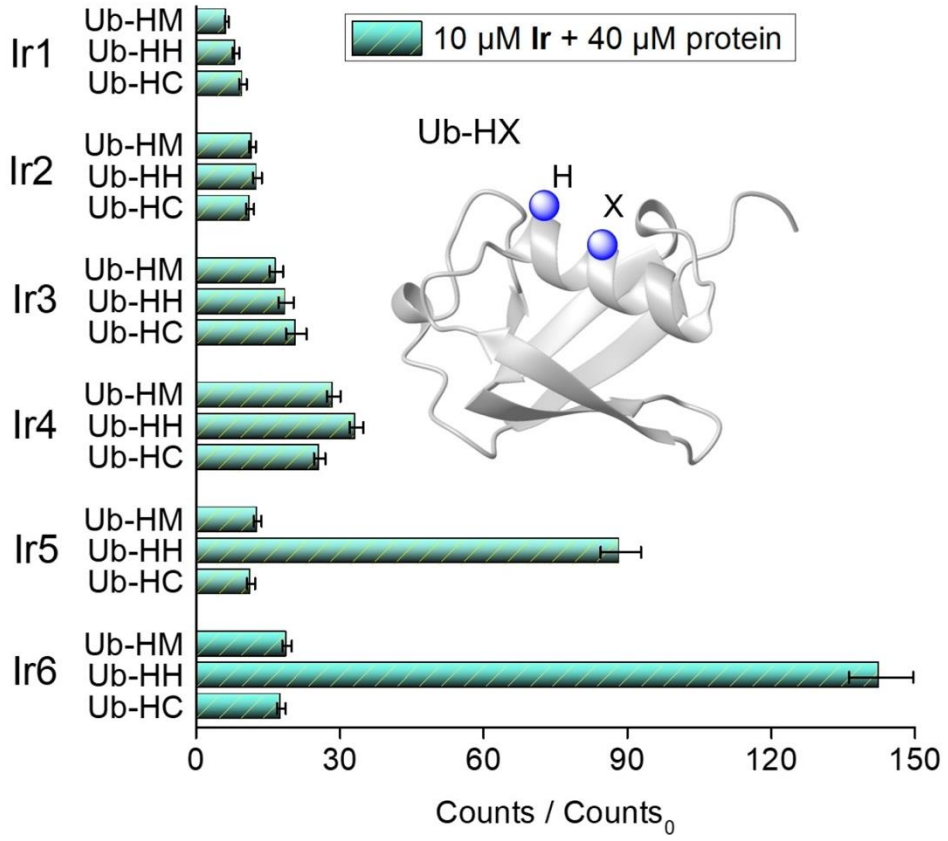
Iridium(Ⅲ) Complex

- **high cell permeability**
- **low cytotoxicity**
- **long emission lifetimes**
- **superior photostability**

site-specific labeling of a protein with non-emissive iridium(III) complex

Su, *et al. ChemBioChem* **2024**, *25*, 2-8

I -(2)Anchoring a luminescent tag in a Protein



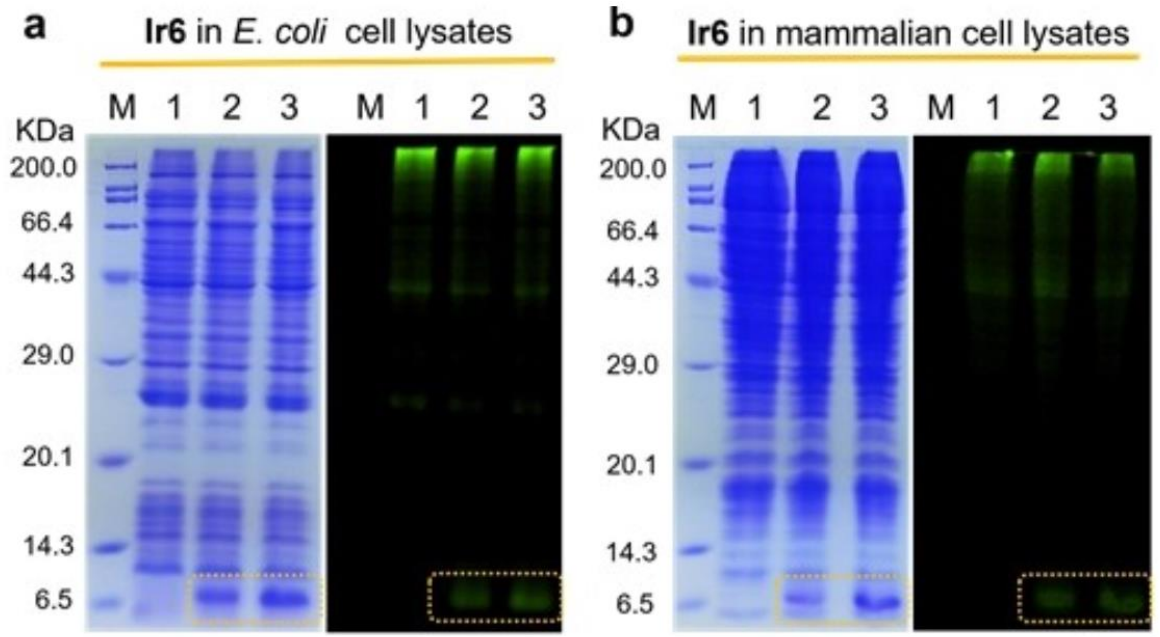


Figure 6. SDS-PAGE in-gel analysis of 100 mg/mL *E. coli* cell lysates (a) or 200 mg/mL mammalian cell lysates (b) treated with 30 μM **Ir6** and varing concentrations of Ub E24H/A28H (Ub-HH, Lane1: 0 μM, Lane2: 30 μM, Lane3: 60 μM) for 1.5 h at room temperature. The gel was analyzed by in -gel fluorescence imaging (right) and stained with Coomassie Brilliant Blue (left).

- **coordinating to His/His that are separated by i and i+4 in α-helix**
- **demonstrated in a specific and efficient manner in many proteins**

Su, *et al. ChemBioChem* **2024**, *25*, 2-8

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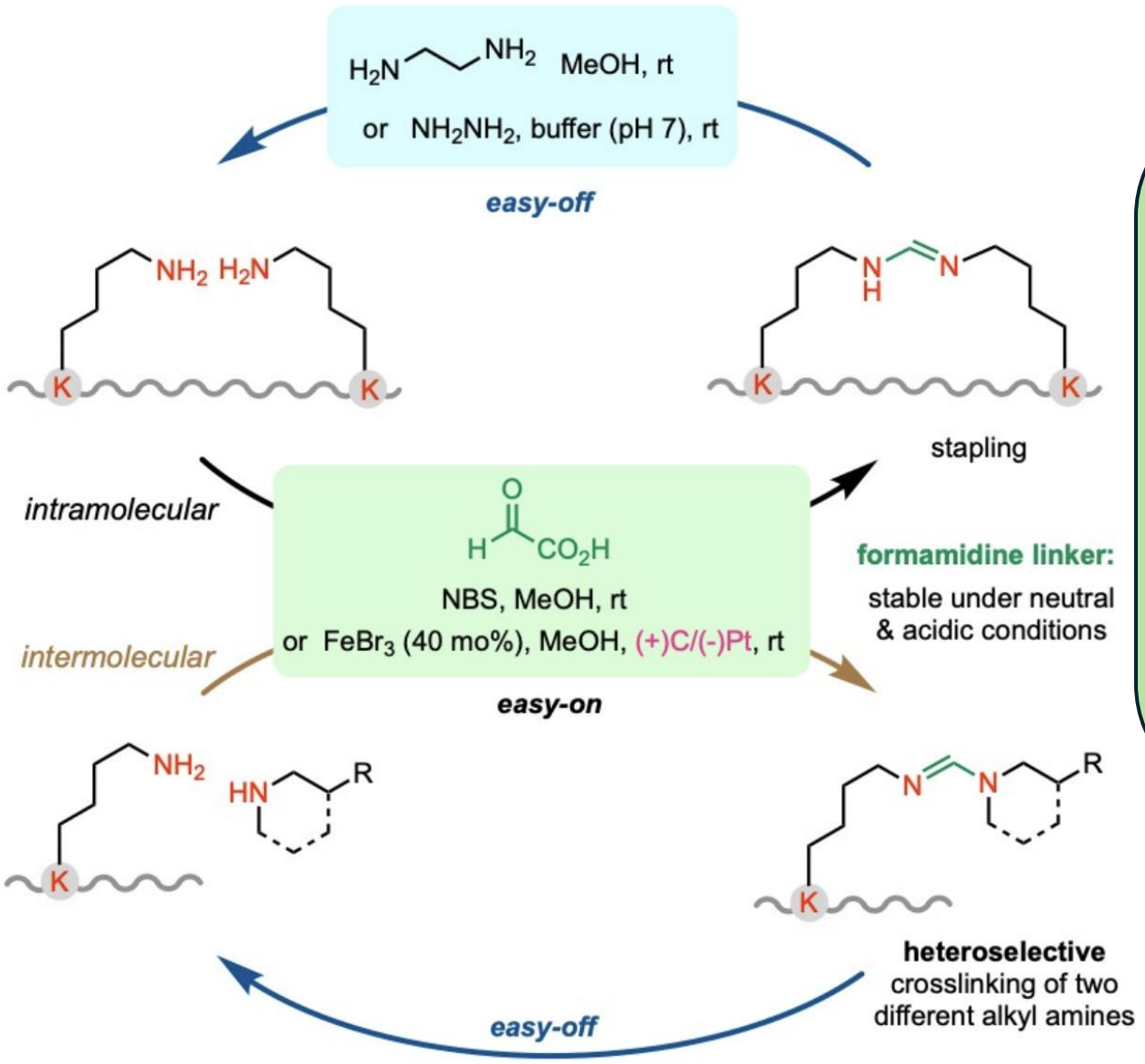
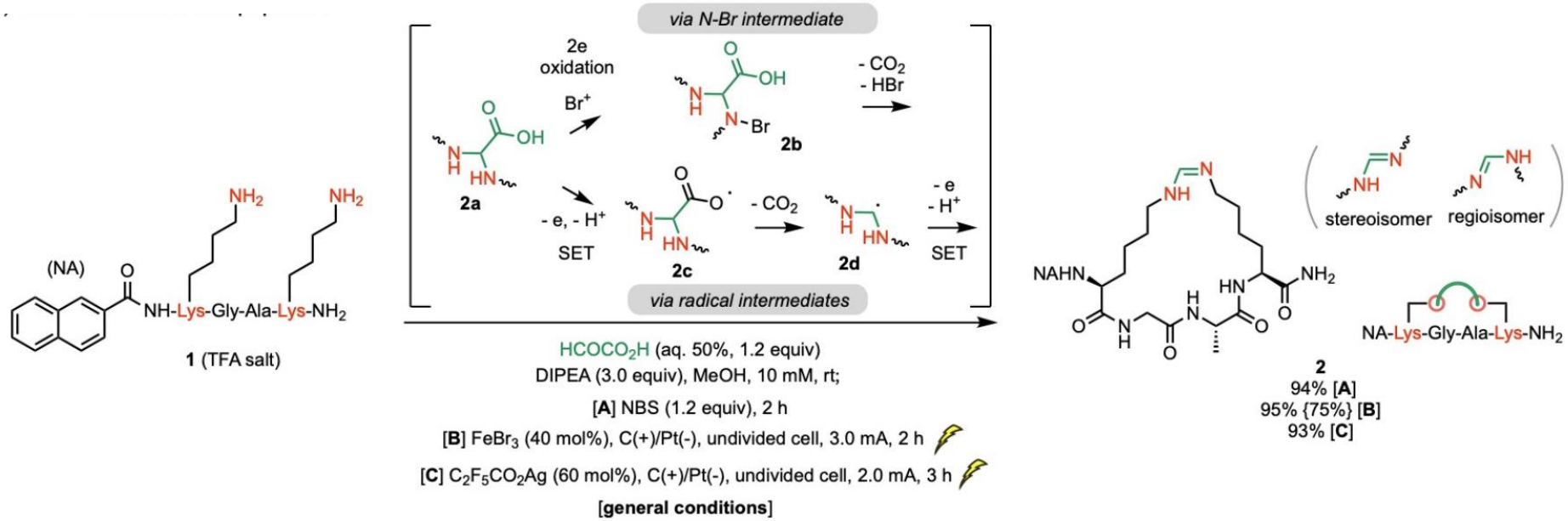
Ⅱ-(1)Reversible Lys-Lys linker

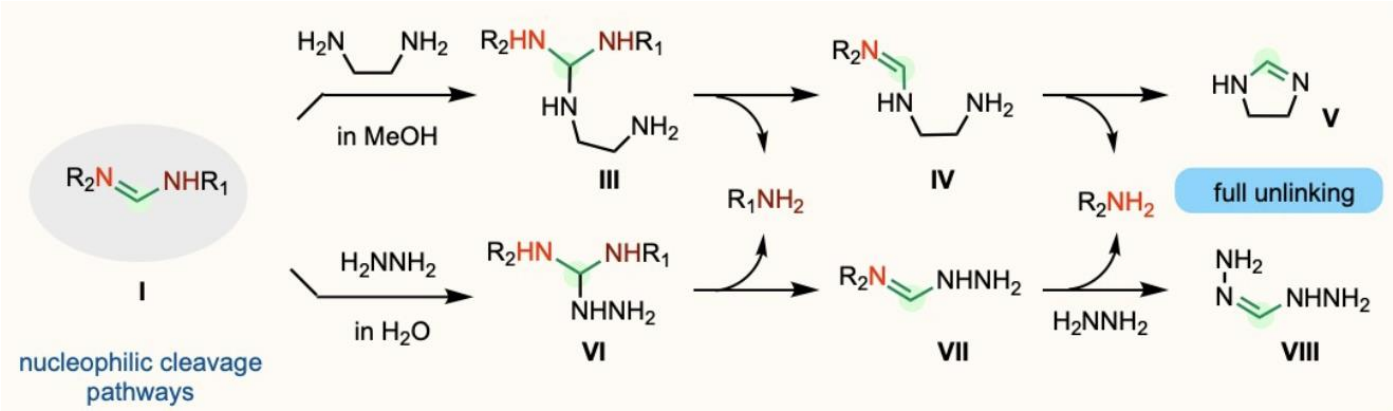


- the use of oxidative decarboxylative condensation of glyoxylic acid
- the formamidine linkage is stable under pH- neutral buffers and acidic conditions
- it can be readily cleaved with ethylenediamine or hydrazine under mild conditions
- two native amines(Lys) in peptide stapling
- easy-ON and easy-OFF linker

