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A Sulfonium Triggered Thiol-yne Reaction for Cysteine Modification

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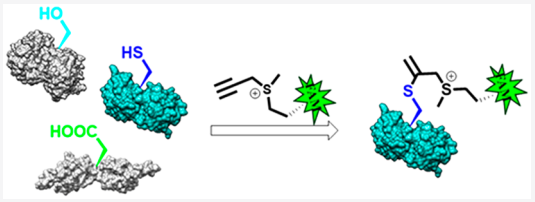
ABSTRACT: We report a facile thiol-yne type reaction triggered by the sulfonium center. After facile propargylation of thiolethers, the resulting sulfonium could undergo facile addition with thiols in aqueous media at ambient temperature. Further applying this reaction in unprotected peptides bearing neighboring methionine and cysteine could enable a facile intramolecular addition to construct cyclic peptides with better stability, good glutathione resistance, and increased cellular uptakes. Also, the propargylated sulfonium may be used as robust and versatile probes to target cysteines containing biomolecules.

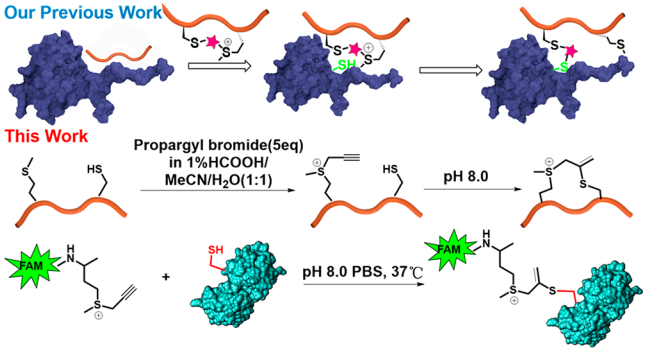


Post translational modifications (PTMs) of proteins play key roles for diversified protein functions, which also creates interest in designing various synthetic methods to modify proteins.[1−4] Various site-selective and/or chemoselective modifications of different amino acids of proteins were reported, including cysteine,[5−8] methionine,[9−12] lysine,[13−16] tyrosine,[17] tryptophan,[18] arginine,[19] etc. Thanks to thiol's high nucleophilicity, cysteine is widely utilized for selective modifications for various applications, including activity-based protein profiling (ABPP),[20] cell imaging,[21] covalent inhibitors,[22−25] and so on.

Thiol-yne coupling is an emerging "click" reaction and extensively used in peptide and protein modification.[26−28] Most of such reactions were initiated by radicals, either with UV light or photoinitiators.[29] Recently, Mootz et al.[30] reported a plethora of cellular applications of terminal alkynes in combination with click chemistry. Ovaa et al.[31] replaced the C-terminal carboxylate of ubiquitin by an alkyne which would only react with nearby cysteines upon target recognition. Pentelute et al.[32] reported a site-selective cysteine−cyclooctyne addition between a DBCO peptide tag at the N- or C-terminus of protein under radical-free conditions.

We recently reported a sulfonium tethered peptide which could rapidly and selectively conjugate with nearby cysteine of its target protein, as shown in Scheme 1.[33] Inspired by the unique reactivity of the sulfonium center, we attached a propargyl group on protected methionine to test the reactivity of the sulfonium center. We found the formed propargyl sulfonium center could trigger a very facile thiol-yne addition in aqueous media at ambient temperature. Unlike conventional sulfur ylides, we suspect that the propargylsulfonium would undergo spontaneous conversion into allenylsulfonium with the presence of nucleophiles and the highly electrophilic allenylsulfonium center would then undergo nucleophilic addition at β-carbon atoms.[34,35] We first utilized this reaction

Scheme 1. Schematic Presentation of Our Previous Cysteine Modification and the Thiol-yne Type Reaction for Selective Cysteine Modification



to construct exemplary cyclic peptides with different functional groups to test the reaction's scope. The serum stability, reductive conditions, and cellular uptake of a simple cyclic peptide (to avoid possible sequence influences) were tested for the cyclization influences. Then, a cyclic peptide with multiple functional amino acids was constructed and its binding affinity to target protein was tested. All of these proof-of-concept studies supporting this method could be utilized to construct protein−protein interaction modulators. In addition to peptides, we also constructed a simplified small molecule probe. Model studies showed that it could efficiently react with different free Cys-containing proteins, indicating its potential

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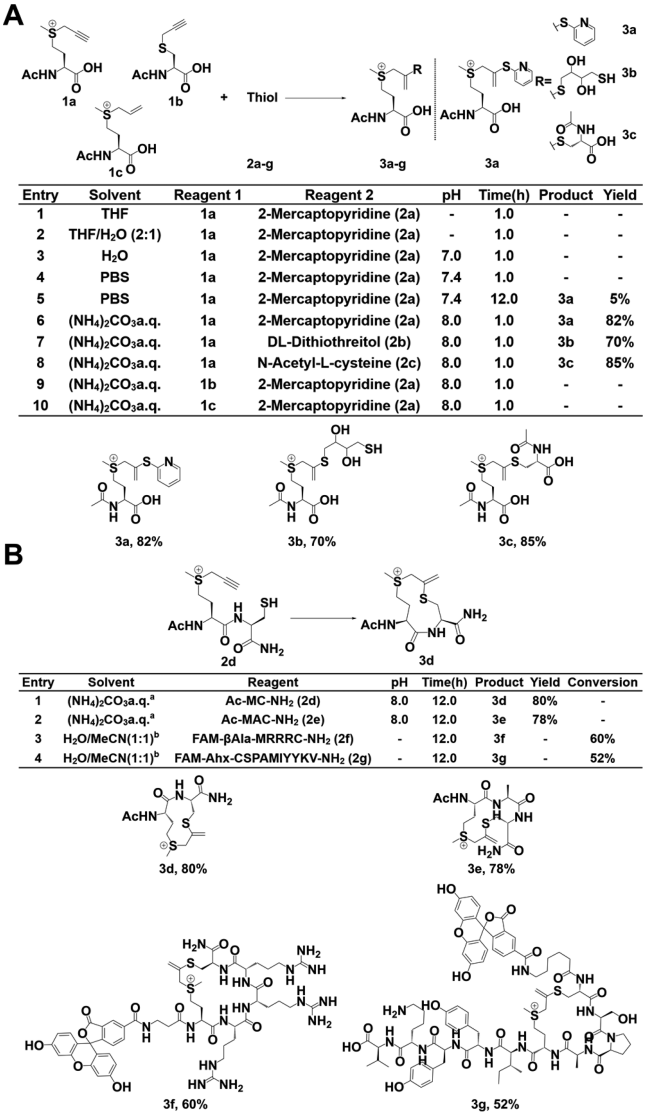
application in ABPP. With further studies, we believe this method could be extended to other amino acid residues and more biorelated applications, such as protein profiling, covalent inhibitor design, and antibody conjugated drug development.

