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Cleavable and tunable cysteine-specific arylation modification with aryl thioethers†

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Cysteine represents an attractive target for peptide/protein modification due to the intrinsic high nucleophilicity of the thiol group and low natural abundance. Herein, a cleavable and tunable covalent modification approach for cysteine containing peptides/proteins with our newly designed aryl thioethers *via* a S_NAr approach was developed. Highly efficient and selective bioconjugation reactions can be carried out under mild and biocompatible conditions. A series of aryl groups bearing different bioconjugation handles, affinity or fluorescent tags are well tolerated. By adjusting the skeleton and steric hindrance of aryl thioethers slightly, the modified products showed a tunable profile for the regeneration of the native peptides.

Introduction

Peptide/protein modification is an important approach for profiling their structures and functions, studying protein–protein interactions (PPIs), monitoring cellular biological processes, and developing therapeutic agents.[1] In comparison to *de novo* peptide synthesis, site-selective modification of existing peptides provides a more straightforward and effective option to diversify peptides for functional studies.[2] Despite various methodologies developed to modify native amino acid residues, a tunable, biocompatible, highly selective and efficient protein modification approach is still highly in demand.[2c,3] Among the 20 proteogenic amino acids, cysteine represents an attractive target for post-translational modification due to the intrinsic high nucleophilicity of the thiol group and low natural abundance (1.9%), and could be easily incorporated into specific sites by site-directed mutagenesis.[4]

To achieve cysteine-specific modification, numerous efforts have been made towards the development of modification reagents that enable cysteine labelling with good efficiency and selectivity, including halogenoalkanes,[5] maleimide,[6] alkenes,[7] alkynes,[4b] allenamides,[4d] and hypervalent iodine compounds.[8] Among the established methods, the arylation strategy was underdeveloped to label peptides which have multiple functional groups and a large molecular weight.[9] The nucleophilic aromatic substitution (S_NAr) strategy provides the possibility for peptide arylation with easy manipulation and no need for extra additives, in comparison with metal- or photo-catalyzed arylation chemistry.[10] In recent years, several arylation reagents based on S_NAr were developed and showed good efficiency in labelling peptides, including aryl halides,[11] perfluoroaromatic molecules,[12] heteroaryl methylsulfone and methylsulfoxide reagents (Fig. 1A).[13] However, the methods mentioned above were mainly focused on improving the modification efficiency or deliberately optimizing the stability of the Cys-bioconjugated linkage. Cleavable and tunable Cys-selective modification,

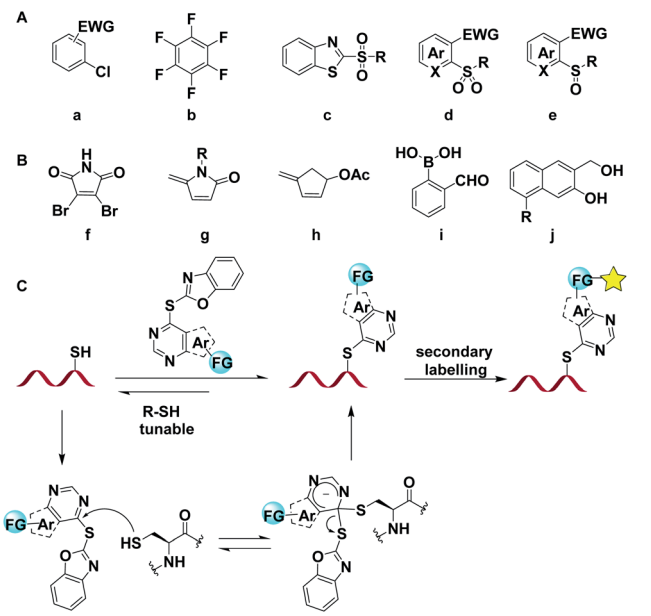

Fig. 1 (A) Representative reagents for Cys modification based on nucleophilic aromatic substitution. (B) Representative cleavable cysteine specific modification reagents. (C) Cleavable and tunable cysteine arylation strategy *via* a S_NAr mechanism.

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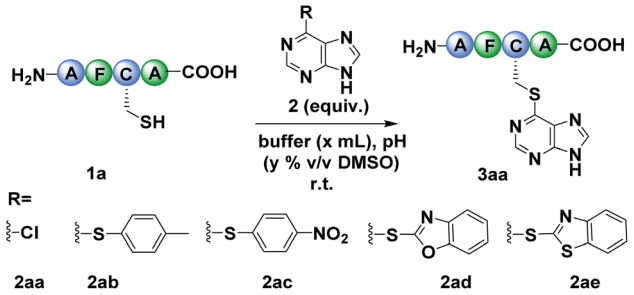
which could enable the regeneration of the native peptides/proteins, was desired in areas such as antibody–drug conjugates (ADCs) and epigenetic modifications, but less studied.[14] Maleimide derivatives **f–h**, formyl benzeneboronic acid **i**, and naphthol derivatives **j** could be applied to regenerate the native peptides or proteins; however the acidic or basic regeneration conditions and the necessity of well-defined triggers/stimuli prevented their further applications (Fig. 1B).[6a,15] The pursuit of developing an efficient, biocompatible and regeneration tunable cysteine-specific modification strategy is still in high demand, especially because the modification could facilitate further decoration of peptides or proteins. Herein, we reported a cysteine selective, transition-metal free, regeneration tunable peptide/protein modification approach with aryl thioethers with good chemo-selectivity and reactivity, and the arylation could be applied to secondary decoration of peptides (Fig. 1C).