Chen, *et al. Angew. Chem. Int. Ed.* **2025**, e202422844

Reaction mechanism





Chen, *et al. Angew. Chem. Int. Ed.* **2025**, e202422844

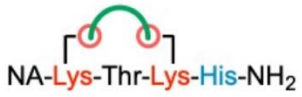
Ⅱ-(1)Reversible Lys-Lys linker

Scope

6, 95% [**A**]
94% {71%} [**B**]

7, 84% [**A**]
95% {74%} [**B**]

8, 95% [**A**]
93% {65%} [**B**]

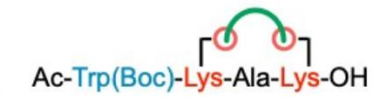


9, 76% [**A**]
91% {67%} [**B**][c]

10, 56% [**A**]
81% {58%} [**B**][d]

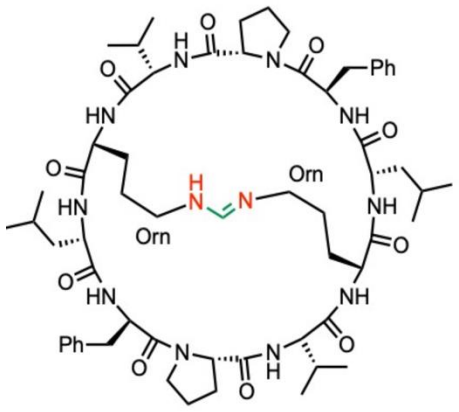
11, 82% [**A**][j]
88% {60%} [**B**][j]

12, 92% [**A**]
90% {65%} [**B**]



13, 88% [**A**]
95% {73%} [**B**][e]

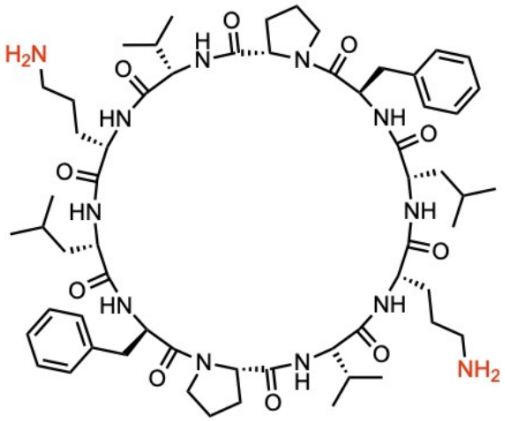
gramicidin S



20, 93% [**A**]
95% {70%} [**B**]

$N_2H_4 \cdot H_2O$ (10.0 equiv)
PBS (pH 7.0)/MeOH (4/1),
20 mM, 4 h

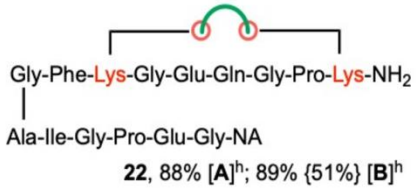
cleavage



54 gramicidin S, 95% {59%}

Reversible crosslinking system

Type II collagen fragment



22, 88% [**A**][h]; 89% {51%} [**B**][h]

$N_2H_4 \cdot H_2O$ (50 equiv)
PBS (pH 7.0), 20 mM, 2 h

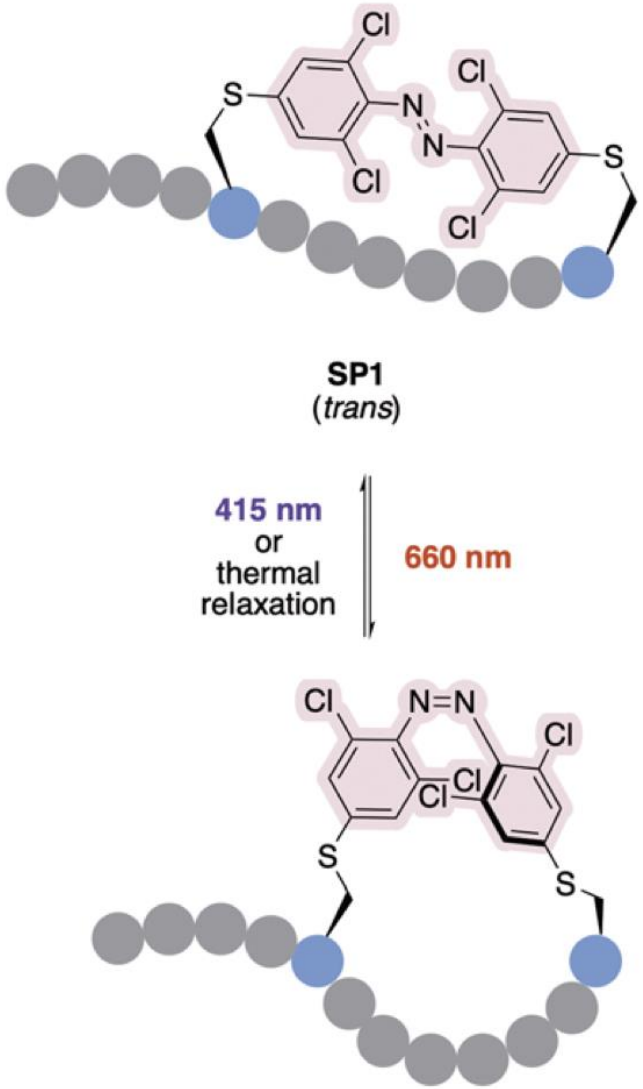
cleavage

NA-Gly-Glu-Pro-Gly-Ile-Ala-Gly-Phe-Lys-Gly-Glu-Gln-Gly-Pro-Lys-NH_2

Type II collagen fragment **55**, ~95%

Chen, *et al. Angew. Chem. Int. Ed.* **2025**, e202422844

Ⅱ-(2) Photoswitch peptide stapling

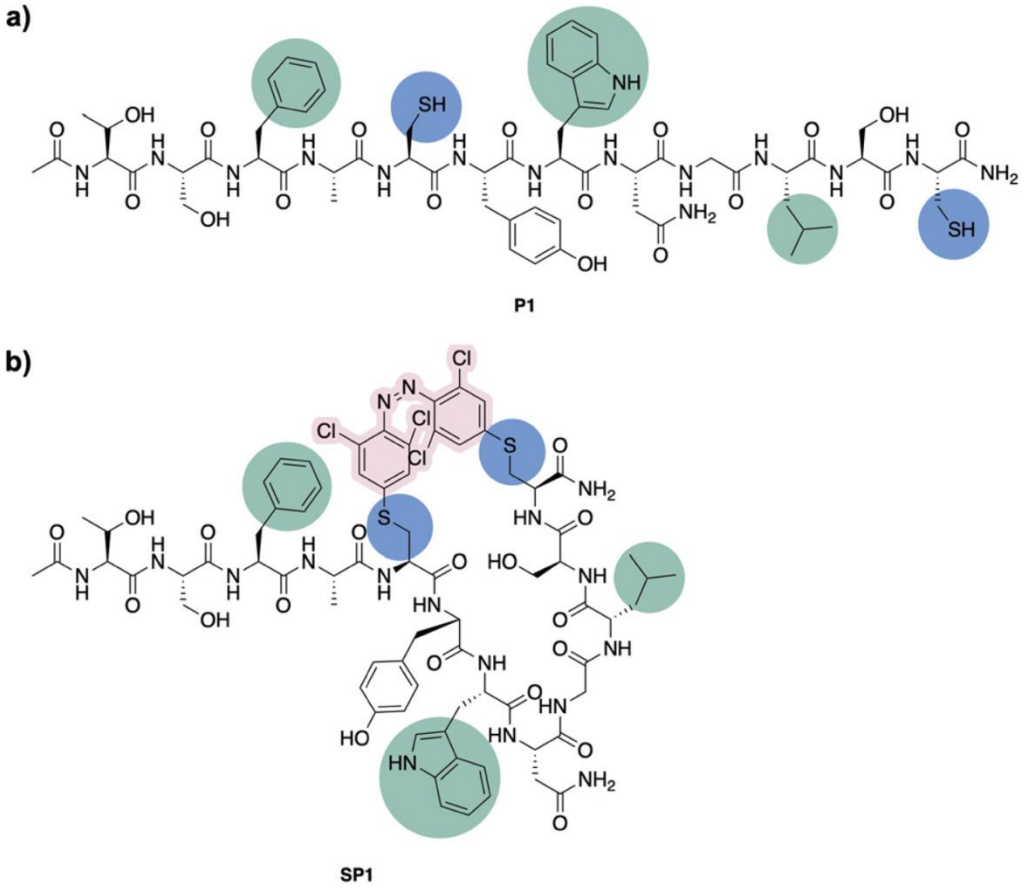


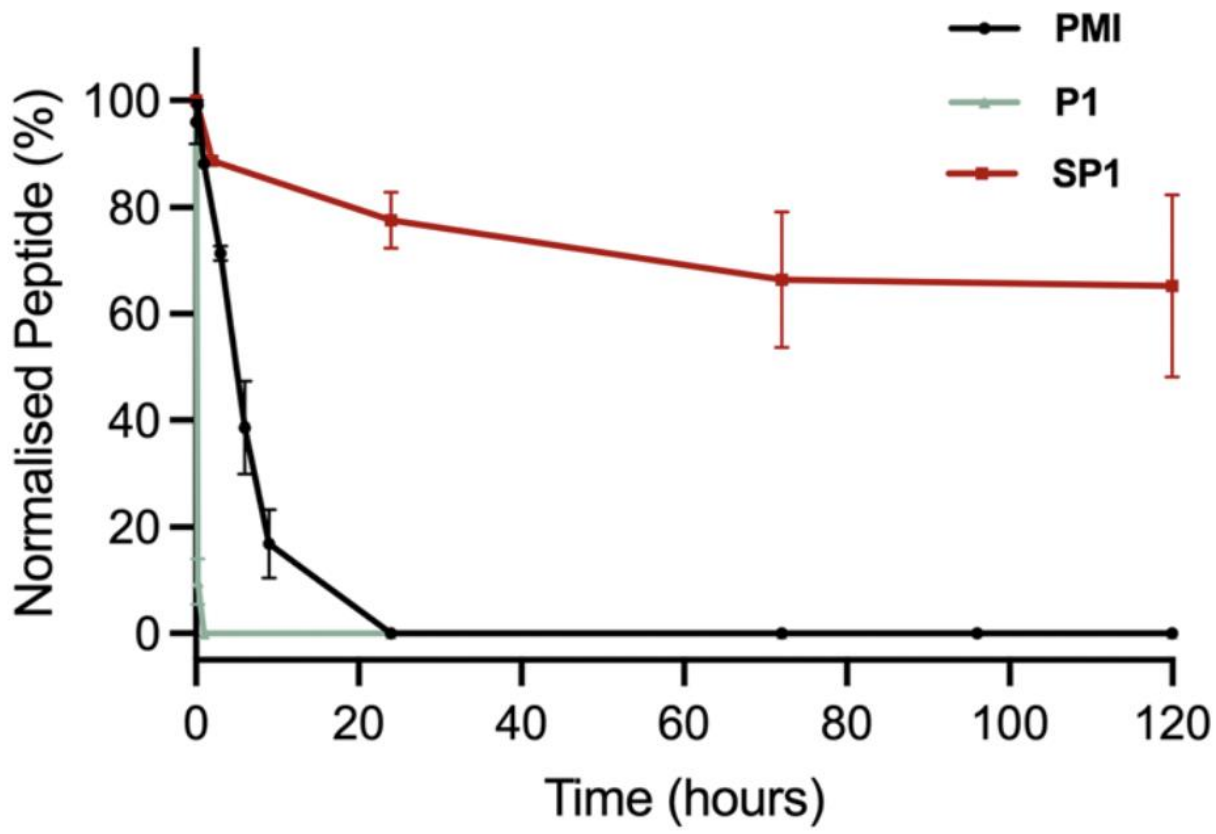
- use of red light
- cysteine-selective peptide stapling
- light induced changes in peptide conformation
- >240-fold difference in binding affinity for MDM2
- can rescue p53 from degradation？

Spring, *et al. RSC Chem. Biol.* **2024**, *5*, 49–54

Ⅱ-(2) Photoswitch peptide stapling

PMI (p53/MDM2 inhibitor; TSFAEYWNNLSP)





SP1 showed markedly improved stability compared P1 and PMI
→ can be attributed to the conformational rigidity

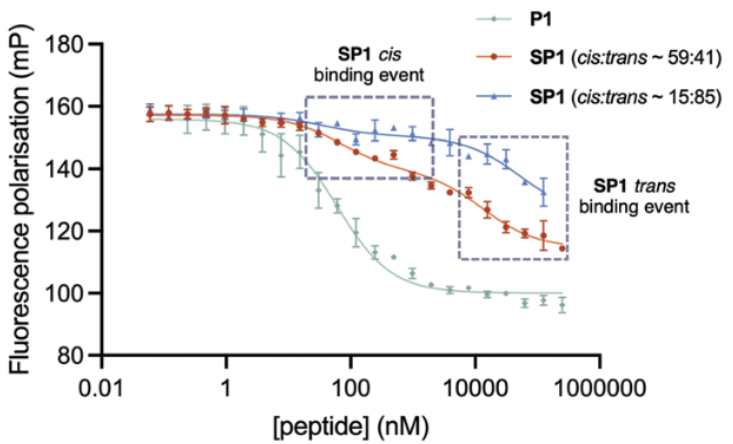
Spring, *et al. RSC Chem. Biol.* **2024**, *5*, 49–54