The reaction between propargylated methionine **1a** and 2-mercaptopyridine **2a** was tested as the model reaction shown in Figure 1A. **1** and **2a** (1:1) were allowed to react in different



Entry	Solvent	Reagent 1	Reagent 2	pH	Time(h)	Product	Yield
1	THF	1a	2-Mercaptopyridine (2a)	-	1.0	-	-
2	THF/H_2O (2:1)	1a	2-Mercaptopyridine (2a)	-	1.0	-	-
3	H_2O	1a	2-Mercaptopyridine (2a)	7.0	1.0	-	-
4	PBS	1a	2-Mercaptopyridine (2a)	7.4	1.0	-	-
5	PBS	1a	2-Mercaptopyridine (2a)	7.4	12.0	3a	5%
6	$(NH_4)_2CO_3$a.q.	1a	2-Mercaptopyridine (2a)	8.0	1.0	3a	82%
7	$(NH_4)_2CO_3$a.q.	1a	DL-Dithiothreitol (2b)	8.0	1.0	3b	70%
8	$(NH_4)_2CO_3$a.q.	1a	N-Acetyl-L-cysteine (2c)	8.0	1.0	3c	85%
9	$(NH_4)_2CO_3$a.q.	1b	2-Mercaptopyridine (2a)	8.0	1.0	-	-
10	$(NH_4)_2CO_3$a.q.	1c	2-Mercaptopyridine (2a)	8.0	1.0	-	-

Entry	Solvent	Reagent	pH	Time(h)	Product	Yield	Conversion
1	$(NH_4)_2CO_3$a.q.[a]	Ac-MC-NH_2 (2d)	8.0	12.0	3d	80%	-
2	$(NH_4)_2CO_3$a.q.[a]	Ac-MAC-NH_2 (2e)	8.0	12.0	3e	78%	-
3	H_2O/MeCN(1:1)[b]	FAM-βAla-MRRRC-NH_2 (2f)	-	12.0	3f	-	60%
4	H_2O/MeCN(1:1)[b]	FAM-Ahx-CSPAMIYYKV-NH_2 (2g)	-	12.0	3g	-	52%

Figure 1. (A) Screening of reaction conditions. Reactions of **1a** (46 mg, 0.20 mmol, 1.0 equiv) and **2a** (22 mg, 0.20 mmol, 1.0 equiv) were carried out in 1 mL of solvent at 25 °C and let react for a given time. (B) Intramolecular peptide cyclization. [a]1 mL of 1 M $(NH_4)_2CO_3$ aq solution. [b]1% Et_3N in 1 mL of MeCN/H_2O (1:1). Ahx, 6-Aminocaproic acid.

solvents as entries 1−4 of Figure 1A. After a 1 h reaction in THF, THF/H_2O (1:1), H_2O (pH 7.0), or PBS (pH 7.4), no adduct was observed via LC-MS. When the reaction time was extended to 12 h, we observed ∼5% adduct in PBS (pH 7.4) (Figure 1A, entry 5). As the reaction pH was increased to 8.0 with addition of $(NH_4)_2CO_3$, we observed **3a** with an 82% isolated yield after 1 h of reaction (Figure 1A, entry 6). Further screening of bases (PBS and Et_3N) and solvents (THF and MeCN) did not give a better yield. Different thiols were then tested; both aromatic thiols and aliphatic thiols could readily undergo this reaction and give good isolated yields (Figure 1A, entries 6−8). This reaction is solely triggered by the sulfonium center; propargyl thiol ether **1b** could not react with **2a** under all conditions tested. Notably, the reaction between alkylated methionine **1c** and **2a** could only provide trace conversion under all conditions tested (Figure 1A, entries 9 and 10).

Then, this reaction was successfully translated into an intramolecular ring closure for peptides, as shown in Figure 1B. For simple peptides shown in entries 1 and 2 of Figure 1B, the standard condition could afford satisfying isolated yields. However, for peptides poorly soluble in water (entries 3 and 4, Figure 1B), a mixed solvent (1% Et_3N solution in 50% MeCN/H_2O) was used to get satisfying conversions. Notably, peptides **2f** and **2g** were used without any protection for both methionine propargylation and following Cys addition. The exemplary peptides contained multiple functional groups including alcohol, phenol, carboxylic acid, and amines. The selective and efficient Cys addition in peptides **2f** and **2g** clearly showed this reaction is generally applicable for peptide modification. Peptide **2f** is designed for testing the cyclization influence on serum stability and cellular uptake; peptide **2g** is reported to selectively bind the PDZ domain of PSCK9 and is designed for testing the cyclization influence on target binding.[33,36]

To fully elucidate the product structure and the underlying reaction mechanism, careful nuclear magnetic resonance (NMR) experiments were performed, including ^{1}H NMR, heteronuclear singular quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), and total correlation spectroscopy (TOCSY). All spectroscopic experiments clearly showed that the two alkene protons are on the same carbon atom, which suggested the thiol added at the β position[37] (Figure 2A, HMBC and TOCSY in the Supporting Information). According to the structure of products and previous reports,[37−39] we proposed that the propragylsulfonium salts would undergo spontaneous conversion into allenylsulfonium in weakly alkaline solution (pH 8.0).[40−42] The highly electrophilic allenylsulfonium center would then undergo a 1,4-Michael type thiol addition. The nonconjugated alkene product **3d** with two terminal protons on the γ-carbon atom was obtained as the final product[38] (Figure 2B). We tried to open the sulfonium ring, but we found that it was difficult to open the ring after 24 h of reaction with **3d** and nucleophilic reagents (dithiothreitol, 2-mercaptopyridine, and benzylamine) and no addition or ring-opening products was identified (Figure 2C and D and Figures S1 and S2). We think the reactivity of the sulfonium center may be influenced by the vinyl sulfide. This phenomenon is consistent with the results reported by Deming's group.[43]

The cyclization influences were then characterized. We first tested the serum stability of peptide **2f**/**3f**, and cyclized peptide **3f** was found to have a significantly enhanced serum stability (Figure 3B). In cellular content, glutathione often presented at high concentration.[44−46] Thus, we tested the stability of the cyclized peptide **3f** in the presence of two different nucleophiles,[43] glutathione (GSH) and 2-mercaptopyridine. The reaction kinetics were tested by letting peptide **3f** (1 mM) react with GSH (10 mM) and 2-mercaptopyridine (10 mM) in PBS (pH 7.4), respectively. The results were monitored by LC-MS analysis at different time points and summarized in Figure 3C. Notably, about 90% of peptide **3f** was retained after 24 h. Peptides' cellular permeability is crucial for their potential applications as PPI modulators. The cellular uptakes of peptide

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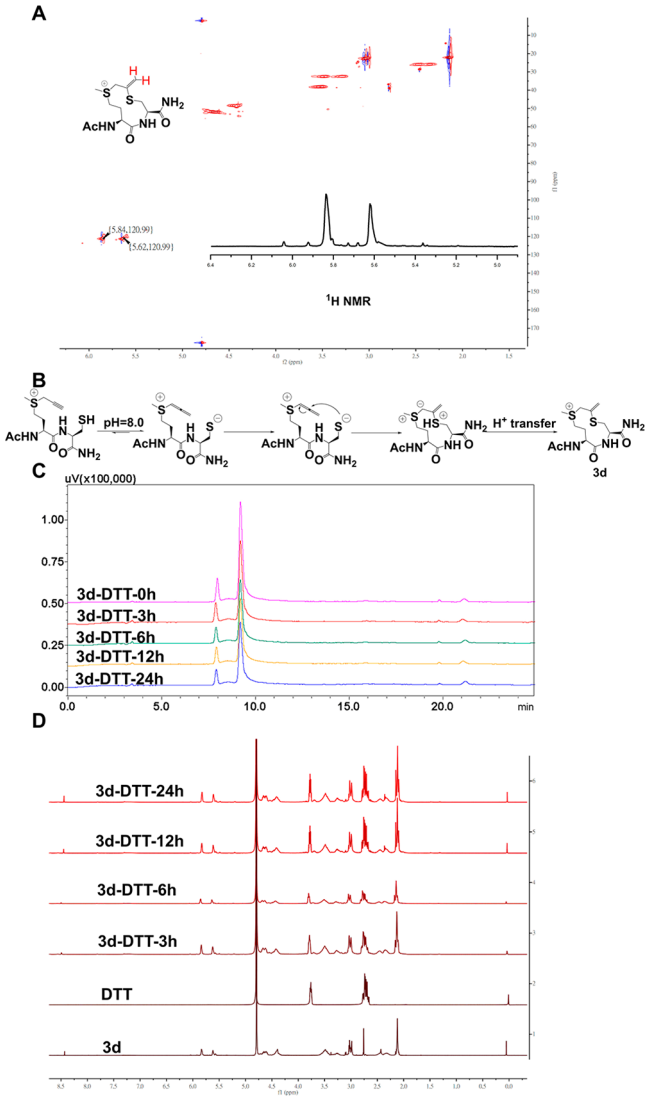

Figure 2. (A) ^{1}H NMR and HSQC spectra of **3d** (400 MHz in D_2O). (B) Proposed reaction pathway. (C) HPLC traces of the time dependent conversion between peptide **3d** and DTT for 0, 3, 6, 12, and 24 h. (D) ^{1}H NMR spectra of the time dependent conversion between peptide **3d** and DTT in D_2O for 3, 6, 12, and 24 h.