Ⅱ-(2) Photoswitch peptide stapling

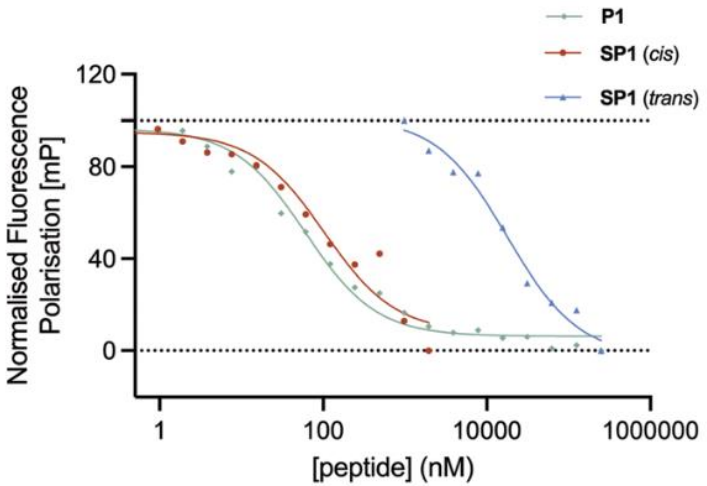
a)

Peptide	PMI	P1	SP1 (*trans*)	SP1 (*cis*)
K_i (nM)	0.78 ± 0.36	7.93 ± 3.83	> 13000	54 ± 4.9

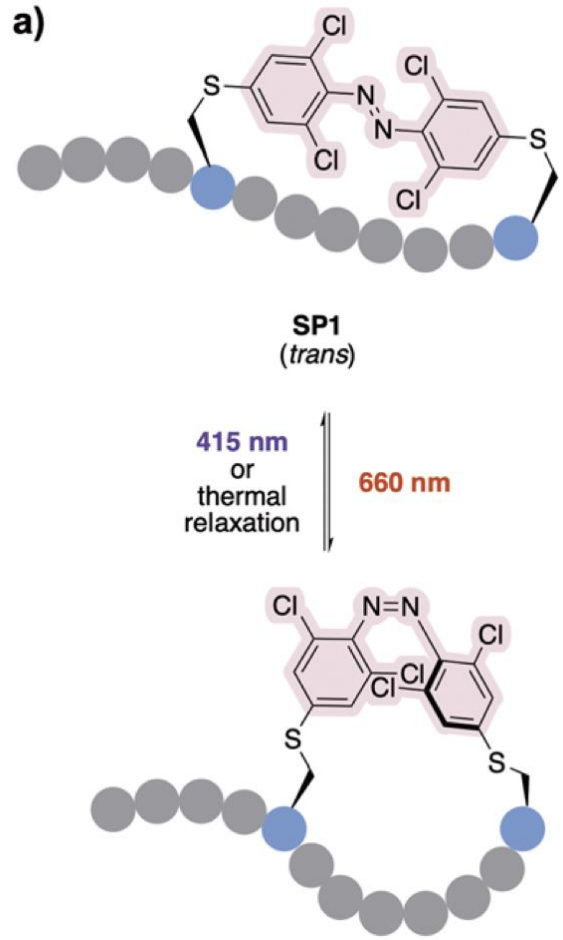
b)



c)



a)



the trans and cis isomers of SP1 displayed significantly different binding affinities for MDM2

a photoswitchable therapeutic stapled peptide between its different isomers

Spring, *et al. RSC Chem. Biol.* **2024**, *5*, 49–54

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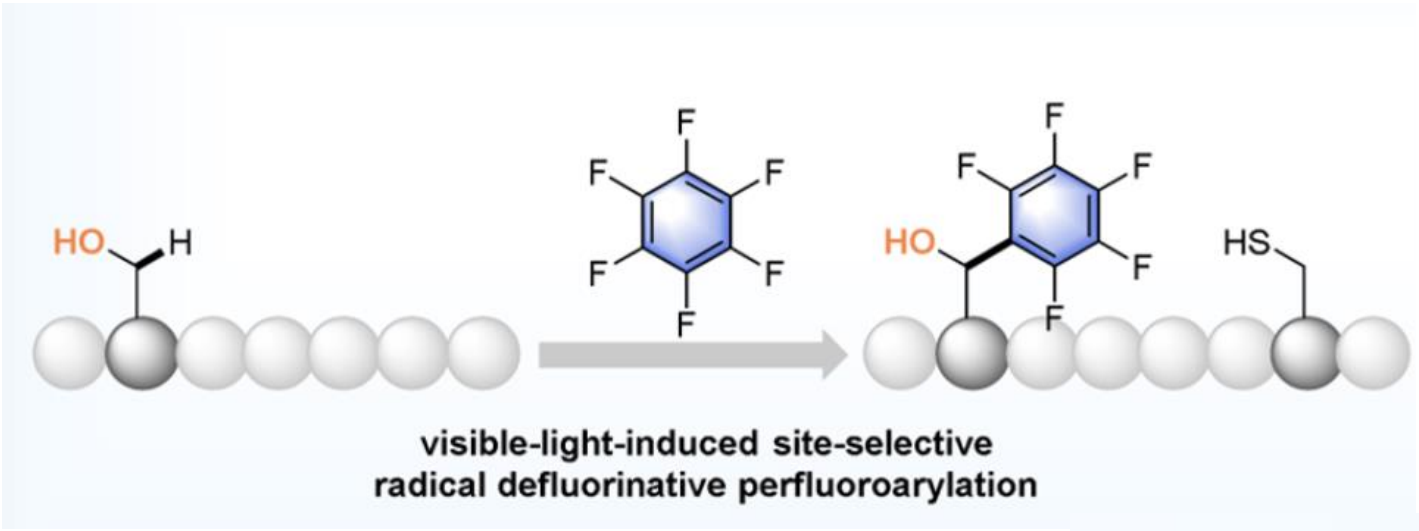
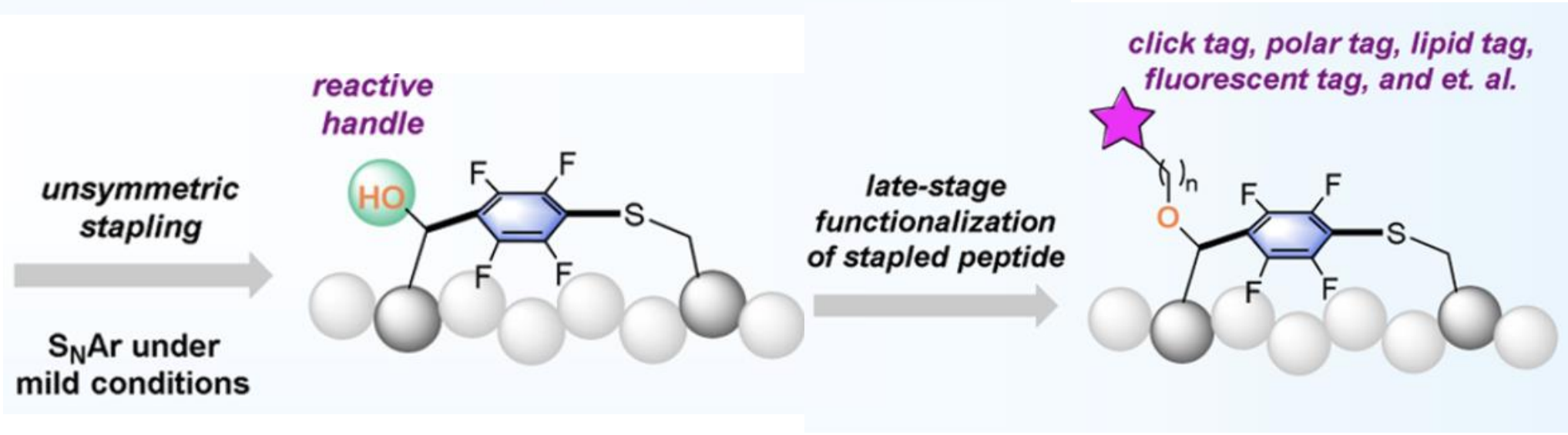
3. Summary

Ⅲ-(1) Retaining a modification handle

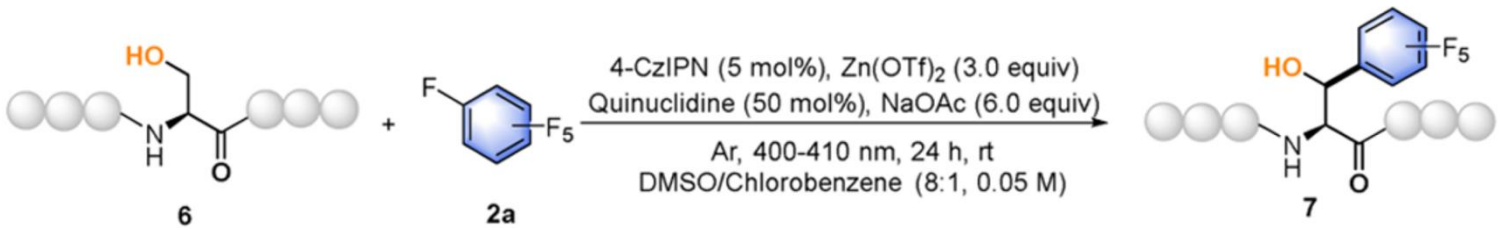


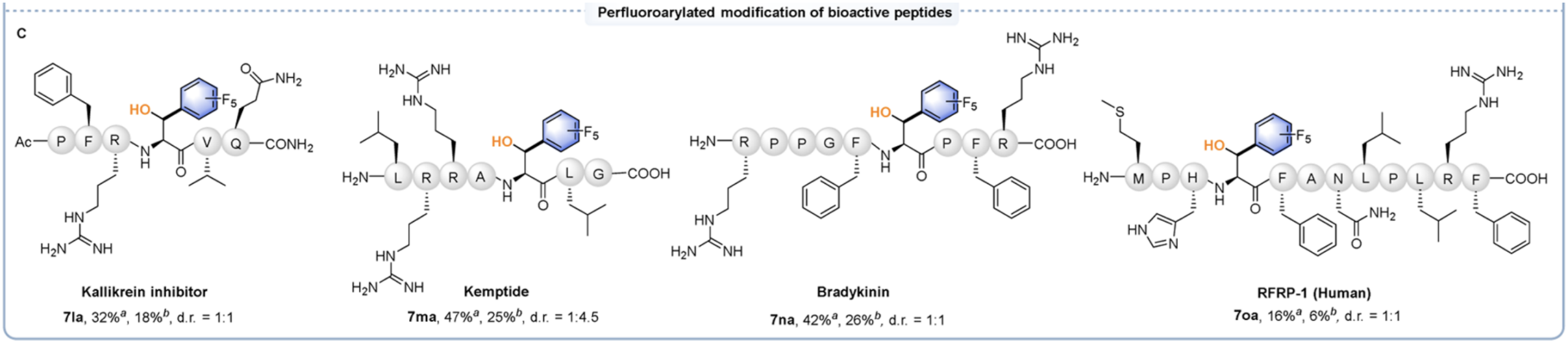
- unsymmetric stapling of unprotected peptides
- the unreacted –OH to serve as a reactive handle



Xu, *et al. J. Am. Chem. Soc.* **2024**, *146*, 6675－6685.

Ⅲ-(1) Retaining a modification handle

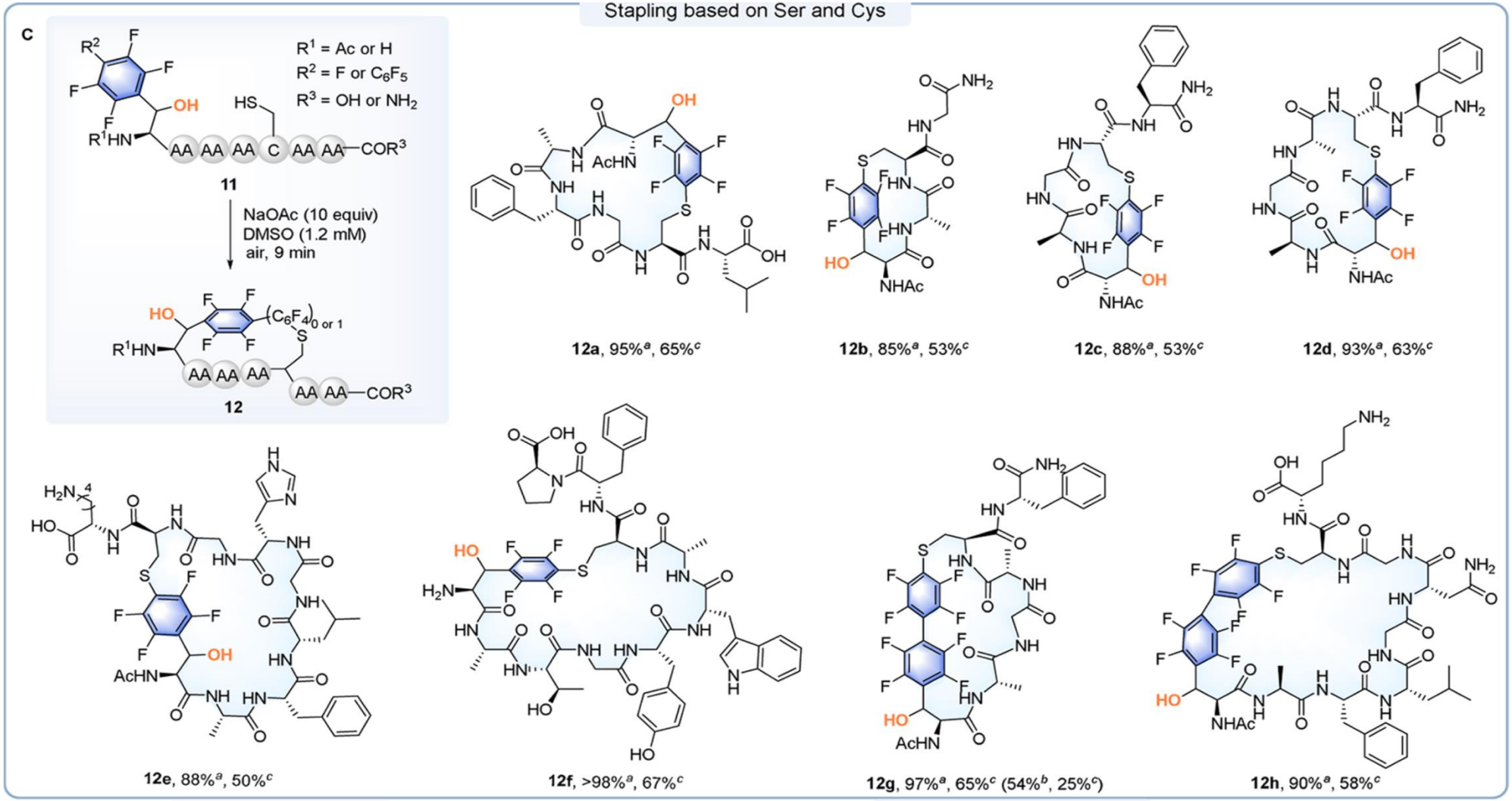




- site-selective perfluoroarylation of Ser β–C–H in peptide
- perfluoroarylated modification of unprotected peptides
- limited to smaller peptides

Xu, *et al. J. Am. Chem. Soc.* **2024**, *146*, 6675－6685.