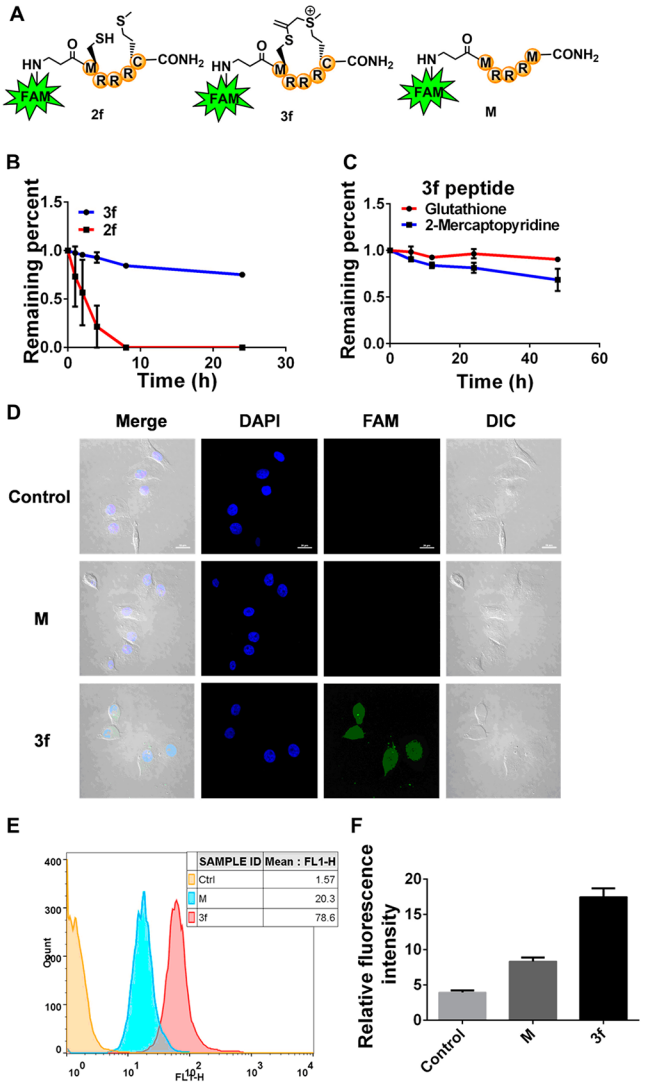

Figure 3. (A) Structures of peptides. (B) In vitro serum digestion assay of **2f** and **3f** peptides. Peptides were incubated with 25% fresh mouse serum (v/v) in PBS to a final concentration of 100 μM at 37 °C for 24 h. (C) Dealkylation of the peptide **3f** (1 mM) in the presence of PyS (10 mM) or GSH (10 mM) in PBS (pH 7.4) at 37 °C for 48 h. (D) Confocal microscopy images of HeLa cells after treatment with 5 μM peptide **3f** and linear cell penetrating peptide **M** for 4 h; the sequence of peptide **M** is FAM-βAla-MRRRM. (E) FACS analysis of HeLa cells treated with 5 μM peptide **3f** and linear cell penetrating peptide **M** at 37 °C for 4 h. (F) Quantitative analysis of cell uptake with 5 μM peptide **3f** and linear cell penetrating peptide **M** by Multilabel Reader.

M and **3f** were then tested in HeLa cells. The confocal microscopy imaging, flow cytometry analysis, and quantitative fluorescent analysis showed increased cellular uptake upon cyclization (Figure 3D–F).[33,47,48]

PDZ$^{\Delta RGS3}$ plays an important role in ephrin-B reverse signaling which is associated with SDF-1 (stromal derived factor 1) neuronal chemotaxis.[49] Peptides **2g**/**3g** were prepared based on reported PDZ binding sequences, and the peptides' binding affinity with PDZ$^{\Delta RGS3}$ was tested by fluorescence anisotropy assays[50] (Figure 4A). Peptide **3g** maintained the binding affinity to PDZ$^{\Delta RGS3}$ (K_D = 108.94 ± 25.40 nM) after the cyclization, which is comparable to the linear peptide **2g** (K_D = 125.59 ± 17.59 nM) (Figure 4B). Combining the characterization of peptides' stability and cellular uptakes, all results suggested this cyclization method could be potentially useful for constructing peptide PPI modulators. Notably, as shown in Scheme 1, sulfonium peptide may react with nearby cysteines of its target such as PDZ$^{\Delta RGS3}$. However, the sulfonium center formed after this thiol-yne type reaction is less reactive and showed much slower conjugation with cysteines, comparing with our previous report.[33]

In addition to enriching the peptide cyclization toolbox, we prepared a simplified model compound FAM-Met **4** and let it react with different Cys-containing proteins, as shown in Figure 5A. We successfully labeled all four Cys-containing proteins (SarA, PDZ$^{\Delta RGS3}$, BFL-1, MgrA), as shown in Figure 5B. Logically, we believe that the structurally reasonable probes modified by this method could be utilized for protein profiling and classification. We also propose this method might be used to make probes to monitor protein–protein interactions and may be extended to multiple other amino acid profiling.

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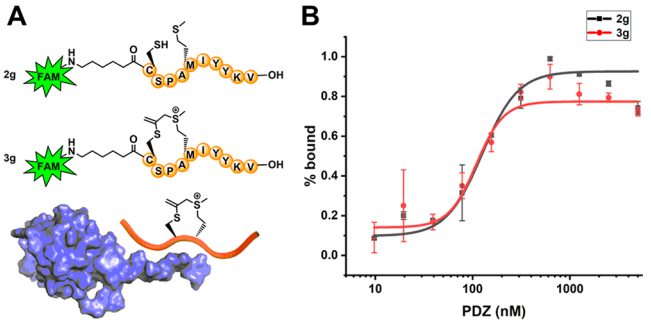

Figure 4. (A) A schematic presentation of the interaction between peptide **3g** and $PDZ^{\Delta RGS3}$. (B) Binding of **2g** and **3g** with $PDZ^{\Delta RGS3}$. The binding affinities were measured using fluorescence anisotropy at 25 °C. Fluorescein-labeled peptides (5 nM) in assay buffer (PBS, pH 7.4) were mixed.

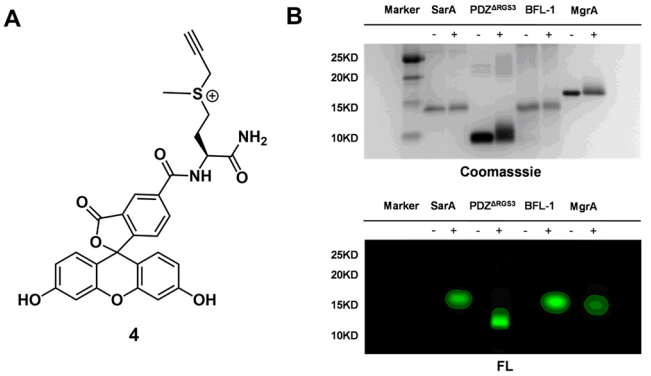

Figure 5. (A) Structure of FAM-Met **4**. (B) **4** (20 μM) with SarA (10 μM), $PDZ^{\Delta RGS3}$ (10 μM), BFL-1 (10 μM), and MgrA (10 μM) were incubated in 20 μL of PBS (pH 8.0) at 37 °C for 24 h.

In summary, we report a facile and highly efficient thiol-yne type reaction triggered by the propargyl sulfonium center in aqueous solution and at ambient temperature. This method could be performed both intermolecularly or intramolecularly with satisfying functional group tolerance. It could be translated into a facile peptide cyclization methodology of unprotecting peptides. Cyclic peptides prepared with this method showed increased serum stability, good GSH resistance, and increased cellular uptakes. In a model study with PDZ protein, cyclized peptide showed a comparable binding affinity with its linear analogue. All of these features indicated the method may be potentially useful for making peptide modulators for various protein targets. The propargyl sulfonium center could react with Cys-containing proteins under biorelevant conditions, which suggested this method might be utilized for making probes with proper structures for protein profiling and protein−protein interactons.

The facileness, high efficiency, and bio-orthogonal nature of this reaction could substantially enrich the chemical toolbox of peptide/protein modification. We believe sulfonium centers played more roles in tuning the reactivity of the attached groups, and further research is under current investigation in our laboratory and will be reported in due course.

EXPERIMENTAL SECTION

Reagents and Materials. For peptide synthesis, all amino acids and resins were purchased from GL Biochem (Shanghai). Dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIPEA), 2-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), trifluoroacetic acid (TFA), triisopropylsilane (TIS), propargyl bromide, 5-carboxyfluorescein, and ammonium carbonate were purchased from Energy Chemical. The reagents used for biological assays were purchased from Sigma-Aldrich and Thermo Fisher.

NMR spectra were measured on a Bruker AVANCE-III 400 instrument. The reverse phase high performance liquid chromatography (HPLC) was performed on a Shimadzu prominence LC-20AT instrument equipped with a C18 column (Kromasil 100-5-C18 4.6 × 250 mm^2) and acetonitrile/water (0.1% TFA) as the eluent condition. HPLC fractions containing product (screened by Electrospray ionization (ESI)) were combined and lyophilized. Molecular weights were measured on an ABI QSTAR Elite instrument. For all of the HRMS measurements, the ionization method is ESI and the mass analyzer type is TOF. Confocal microscopy imaging assays were visualized on a Nikon A1R.

General Procedures for SPSS. Peptides were synthesized on Rink Amide MBHA Resin or Fmoc-Val-Wang Resin by standard manual Fmoc solid phase synthesis (SPSS). Rink amide resin was preswelled with DCM for 30 min and filtered. Then, the Fmoc (9-fluorenylmethyloxycarbonyl) group was removed with 50% (v/v) morpholine for 30 min × 2; the resin was sequentially washed with DCM and DMF three times. For amino acid coupling, Fmoc-protected amino acid (5.0 equiv) and HATU (5.0 equiv) were dissolved in DMF, followed by DIPEA (10.0 equiv). The mixture was preactivated for 1 min, added to the resin, and allowed to react for 1 h under N_2 bubbling. The resin was washed sequentially with DCM and DMF three times. Crude peptides were cleaved from the resins by TFA/TIS/H_2O (95:2.5:2.5, v/v) for 2 h. Peptides were purified and characterized by HPLC and LC-MS.

Reactions of Compounds with Propargyl Bromide. To a solution of compound (0.2 mmol, 1.0 equiv) in 0.2 mL of MeCN/H_2O (1:1, v/v), 1% HCOOH aqueous solution (in volume) and propargyl bromide (1.0 mmol, 5.0 equiv) were added. The reaction was shaken at room temperature for 12 h. Then, the solution was purified by reversed phase HPLC immediately.

General Procedure for the Addition of Thiols to Propragylsulfonium Salts in Aqueous Media. Thiol (0.20 mmol, 1.0 equiv) was dissolved in ammonium carbonate solution (0.5 mL); **1** (0.20 mmol, 1.0 equiv) was added. The reaction pH value was maintaining at 8.0. The reaction mixture was stirred for 1 h at 25 °C. The solution was immediately purified by HPLC.

General Procedure for Synthesis of Cyclic Peptides. To a solution of peptide in 0.5 mL of MeCN/H_2O (1:1, v/v) in an Eppendorf tube, 1% Et_3N (in volume) was added. The reaction was shaken at room temperature for 12 h. Then, the solution was lyophilized to remove Et_3N, and the crude product was purified by HPLC.

NMR Spectroscopy. ^1H, $^{13}C\{^1H\}$ NMR data and 2D NMR data were recorded on a Bruker AVANCE III 400 MHz spectrometer. For these experiments, the compounds were dissolved in D_2O (or DMSO-d_6, CD_3CN). All of the NMR spectra were processed by Mestre Nova 12.0 software.

Dealkyation of the Cyclic Peptide. The cyclic peptide was dissolved in nucleophilic reagents (DTT, PyS, and $BnNH_2$) in D_2O and incubated at 37 °C for 0, 3, 6, 12, and 24 h. An aliquot of each reaction solution was removed and monitored by LC-MS and NMR.

Molecular Cloning, Protein Expression, and Purification. *E. coli* strains DH5*α* and BL21 star (DE3) (Laboratory stock) were used for DNA manipulation and protein expression, respectively. Vector pET28a (Novagen), pET22b (Novagen), and pCold I (Takara) were used for cloning in *E. coli* (the $PDZ^{\Delta RGS3}$ fragment was a gift from Prof. Jiang Xia's group, Hong Kong Chinese University). *SarA* and *mgrA* were amplified from chromosomal DNA of Newman using primers SarA-NdeI-F/SarA-XhoI-R and MgrA-NdeI-F/MgrA-XhoI-R. The *sarA* and *mgrA* amplicons were ligated into NdeI/XhoI sites of pET28a. The gene *BFL-1* encoding 1−152 amino acids were ligated into NdeI/XhoI sites of pET28a.