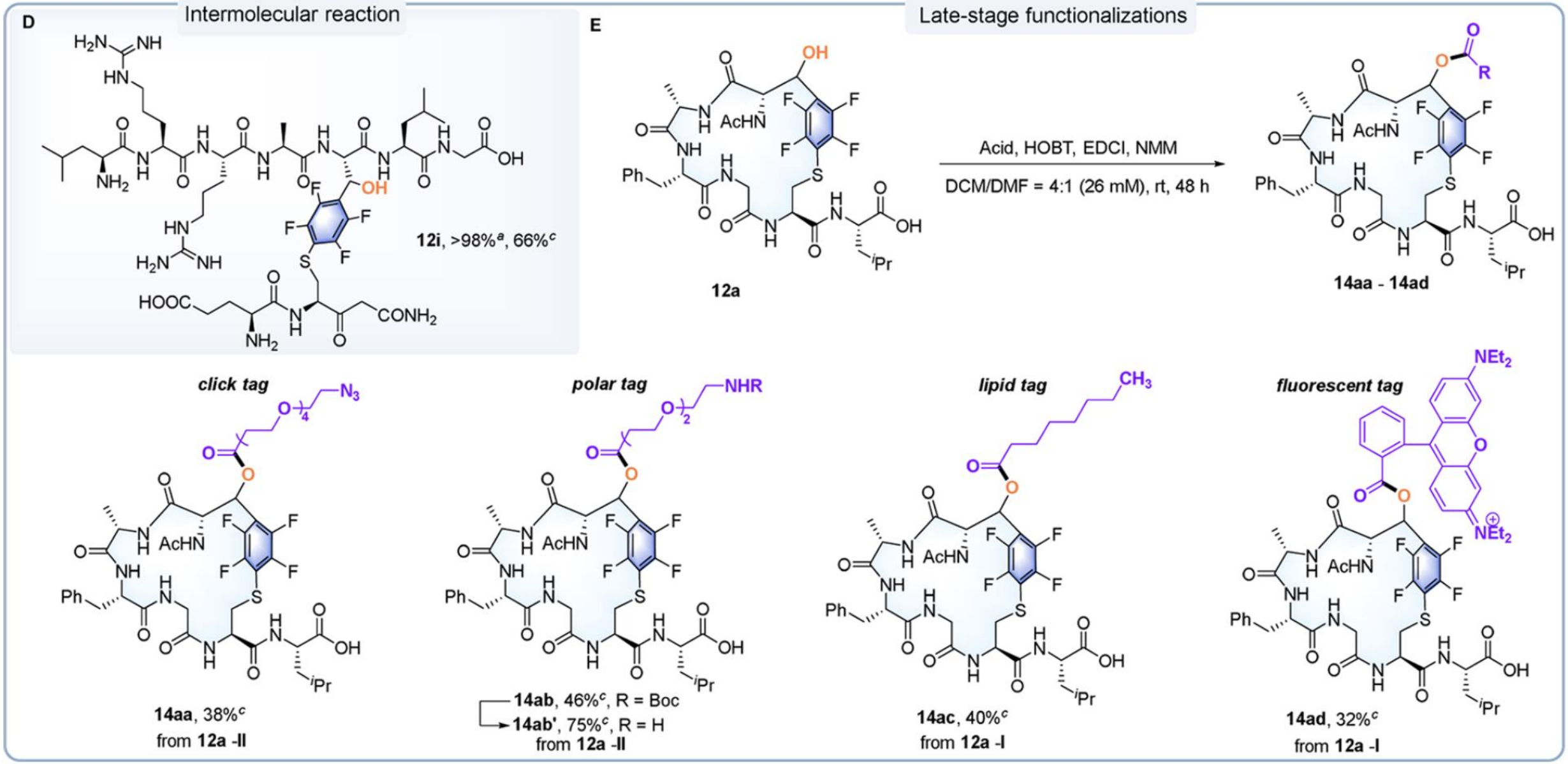
Ⅲ-(1) Retaining a modification handle



unsymmetric perfluoroaryl stapling of unprotected peptides based on Ser and Cys

Xu, *et al. J. Am. Chem. Soc.* **2024**, *146*, 6675－6685.

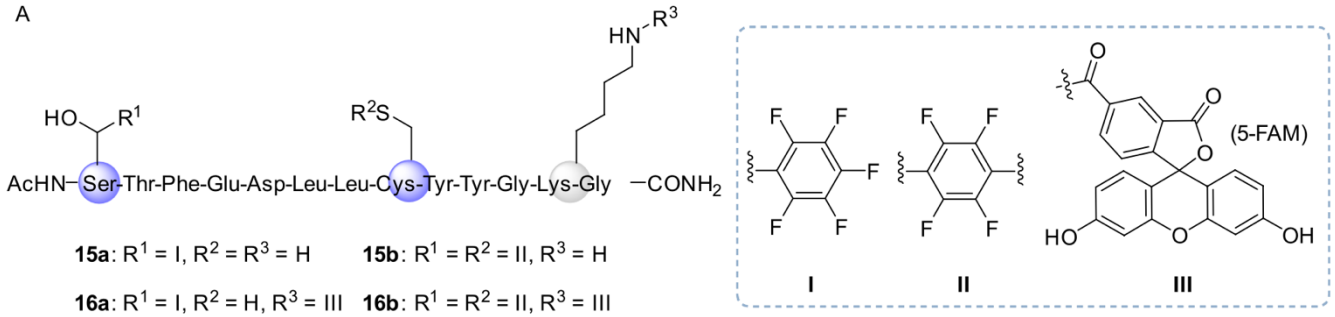
Ⅲ-(1) Retaining a modification handle



Xu, *et al. J. Am. Chem. Soc.* **2024**, *146*, 6675－6685.

Ⅲ-(1) Retaining a modification handle

A

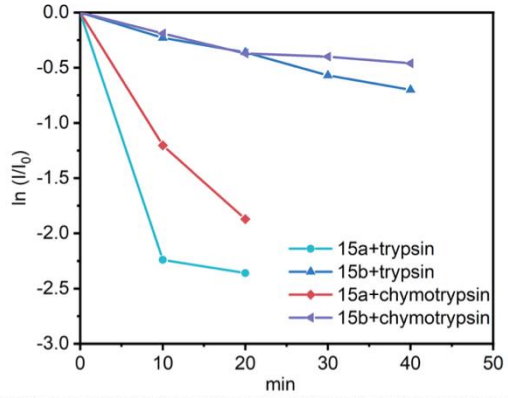


15a: R^1 = I, R^2 = R^3 = H　**15b**: R^1 = R^2 = II, R^3 = H

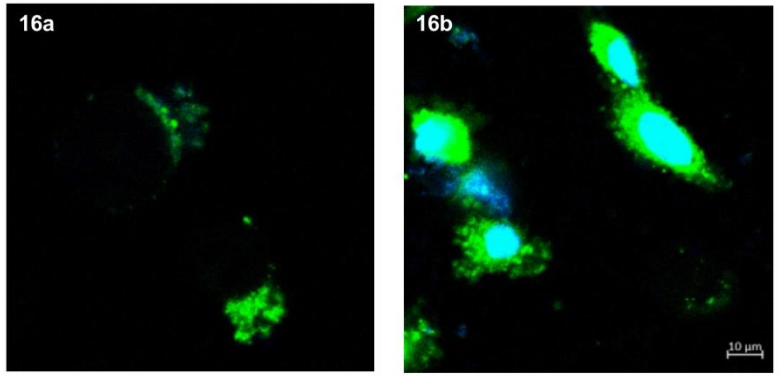
16a: R^1 = I, R^2 = H, R^3 = III　**16b**: R^1 = R^2 = II, R^3 = III

stapled peptides (15b, 16b)
linear precursor (15a, 16a)

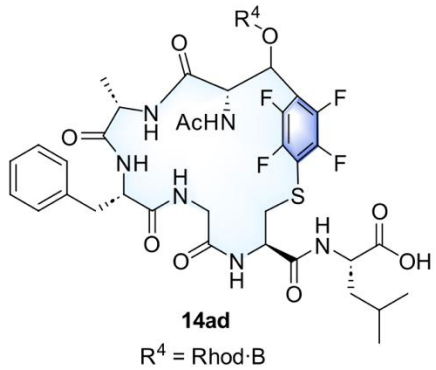
B

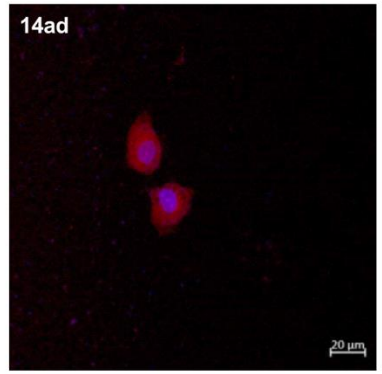


C



D





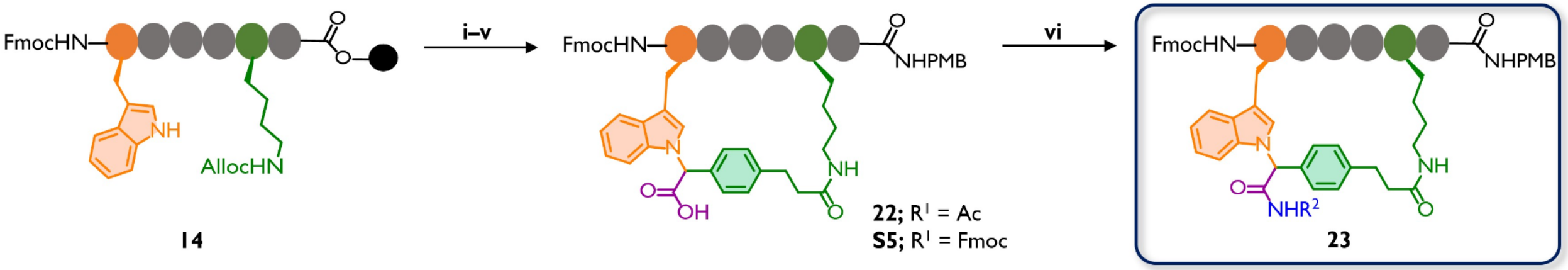
stapled peptide

(B)→enhanced stability
(C)→different localization

late-stage functionalizations
→various applications（intracellular tracking）

Xu, *et al. J. Am. Chem. Soc.* **2024**, *146*, 6675－6685.

Ⅲ-(2) Tryptophan stapling & late-stage functionalization



Reagents and conditions:

i) 2 % DBU in CH_2Cl_2, 20 min, r.t., then acetic anhydride, DMAP, CH_2Cl_2, r.t., 16 h
ii) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, r.t., 3 h, then 4-(2-carboxyethyl)benzeneboronic acid, HATU, DIPEA, r.t., 16 h
iii) CH_2Cl_2/HFIP 4 : 1, r.t.;
iv) *para*-methoxybenzyl amine, HOBt, DIC, DMF, r.t., 16 h
v) glyoxylic acid, 80 °C, 16 h
vi) *N*-hydroxysuccinimide, DIC, DMF, r.t., 5–8 h then corresponding amine, DIPEA, DMF, r.t., 16 h
vii) TFA/DMSO/TIPS 9 : 1:0.1, 80 ◦ C, 16 h

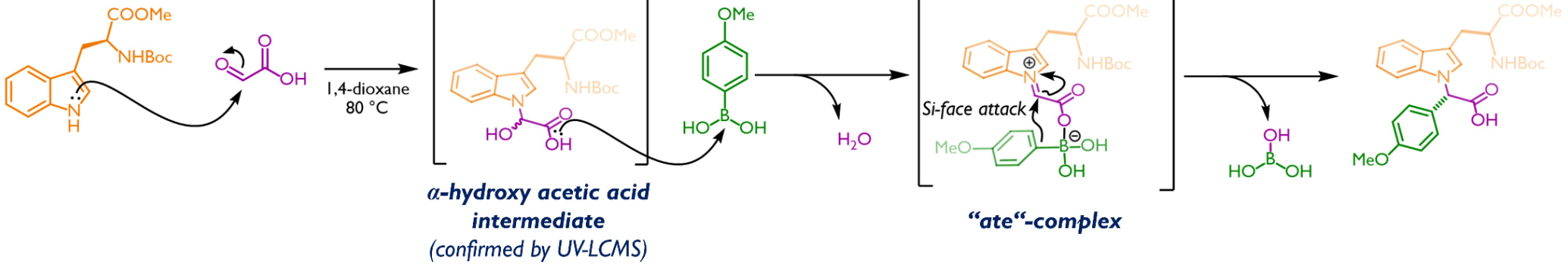
peptide stapling using the tryptophan-mediated Petasis reaction