Cloning, expression, and purification of SarA, $PDZ^{\Delta RGS3}$, BFL-1, and MgrA were transformed into *E. coli* BL21 (DE3) cells, and they

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were grown overnight at 37 °C in LB media supplemented with 100 μg/mL ampicillin. When cultures were grown at 37 °C to reach OD_{600} ~ 0.6, cultures were removed to 16 °C with 1 mM IPTG to induce protein expression for 8 h. The cultures were harvested and resuspended in 50 mL of lysis buffer (containing 20 mM Tris, 500 mM NaCl, 3 mM DTT, 0.1 mM PMSF, pH 7.5), sonicated, and centrifugated at 14000*g* for 1 h to obtain the supernatant and remove cell debris. Recombinant proteins were extracted from cleared lysates by nickel nitrilotriacetic acid-agarose (Qiagen) followed by washes with PBS containing 10 mM imidazole. The fusion protein was eluted with 250 mM imidazole and dialyzed against PBS. More construction details were also enclosed in our previous report.[33,51]

In Vitro Protein FAM-Met Covalent Conjugation Experiment. For FAM-Met 4 protein conjugation, the purified SarA, $PDZ^{\Delta RGS3}$, BFL-1, and MgrA (10 μM) were allowed to react with FAM-Met 4 (20 μM) in 20 μL of pH 8.0 PBS buffer at 37 °C for 24 h. Then, the reaction mixtures were analyzed with 15% tricine gels.

In Vitro Serum Stability. Peptides were incubated with 25% fresh mouse serum (v/v) in PBS to a final concentration of 100 μM at 37 °C. Aliquots (10 μL) were taken periodically at 0, 1, 2, 4, 8, and 24 h, and then, 100 μL of 12% trichloroacetic acid in H_2O/MeCN (1:3) was added and cooled to 4 °C for 30 min to precipitate serum proteins. Then, all of the samples were removed by centrifugation at 14000 rpm for 15 min. The supernatant was analyzed by HPLC. The peptide serum half-life was calculated by HPLC peak integration.

Reduction Experiment of the Cyclic Peptide. The cyclic peptides (1 mM) were dissolved with 10 mM nucleophile solution (2-mercaptopyridine or GSH) in PBS (pH 7.4) and incubated at 37 °C for 0, 6, 12, 24, and 48 h; an aliquot of each reaction solution was removed and monitored by HPLC. The peptide serum half-life was calculated by HPLC peak integration.

Fluorescence Anisotropy. $PDZ^{\Delta RGS3}$ peptide fluorescence anisotropy experiments were performed in 96-well plates (PerkinElmer Optiplate96F) on a plate reader (PerkinElmer, Envision, 2104 Multilabel Reader). Concentrations of peptides were determined by 485 nm absorption of FAM. Purified $PDZ^{\Delta RGS3}$ and fluorescein-labeled peptides (5 nM) in assay buffer (PBS = 7.4) were mixed. The fluorescence anisotropy of the labeled peptides was measured at 25 °C with excitation at 485 nm and emission at 520 nm and then plotted against the concentrations of the $PDZ^{\Delta RGS3}$

$$r = \frac{S - GP}{S + 2GP} \tag{1}$$

where S is parallel intensities, P is perpendicular intensities, and G is the G factor usually attributed to Azumi and McGlynn

$$F_{SB} = \frac{r - r_{free}}{r_{bound} - r_{free}} = 1 - \frac{L_S}{L_{ST}} \tag{2}$$

where F_{SB} is the fraction of bound labeled ligand, r_{free} is the anisotropy of the free labeled ligand, and r_{bound} is the anisotropy of the ligand–protein complex at saturation

$$K_D = \frac{R \times L_S}{[RL_S]} \tag{3}$$

$$R_T = R + [RL_S] \tag{4}$$

$$L_{ST} = L_S + [RL_S] \tag{5}$$

where R, L_S, and RL_S denote the free concentrations of the receptor, labeled ligand, and labeled ligand–receptor complex, respectively. K_D is the dissociation constant of the interaction. R_T and L_{ST} are the total input concentrations of the receptor and labeled ligand, respectively. Solving eqs 2–5 for F_{SB} analogously, we find the well-known eq 6.

$$F_{SB} = \frac{K_D + L_{ST} + R_T - \sqrt{(K_D + L_{ST} + R_T)^2 - 4L_{ST}R_T}}{2L_{ST}} \tag{6}$$

K_D is derived from eq 6, and the data points were fitted by OriginPro 2018C.[52,53]

Confocal Microscopy Imaging. HeLa cells were cultured with DMEM with 10% FBS (v/v) in a humidified incubator containing 5% CO_2 at 37 °C. Then, cells were incubated with 5 μM FAM-labeled peptides in DMEM medium for 4 h at 37 °C. Cells were washed three times with PBS and then fixed with 4% (wt/v) formaldehyde in PBS for 3 min. They were then washed three times with PBS and stained with DAPI (4′,6-diamidino-2-phenylindole) for 10 min. The coverslips were mounted onto slides and visualized by a confocal laser scanning microscope (Nikon A1R).

Flow Cytometry Analysis. HeLa cells were seeded in 24-well dishes for 24 h in DMEM medium with 10% FBS containing 5% CO_2 at 37 °C. The plates were incubated with 5 μM FAM-labeled peptides in DMEM medium for 4 h at 37 °C. After washing with media, the cells were washed with PBS and resuspended in PBS. Cellular fluorescence was analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson) and CFlow plus.

Quantitative Fluorescent Analysis. Intracellular fluorescence intensity quantification was measured using a plate reader (PerkinElmer, Envision, 2104 Multilabel Reader). The cellular uptake of FAM-labeled peptides was performed as described previously.[54] Cells were washed and then lysed by adding 300 μL of lysing buffer (0.1 M NaOH) for 10 min at 37 °C. The cell lysates were harvested and centrifuged (14000 rpm for 10 min), and the supernatant was used to measure the fluorescence intensity.

HPLC Purifications of Compound Methods. *HPLC Method A.* Kromasil C18 column, 5 μm, 4.6 × 250 mm^2 (Kromasil 100-5-C18.), flow rate 0.8 mL/min, solvent A 0.1% TFA + water, solvent B 100% acetonitrile, gradient (A:B) 80:20 (0 min) to 20:80 (16 min) to 2:98 (20 min).

HPLC Method B. Kromasil C18 column, 5 μm, 4.6 × 250 mm^2 (Kromasil 100-5-C18.), flow rate 0.8 mL/min, solvent A 0.1% TFA + water, solvent B 100% acetonitrile, gradient (A:B) 95:5 (0–3 min) to 60:40 (20 min) to 2:98 (25 min).

Synthesis of Compounds. *((S)-3-Acetamido-3-carboxypropyl)(methyl)(prop-2-yn-1-yl)sulfonium (**1a**).* It is obtained as a white solid. Yield: 214 mg, 93%. mp: 122–124 °C. ^{1}H NMR (400 MHz, D_2O) δ 4.64–4.55 (m, 1H), 4.40 (t, 2H), 3.53–3.43 (m, 2H), 3.25 (td, 1H), 2.99 (s, 3H), 2.54–2.41 (m, 1H), 2.35–2.20 (m, 1H), 2.08 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, D_2O) δ 174.5, 173.5, 80.8, 51.2, 37.2, 25.6, 25.5, 22.1, 22.0. HRMS (ESI-TOF): m/z calculated for $C_{10}H_{16}NO_3S^+$ $[M]^+$, 230.0845; found, 230.0848.

*((S)-4-Amino-3-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-carboxamido)-4-oxobutyl)(methyl)(prop-2-yn-1-yl)sulfonium (**4**).* It is obtained as a yellow solid. Yield: 39 mg, 90%. mp: 394–396 °C. ^{1}H NMR (400 MHz, D_2O/CD_3CN) δ 7.85 (d, 1H), 7.51 (dd, 1H), 6.63 (d, 1H), 6.20–6.17 (m, 4H), 6.04 (dd, 2H), 4.08–4.04 (m, 1H), 3.63 (d, 2H), 2.79–2.75 (m, 2H), 2.50 (t, 1H), 2.22 (d, 3H), 1.79–1.74 (m, 1H), 1.68–1.62 (m, 1H). ^{13}C{^{1}H} NMR (101 MHz, D_2O/CD_3CN) δ 173.1, 168.2, 167.1, 162.5, 154.1, 134.7, 133.3, 129.8, 127.6, 126.0, 117.4, 114.7, 111.2, 102.1, 80.2, 67.9, 51.7, 38.3, 36.7, 29.8, 29.6, 25.3, 25.2, 21.3. HRMS (ESI-TOF): m/z calculated for $C_{29}H_{25}N_2O_7S^+$ $[M]^+$, 545.1377; found, 545.1376.

*((S)-3-Acetamido-4-(((R)-1-amino-3-mercapto-1-oxopropan-2-yl)amino)-4-oxobutyl)(methyl)(prop-2-yn-1-yl)sulfonium (**2d**).* It is obtained as a white solid. Yield: 60 mg, 89%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (dd, 1H), 8.19 (dd, 1H), 7.45 (s, 1H), 7.21 (s, 1H), 4.57 (t, 2H), 4.52–4.43 (m, 1H), 4.30 (td, 1H), 3.94 (t, 1H), 3.37 (t, 2H), 2.94 (s, 3H), 2.88–2.71 (m, 2H), 2.40 (t, 1H), 2.23–2.15 (m, 1H), 2.09–2.00 (m, 1H), 1.90 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ 171.3, 170.3, 170.0, 81.6, 70.5, 55.0, 51.1, 37.4, 30.3, 26.4, 26.0, 22.7, 22.2. HRMS (ESI-TOF): m/z calculated for $C_{13}H_{22}N_3O_3S_2^+$ $[M]^+$, 332.1097; found, 332.1098.

*((S)-3-Acetamido-3-carboxypropyl)(methyl)(2-(pyridin-2-ylthio)-allyl)sulfonium (**3a**).* It is purified by HPLC method A. The retention time is 20.896 min. It is obtained as a pale yellow solid. Yield: 55 mg, 82%. mp: 113–116 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.48 (dd, 1H), 8.33 (d, 1H), 7.78–7.75 (m, 1H), 7.43 (dd, 1H), 7.28–7.25 (m, 1H), 6.17 (s, 1H), 5.99 (s, 1H), 4.58–4.50 (m, 2H), 4,35–4.31 (m, 2H), 3.43–3.31 (m, 2H), 2.93 (s, 3H), 2.27–2.18 (m, 1H), 2.06–1.98 (m, 1H), 1.86 (s, 3H). ^{13}C{^{1}H} NMR (101 MHz, DMSO-d_6) δ

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169.5, 168.7, 157.4, 149.9, 137.4, 124.0, 123.0, 121.0, 112.8, 65.3, 53.3, 48.0, 30.0, 22.8, 14.7. HRMS (ESI-TOF): m/z calculated for $C_{15}H_{21}N_2O_3S_2^+$ $[M]^+$, 341.0988; found, 341.0984.

*((S)-3-Acetamido-3-carboxypropyl)(2-((2,3-dihydroxy-4-mercaptobutyl)thio)allyl)(methyl)sulfonium (**3b**).* It is purified by HPLC method A. The retention time is 13.842 min. It is obtained as a white solid. Yield: 53 mg, 70%. mp: 75−77 °C. ^{1}H NMR (400 MHz, CD_3OD) δ 5.66 (s, 1H), 5.48 (s, 1H), 4.59−4.56 (m, 1H), 4.55−4.51 (m, 2H), 4.40−4.28 (m, 2H), 3.94−3.91 (m, 1H), 3.86−3.80 (m, 1H), 3.63−3.54 (m, 3H), 3.51−3.45 (m, 3H), 2.93 (d, 3H), 2.03 (s, 3H). $^{13}C\{^1H\}$ NMR (101 MHz, CD_3OD) δ 174.0, 172.3, 142.1, 117.3, 71.6, 69.7, 66.2, 51.6, 34.4, 30.9, 30.0, 27.2, 21.5, 24.3. HRMS (ESI-TOF): m/z calculated for $C_{14}H_{26}NO_5S_3^+$ $[M]^+$, 384.0968; found, 384.0965.