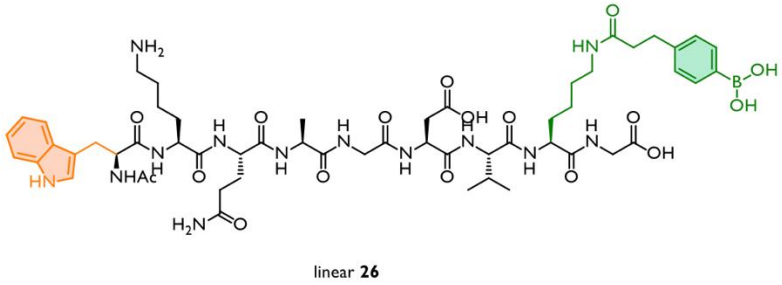
Krajcovicova, *et al. Angew. Chem. Int. Ed.* **2023**, *62*, e202307782

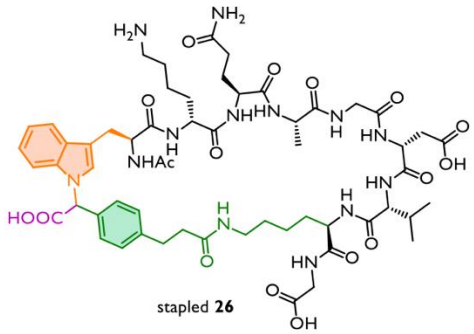
Ⅲ-(2) Tryptophan stapling & late-stage functionalization

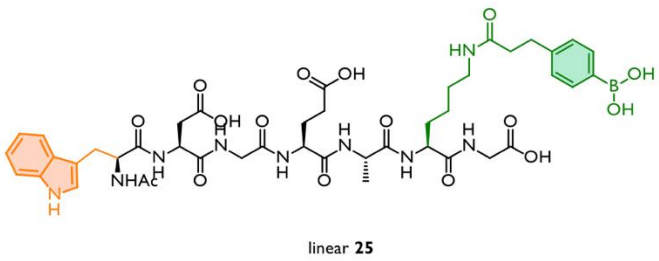
Petasis reaction

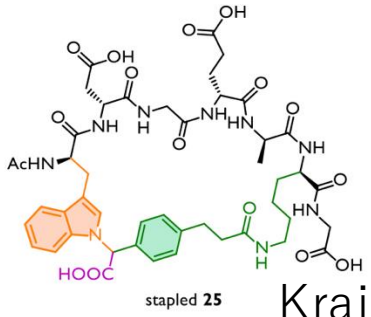
C. Plausible mechanism

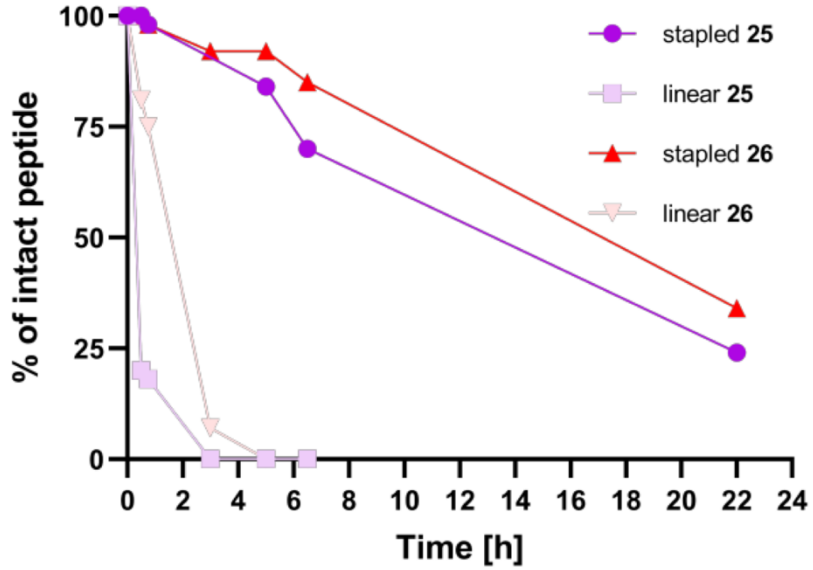








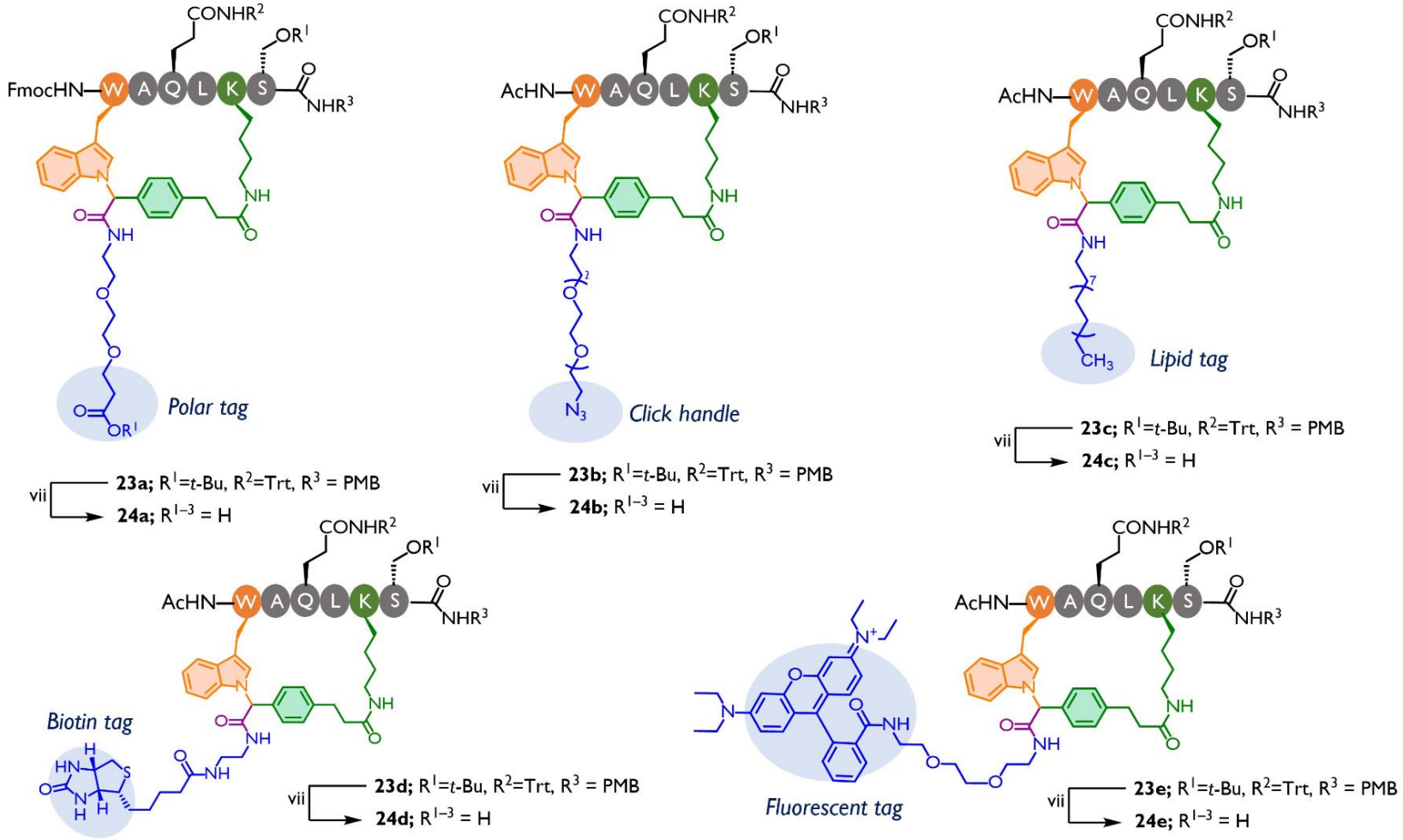




better stability in human serum

Krajcovicova, *et al. Angew. Chem. Int. Ed.* **2023**, *62*, e202307782

Ⅲ-(2) Tryptophan stapling & late-stage functionalization



efficient and diverse late-stage peptide modifications

Krajcovicova, *et al. Angew. Chem. Int. Ed.* **2023**, *62*, e202307782

Today's Contents

1. Introduction
   History of stapled peptide

2. Literature
   Ⅰ. Glowing staple
   Ⅱ. Reversible staple
   Ⅲ. Linkers with different functionality

3. Summary

Summary

Emergence of Peptide Drugs

Problems with the structure → Strategy : Peptide Stapling

Advances in staple technology are not only stabilizing the structure, but also providing additional functionality.

① Intracellular tracking
② On/Off chemical reaction or Light irradiation switching
③ Additional functional group

Stapled peptide has a wide range of applications.
☞ The future is bright for stapling technology !

Thank you
for your kind attention!

[Appendix]

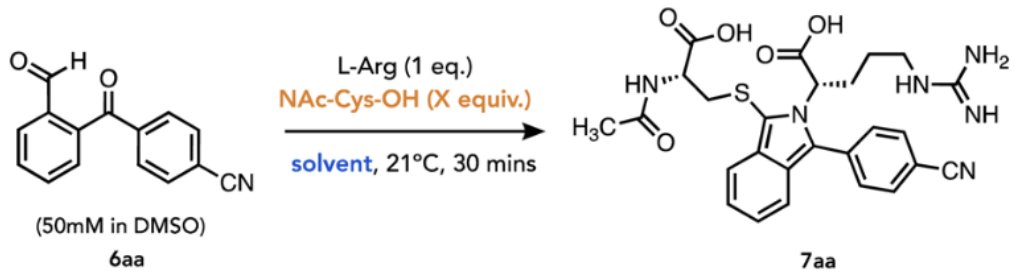
Peptide drug's advantages

Table 1. The comparison properties of three classes of therapeutic molecules

Properties	Small molecules	Stapled peptides	Biologics
Molecular weight	< 1,000	1,000–5,000	> 10,000
Stability	High	High	Low
Binding affinity	Low	High	High
Specificity	Low	High	High
Cellular permeability	High	High	Low
Proteolysis resistance	High	High	Low
Toxicity/side effects	High	Low	Low
Ability to disrupt PPIs	Low	High	High
Manufacturing cost	Low	Low	High

Zhang, *et al. Explor Drug Sci*. **2024**, *2*, 154–89.

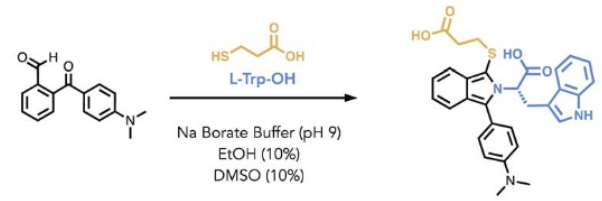
Table 1 Screening of conditions for the conversion of **6aa** to isoindole **7aa**



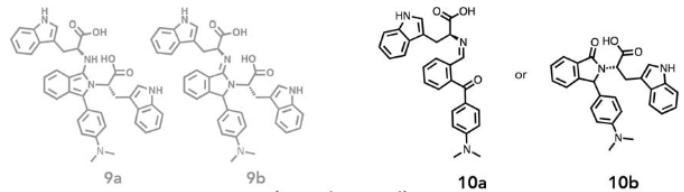
Entry	NAc-Cys	Co-solvent : solvent	% conv. to **7aa**[d]
1[a]	1[b] eq.	Na borate buffer pH 9 (aq.)	0%
2	3[b] eq.	Na borate buffer pH 9 (aq.)	0%
3	1[b] eq.	CH_3CN : Na borate buffer pH 9 (aq.)	0%
4	1[c] eq.	DMSO : Na borate buffer pH 9 (aq.)	39%
5	1[c] eq.	EtOH : Na borate buffer pH 9 (aq.)	66%

[a] Baseline run was obtained by injecting the crude reaction mixture immediately after all reagents were added, amounting to <2 minutes of reaction time. [b] Prepared as 50 mM solution in H_2O. [c] Prepared as 50 mM solution in H_2O (0.1% formic acid). [d] % conversion was determined by HPLC peak integration of **7aa** relative to peaks observed in the baseline run (entry 1).

Table 2 Chemoselectivity of 3-component intermolecular FliCk reaction with **6w**



Side products:



	Equiv.			Conversion[a] (%)			
Entry	Trp	Thiol	Reaction time	**8**	**9a**	**9b**	**10a/10b**
1	2.0	—	1 hour	0	0	0	0
2	5.0	—	5 hours	0	0	0	16
3	2.0	1.3	5 min	62	0	0	0
4	2.0	1.3	10 min	81	0	0	0
5	2.0	1.3	1 hour	90	0	0	0

[a] Percent conversion is calculated as the area under the curve of the chromatogram observed at 230 nm relative to 1 equivalent of **6w**. Other peaks observable at this wavelength is the DMSO co-solvent and this was not included in peak integration calculations. H-Trp (2.0 equivalents) also not included in percent conversion calculation because it remained in excess. All traces were obtained using the same HPLC method. Solvent A: 40 mM NH_4OH/HCOOH pH 8, solvent B: MeCN. 5–100% over 12 minutes.