*(4R,12S)-4,12-Dicarboxy-9-methyl-7-methylene-2,14-dioxo-6,9-dithia-3,13-diazapentadecan-9-ium (**3c**).* It is purified by HPLC method A. The retention time is 5.992 min. It is obtained as a white solid. Yield: 66 mg, 85%. mp: 160−162 °C. ^{1}H NMR (400 MHz, D_2O) δ 5.24 (s, 1H), 4.98 (s, 1H), 4.28−4.23 (m, 1H), 4.18−4.14 (m, 1H), 3.33−3.08 (m, 4H), 2.95−2.84 (m, 1H), 2.81−2.74 (m, 2H), 2.71−2.62 (m, 1H), 1.92−1.83 (m, 9H). $^{13}C\{^1H\}$ NMR (101 MHz, D_2O) δ 177.0, 176.7, 173.6, 173.6, 173.5, 171.0, 160.3, 113.1, 54.4, 53.9, 38.1, 33.2, 32.9, 22.1, 22.1. HRMS (ESI-TOF): m/z calculated for $C_{15}H_{25}N_2O_6S_2^+$ $[M]^+$, 393.1149; found, 393.1143.

*(6R,9S)-9-Acetamido-6-carbamoyl-1-methyl-3-methylene-8-oxo-1,4-dithia-7-azacycloundecan-1-ium (**3d**).* It is purified by HPLC method B. The retention time is 8.338 min. It is obtained as a white solid. Yield: 47 mg, 80%. mp: 230−233 °C. ^{1}H NMR (400 MHz, D_2O) δ 5.84 (s, 1H), 5.62 (s, 1H), 4.72−4.56 (m, 2H), 4.48−4.34 (m, 2H), 3.53−3.44 (m, 2H), 3.32−3.19 (m, 1H), 3.05−2.95 (m, 3H), 2.80−2.77 (m, 1H), 2.51−2.40 (m, 1H), 2.37−2.24 (m, 1H), 2.12 (s, 3H). $^{13}C\{^1H\}$ NMR (101 MHz, DMSO-d_6) δ 158.8, 158.3, 132.8, 118.8, 116.3, 51.7, 47.9, 38.6, 33.6, 27.0, 23.2, 22.3, 15.2. HRMS (ESI-TOF): m/z calculated for $C_{13}H_{22}N_3O_3S_2^+$ $[M]^+$, 332.1097; found, 332.1098.

*(6R,9S,12S)-12-Acetamido-6-carbamoyl-1,9-dimethyl-3-methylene-8,11-dioxo-1,4-dithia-7,10-diazacyclotetradecan-1-ium (**3e**).* It is purified by HPLC method B. The retention time is 8.952 min. It is obtained as a white solid. Yield: 13 mg, 78%. ^{1}H NMR (300 MHz, D_2O) δ 5.73 (s, 1H), 5.53 (s, 1H), 4.54−4.44 (m, 1H), 4.39−4.27 (m, 2H), 3.47−3.27 (m, 2H), 3.01−2.83 (m, 3H), 2.61−2.48 (m, 2H), 2.33−2.19 (m, 2H), 2.03−1.97 (m, 6H), 1.39−1.27 (m, 5H). $^{13}C\{^1H\}$ NMR (75 MHz, D_2O) δ 174.3, 173.7, 163.0, 162.5, 118.2, 114.3, 113.1, 52.6, 50.0, 41.7, 30.4, 29.2, 29.1, 21.6, 16.0, 14.1. HRMS (ESI-TOF): m/z calculated for $C_{16}H_{27}N_4O_4S_2^+$ $[M]^+$, 403.1468; found, 403.1460.

*(6R,9S,12S,15S,18S)-6-carbamoyl-18-(3-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-carboxamido) propanamido)-9,12,15-tris(3-guanidinopropyl)-1-methyl-3-methylene-8,11,14,17-tetraoxo-1,4-dithia-7,10,13,16-tetraazacycloicosan-1-ium (**3f**).* It is purified by HPLC method B. The retention time is 11.220 min. It is obtained as a yellow solid. Conversion: 60%. HRMS (ESI-TOF): m/z calculated for $C_{53}H_{71}N_{16}O_{12}S_2^+$ $[M]^+$, 1187.4873; found, 1187.4886.

*(3S,6S,14R,17S,22aS)-6-(((3S,6S,9S,12S,15S,16R)-6-(4-aminobutyl)-3-carboxy-9,12-bis(4-hydroxybenzyl)-2,16-dimethyl-5,8,11,14-tetraoxo-4,7,10,13-tetraazaoctadecan-15-yl)carbamoyl)-14-(6-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-carboxamido)hexanamido)-17-(hydroxymethyl)-3,9-dimethyl-11-methylene-1,4,15,18-tetraoxoicosahydro-1H-pyrrolo[1,2-k][1,4]-dithia[8,11,14,17]tetraazacycloicosin-9-ium (**3g**).* It is purified by HPLC method B. The retention time is 12.916 min. It is obtained as a yellow solid. Conversion: 52%. HRMS (ESI-TOF): m/z calculated for $C_{84}H_{107}N_{12}O_{21}S_2^+$ $[M]^+$, 1683.7110, $[M + 1]/2^+$, 842.3555; found, 1683.7074, 842.3529.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.9b02505.

Spectra parameters for ^{1}H, $^{13}C\{^1H\}$, and 2D NMR and mass spectra and HPLC data for all peptides after purification (PDF)

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Notes

The authors declare no competing financial interest.

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