I -(1) Fluorogenic stapling

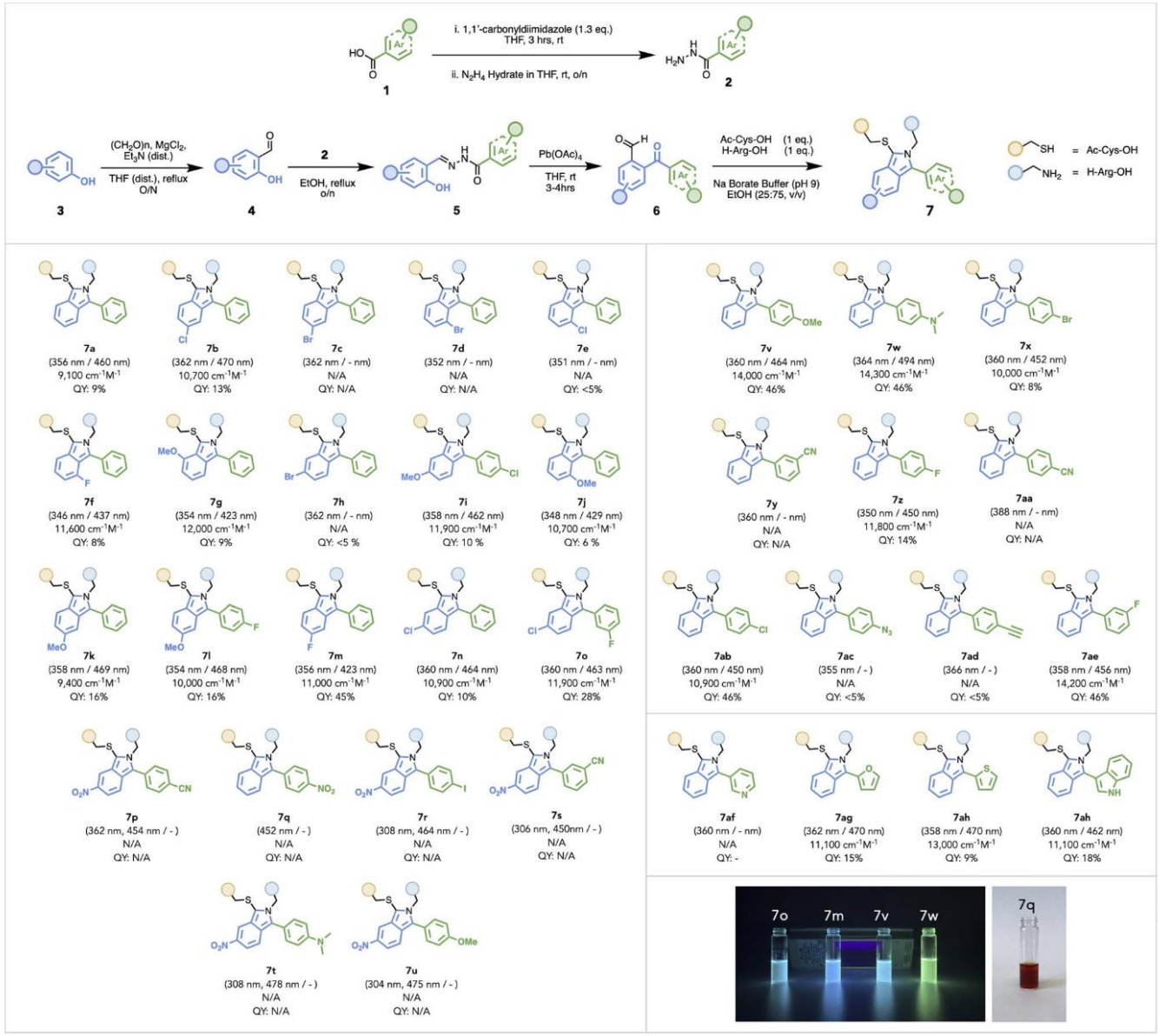


Fig. 3 Synthesis of an array of regioselective thio-isoindoles **7a–7ai**. Extinction coefficient was measured at respective $\lambda_{ex\text{-}max}$ N/A for compounds that did not show any fluorescence observed under 365 nm hand-held UV lamp, or where QY values were found to be <5%.

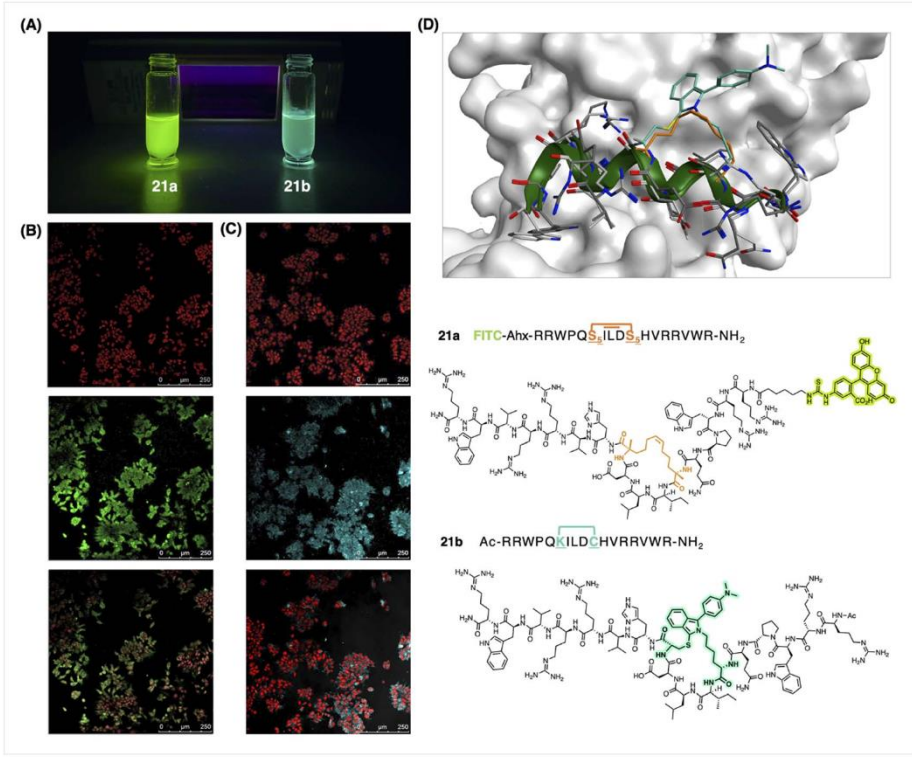


Fig. 6 (A) Solution of **21a** and **21b** in PBS Buffer (pH 7.4), 10% EtOH. (B) Imaging of cellular uptake (DLD-1 cells) of **21a** FITC-labeled peptide (7.5 μM, 1 h) and (C) **21b** FIICked-peptide (5 μM, 1 h) by confocal fluorescence microscopy. Overlaid images, in red: nuclear TO-PRO-3 Iodide, in green: FITC-labeled **21a** positive control, in blue: **21b** FIICked peptide. TO-PRO-3 was excited at 663 nm, FITC at 488 nm, and FIICk-ed peptide at 405 nm. (D) Molecular modelling of **21a** and **21b** (FITC not shown in the model). Orange bonds represent the olefin staple found in **21a**, and teal bonds represent the FIICk found in **21b**.

I -(2)Anchoring a luminescent tag in a Protein

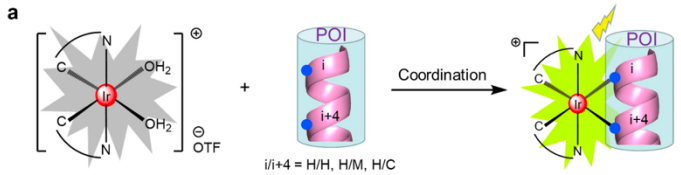


Figure 1. (a) Schematic illustration of site-specific labeling of a protein with non-emissive iridium(III) complex. (b) Chemical structures of the **Ir1**–**Ir6** complexes bearing exchangeable ligands that can be replaced by the sidechain of histidine, cysteine, or methionine in a protein.

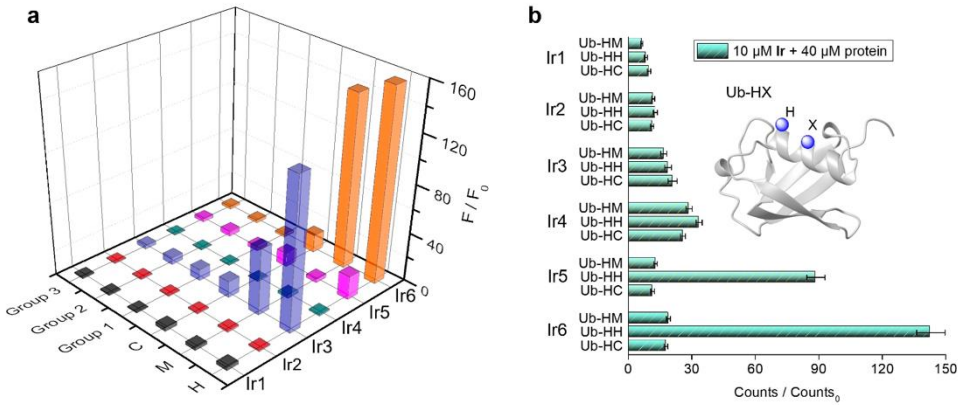


Figure 2. (a) Luminescence changes for the 50 μM Ir(III) complex after incubation with 200 μM amino acid or group of amino acids for 1 h in 20 mM PBS at ph 7.4 containing 1 % DMSO. Group 1: mixture of Gly, Ala, Val, Ser, Thr. Group 2: mixture of Leu, Iso, Asp, Glu, Asn and Gln. Group 3: mixture of Lys, Arg, Pro, Phe, Tyr and Trp. (b) Luminescence changes for the 10 μM Ir(III) complex after incubation 40 μM Ub E24H/A28 M (Ub-HM), Ub E24H/A28H (Ub-HH) and Ub E24H/A28 C (Ub-HC) for 1 h in 20 mM PBS at ph 7.4 containing 1 % DMSO at room temperature. Insertion: 3D structural representation of human ubiquitin and the mutation site at 24 (H) and 28 (X, X denotes M, H and C) [PDB code: 1UBI.[43] The luminescence was measured with λ_{ex} = 400 nm, λ_{em} = 560 ± 30 nm

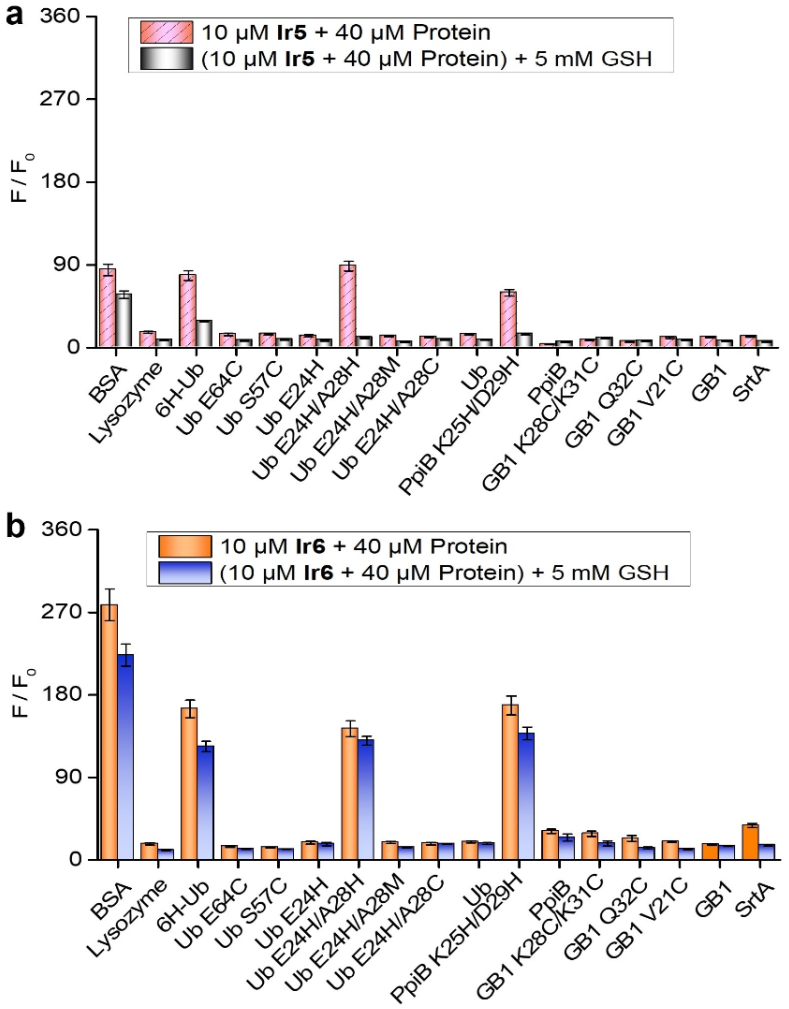


Figure 3. Interaction of **Ir5**/**Ir6** with various proteins. Luminescence intensity changes of 10 μM **Ir5** (a) and **Ir6** (b) in the presence of 40 μM various proteins (containing different numbers of histidine residues) for 1 h, followed by treatment with 5 mM GSH for another 1 h in 20 mM PBS (pH 7.4, containing 1 % DMSO). λex = 400 nm, λem = 560 ± 30 nm under the same buffer condition.

I -(2)Anchoring a luminescent tag in a Protein

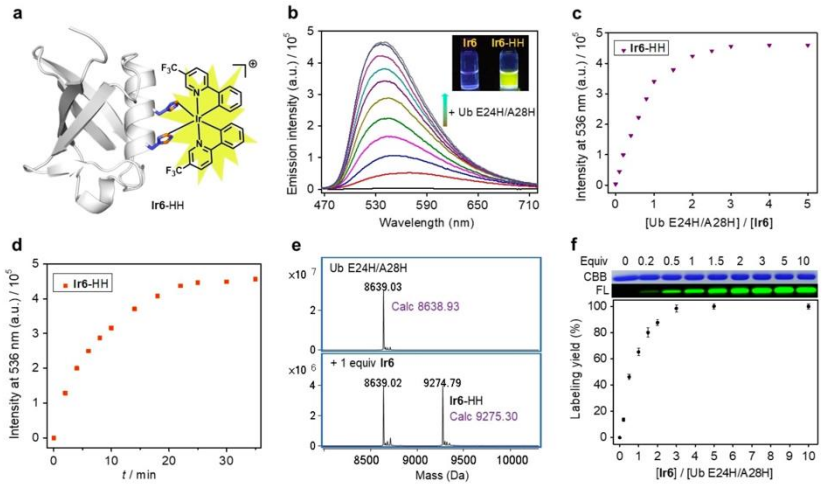


Figure 4. (a) Structural representation of the conjugate formed by Ub E24H/A28H and **Ir6** (**Ir6**-HH). (b) Emission spectra recorded for the solution of **Ir6** (10 μM) in the presence of an increasing concentration of Ub E24H/A28H (0–40 μM) in 20 mM PBS (pH 7.4, containing 1 % DMSO), reaction time: 1 h at each concentration. Insertion: photos of **Ir6** and **Ir6**-Ub E24H/A28H (**Ir6**-HH) under UVA irradiation. (c) Emission intensity recorded for the mixture of **Ir6** at 536 nm with respect to ratio of [Ub E24H/A28H]/[**Ir6**]. (d) Time-dependent emission intensity (at 536 nm) recorded for the mixture of 10 μM **Ir6** upon the addition of 40 μM Ub E24H/A28H in 20 mM PBS (pH 7.4, containing 1 % DMSO) at room temperature. (e) ESI/Q-TOF spectra recorded for the solution of Ub E24H/A28H (20 μM) in the absence or presence of 1 equiv. **Ir6** for 30 min in 20 mM PBS at ph 7.4 containing 1 % DMSO at room temperature. (f) Labeling efficiency of **Ir6** to Ub E24H/A28H (30 μM) monitored by Coomassie brilliant blue (CBB) staining and In-gel fluorescence (up), the corresponding normalized fluorescence intensity changes were plotted against the equivalent ratios (bottom).

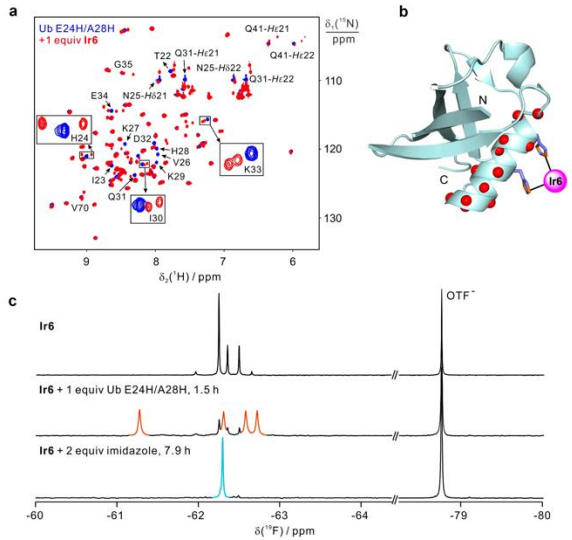


Figure 5. (a) Superposition of ^{15}N-HSQC spectra recorded for the sample of 0.1 mM ^{15}N-labeled Ub E24H/A28H in the absence (blue) and presence of 1 equiv **Ir6** (red). (b) Structural representation of ubiquitin for the residues with significant chemical shift perturbations after interaction with **Ir6**, in which sidechains of H24 and H28 were shown in sticks and the Cα atoms for the residues with large chemical shift perturbations were shown in red spheres and Ir(III) moiety in magenta. (c) 1D ^{19}F NMR spectra recorded for the sample of free 0.1 mM **Ir6** (top), after incubation with 1 equiv. Ub E24H/A28H for 2 h (middle) or 2 equiv. imidazole for 2 h (bottom) in 20 mM PBS at ph 7.4 contains 1 % DMSO. The NMR signals of **Ir6**-Ub E24H/A28H complex were marked in orange, and **Ir6**-imidazole in cyan, respectively. The spectra were recorded at 298 K with a proton frequency of 800 MHz spectrometer.

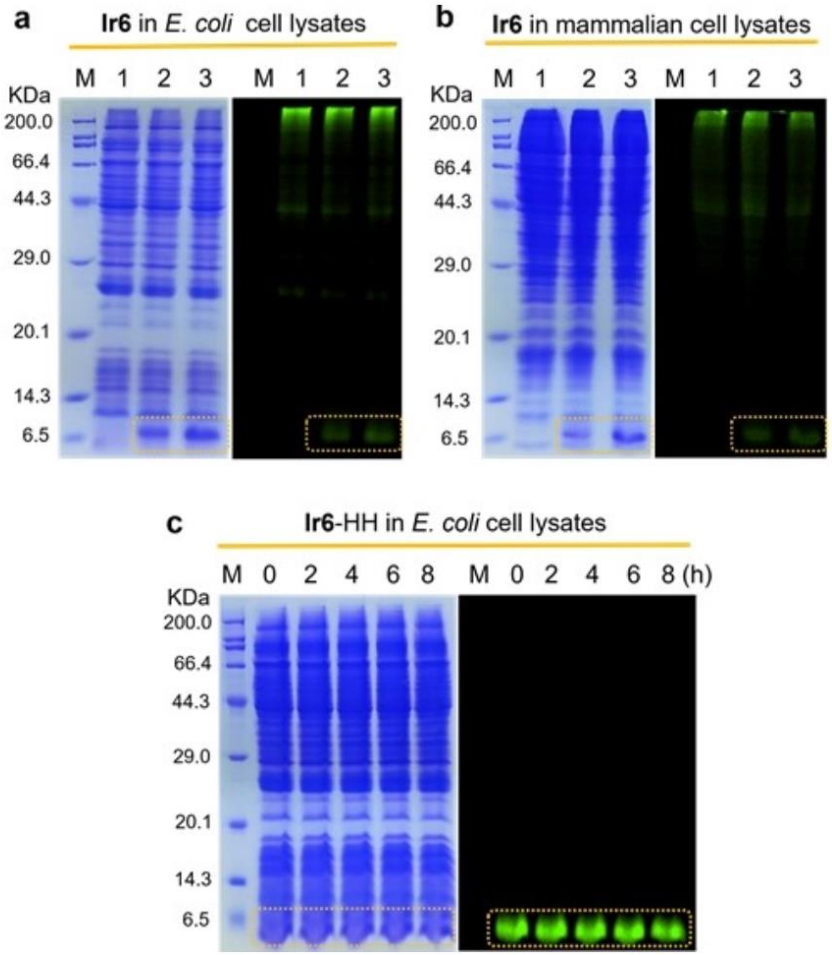


Figure 6. SDS-PAGE in-gel analysis of 100 mg/mL *E. coli* cell lysates (a) or 200 mg/mL mammalian cell lysates (b) treated with 30 μM **Ir6** and varing concentrations of Ub E24H/A28H (Ub-HH, Lane1: 0 μM, Lane2: 30 μM, Lane3: 60 μM) for 1.5 h at room temperature. The gel was analyzed by in -gel fluorescence imaging (right) and stained with Coomassie Brilliant Blue (left). (c) Stability of **Ir6**-Ub E24H/A28H (30 μM) incubated with 200 mg/mL *E. coli* cell lysates for different time (0–8 h) at room temperature and analyzed by SDS-PAGE. 'M' stands for the protein marker.

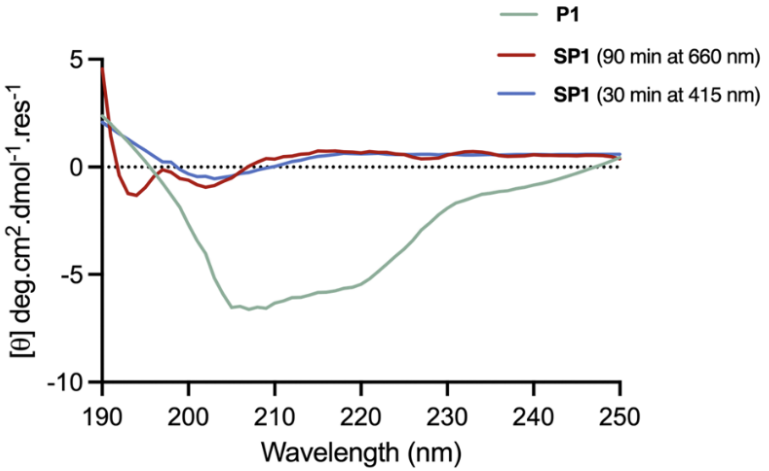


Fig. 5 CD spectra of the linear peptide **P1** in 50 : 50 H_2O/MeCN, and stapled peptide **SP1** in MeCN, recorded across 190–250 nm. *In situ* isomerisation of **SP2** was carried out upon 30 min and 90 min irradiation with 415 nm and 660 nm LED lights, respectively.

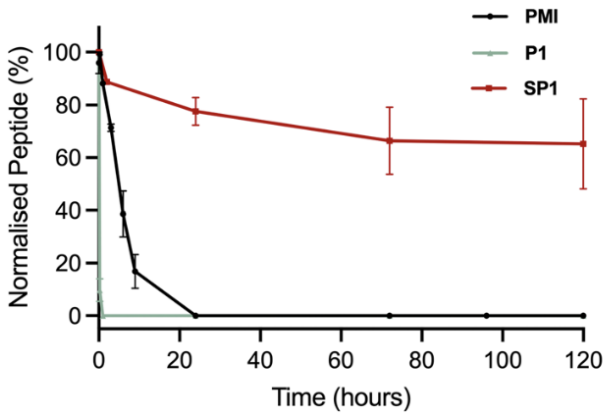
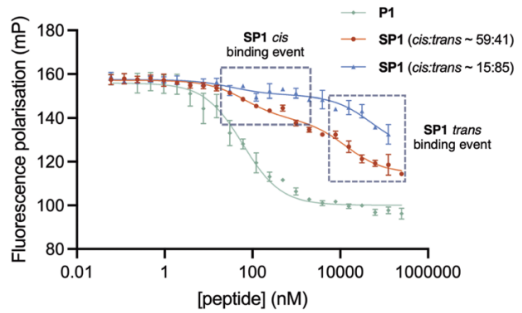


Fig. 6 Stability of the precursor peptide **P1**, the parent **PMI** and **SP1** in human serum at 37 °C, over 5 days, monitored by HPLC at 220 nm.

a)

Peptide	PMI	P1	SP1 (*trans*)	SP1 (*cis*)
K_i (nM)	0.78 ± 0.36	7.93 ± 3.83	> 13000	54 ± 4.9

b)



c)

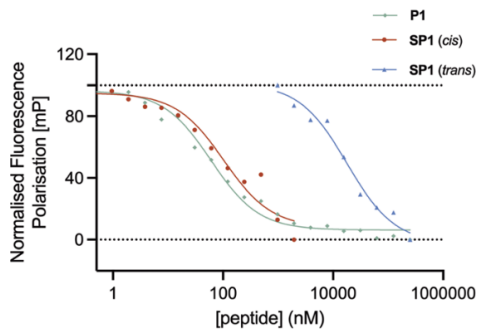


Fig. 7 (a) Binding affinities of **PMI**, **P1** and **SP1** for MDM2. The K_i values are reported as the average of two or three biological replicates each performed as a triplicate and the error bars shown represent the standard error of the mean, determined from the fit for each of the independent experiments and subtracted from the average K_i value. (b) Competitive FP assay of **P1** and **SP1**. Each data point is arithmetic mean of two or three biological replicates each performed as a triplicate and the error bar shown are standard errors of the mean. *In-situ* isomerisation of **SP1** was carried out upon 30 min and 90 min irradiation with 415 nm and 660 nm LED lights, respectively. (c) Normalised FP curves of *cis* and *trans* **SP1** individual binding events, extracted from the **SP1** (*cis* : *trans* ~ 59 : 41) fitted curve, and the normalised FP of **P1** for comparison.

Ⅲ-(1) Retaining a modification handle

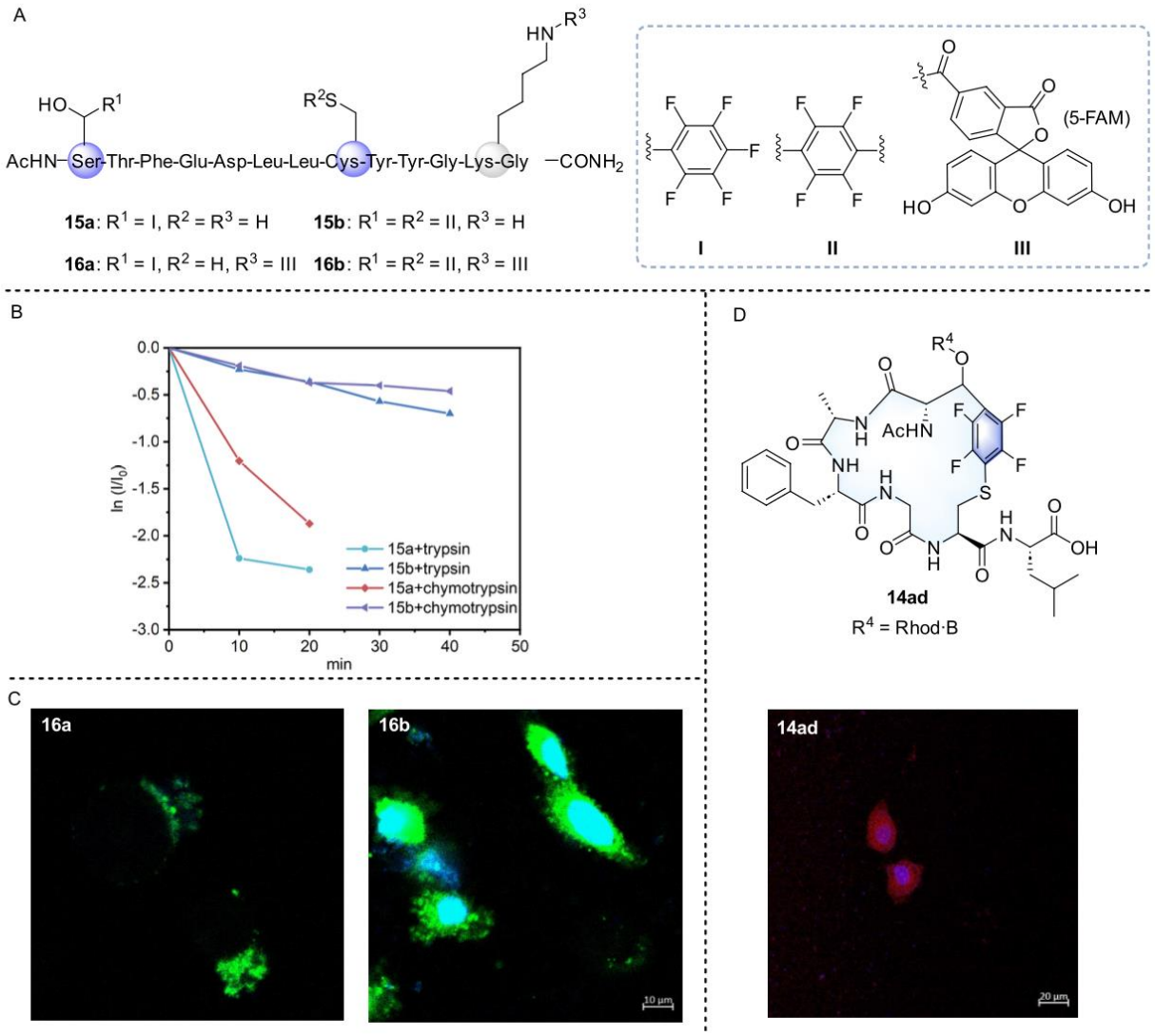


Figure 4. Bioactivity studies of stapled peptides. (A) Structure of stapled peptides (**15b**, **16b**) and linear precursor (**15a**, **16a**). (B) Proteolytic assay of stapled peptides **15b** and their linear precursors **15a**. (C) Confocal microscopy image of NCI-H1975 cells treated with compounds **16a** and **16b** (50 μM). Scale bar, 10 μm. (D) Confocal microscopy image of NCI-H1975 cells treated with compound **14ad** (50 μM). Scale bar: 20 μm.

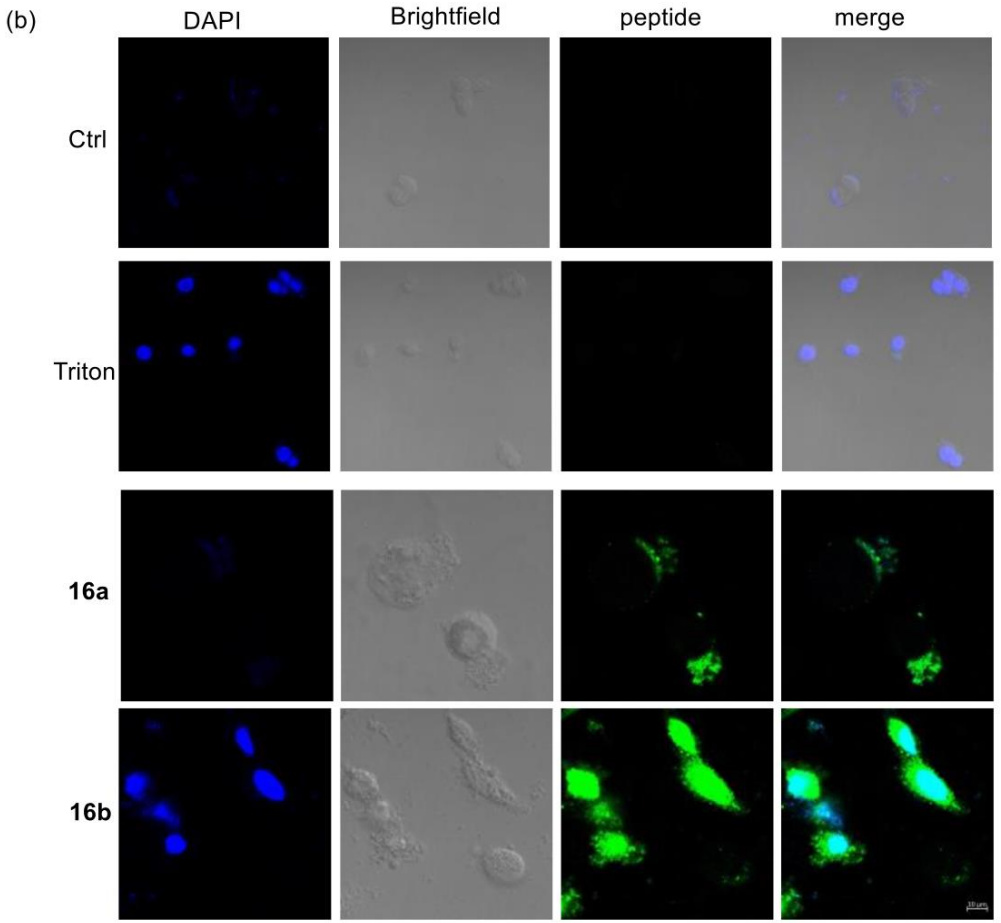
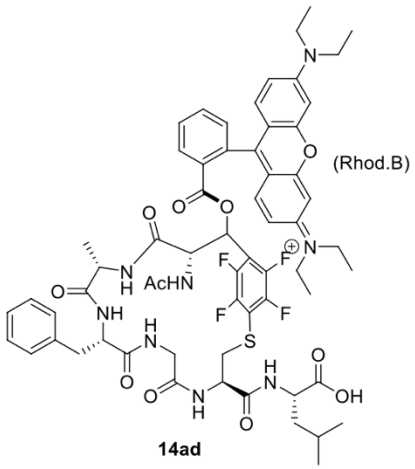


Figure S10. Fluorescent confocal microscopy of cells studied for peptide cell permeability. (a) Structure of stapled peptides **16b** and linear precursor **16a**. (b) Fluorescent confocal microscopy of NCI-H1975 cells treated with compound **16a** and **16b** (50 μM). Scale bar, 10 μm.



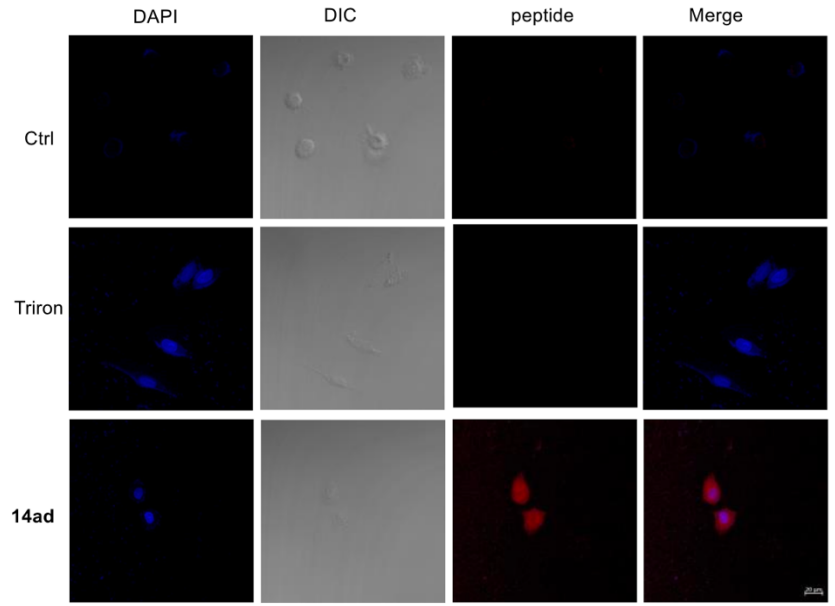
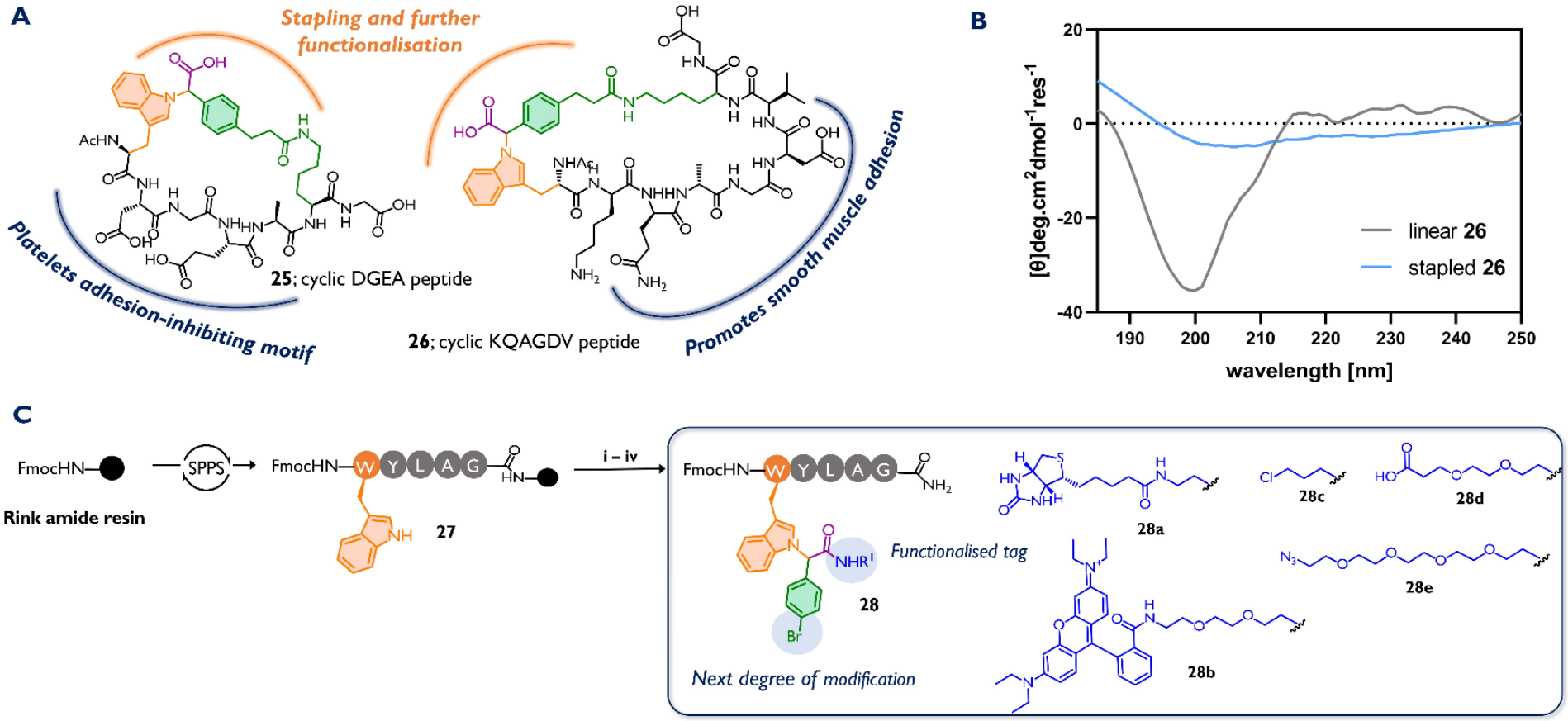


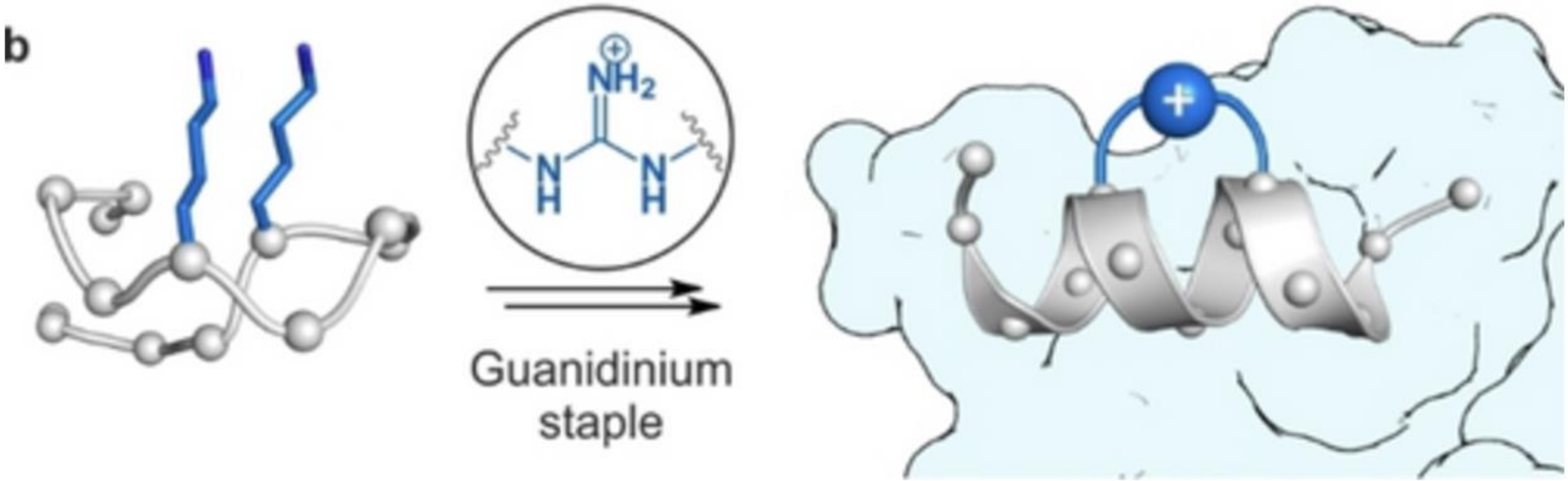
Figure S11. Fluorescent confocal microscopy of cells studied for peptide cell permeability. Fluorescent confocal microscopy of NCI-H1975 cells treated with compound **14ad** (50 μM). Scale bar, 20 μm.

Ⅲ-(2) Tryptophan stapling & late-stage functionalization



Scheme 4. A) Biologically relevant peptides (for full preparation methods see the Supporting Information). B) Circular dichroism (CD) spectra of peptide **26** (linear and stapled). C) Labelling of a model peptide on resin. Reagents and conditions: i) glyoxylic acid monohydrate, 1,4-dioxane, 80 °C, 16 h; ii) 4-bromophenylboronic acid, 1,4-dioxane, 80 °C, 16 h; iii) corresponding amine, HATU, DIPEA, DMF, r.t., 16 h; iv) CH_2Cl_2/TFA 1:1, r.t., 3 h. Overall yields and purities: **28a**: 7%,[a] 96%;[b] **28b**: 6%,[a] 85%;[b] **28c**: 14%,[a] 99%;[b] **28d**: 13%,[a] 98%;[b] **28e**: 9%,[a] 99%.[b] [a] Combined yield of both diastereomers after HPLC. [b] UV–LCMS or HPLC purity.

Ⅲ-(3)Guanidinium-staple helical peptides



Pasco, *et al. Angew. Chem. Int. Ed.* **2025**, *64*, e202416348