Results and discussion

Our research was initiated by testing the reactivity of model peptide **1a** with an array of arylation reagents. Commercially available 6-chloro purine **2aa**, which could react with mercaptan and presents good skeleton functionalization feasibility, was employed initially. However, no reaction occurred, which might be attributed to the inapposite leaving ability of the chlorine atom within **2aa** (Table 1, entry 1).[16] Inspired by the work of Swager, which disclosed the dynamic and self-correcting nature of S_NAr, we replaced the chloro- with a series of aromatic sulphides and investigated their reactivity with **1a** (Table 1, entries 2–5).[17] To our delight, **2ad** and **2ae** could react with **1a** in 100 mM PBS buffer (pH = 8.0), affording the desired modified product **3aa** with the yields of 43% and 17% after 1 h (Table 1, entries 4–5).[18] It worth noting that the free *C*-terminus and *N*-terminus did not react with the thioethers **2ad** and **2ae**, and the chemo-selectivity was determined by 1D ^{1}H NMR spectroscopy (Fig. S1†). Subsequently, different buffers and pH were screened using **2ad** as the arylation agent. 100 mM Tris buffer (pH = 8.0) turned out to be the best solvent for the modification, as it gave the product in 47% yield after 1 h (Table 1, entries 6–9 and Table S1†). To further improve the modification efficiency, we adjusted the equivalent of **2ad**, the reaction concentration and the ratio of DMSO for the reactions (Table 1, entries 10–13 and Table S1†). The results showed that when reducing the ratio of DMSO, the byproduct generated from the dimerization of **1a** was increased accompanied by the decrease of modification yields (Fig. S6†). Interestingly, by fine-tuning the ratio of DMSO and reaction concentration, the dimerized byproduct could be efficiently suppressed and the desired product **3aa** was obtained in 90% yield, despite the slightly increased reaction time (Table S1†). Finally, the optimal reaction conditions were established as performing the reaction at room temperature, with a concentration of peptide of 1 mM and 3 equivalents of arylation reagent in 100 mM Tris buffer (pH 8.0, 1% v/v DMSO) (Table 1, entry 13). Based on previous work and time-course analysis of the arylation process (Fig. S215†), we hypothesized that **2ad** reacted with **1a** *via* a two-step S_NAr mechanism and the first step is rate-limiting (Fig. 1C).[13c]

Table 1 Optimization of reaction conditions for cysteine arylation[a]



Entry	2 (eq.)	Buffer (pH)	*x*	*y*	Yield[b] (%)
1	**2aa** (1.5)	PBS (8.0)	0.5	10	n.d
2	**2ab** (1.5)	PBS (8.0)	0.5	10	n.d
3	**2ac** (1.5)	PBS (8.0)	0.5	10	n.d
4	**2ad** (1.5)	PBS (8.0)	0.5	10	43
5	**2ae** (1.5)	PBS (8.0)	0.5	10	17
6	**2ad** (1.5)	HEPES (8.0)	0.5	10	41
7	**2ad** (1.5)	Tris (8.0)	0.5	10	47
8	**2ad** (1.5)	Tris (7.6)	0.5	10	39
9	**2ad** (1.5)	Tris (7.8)	0.5	10	43
10	**2ad** (3.0)	Tris (8.0)	0.5	10	64 (85)
11	**2ad** (3.0)	Tris (8.0)	0.5	5	62 (87)
12	**2ad** (3.0)	Tris (8.0)	0.5	1	45 (80)
13	**2ad** (3.0)	Tris (8.0)	1.0	1	59 (90, 83[c])

[a] Reaction conditions: 1.0 μmol **1a**, **2** (eq.) in 100 mM non-degassed buffer at room temperature for 1 h. [b] Reported yields are LC-MS yields after 1 h (the yields in parentheses correspond to the yields after a 5 h reaction). [c] Isolated yield.

After establishing the optimal reaction conditions, we set out to exam the diversity of the arylation reagents **2** (Fig. S2†). Benzoxazole sulfides **2** with different heterocycle skeletons, including purine (**2b–2c**, **2h**), pyrimidine (**2d**), quinazoline (**2e**, **2f**), triazole pyrimidine (**2g**) and pyrazolopyrimidine (**2i–2k**), all ran smoothly to afford the desired modified products (Fig. 2, **3ab–3ak**). Generally, arylation reagents **2** bearing electron-withdrawing groups on the purine (Fig. S2,† **2b–2c**), quinazoline (**2e–2f**) or pyrazolopyrimidine (**2i–2k**) could improve the reaction efficiency while electron-donating groups had reverse effects (Fig. 2, **3ab–3ac**, **3ae–3af**, and **3ai–3ak**). Taking reaction efficiency, synthetic feasibility and functional group compatibility into consideration, we chose purine and pyrazolopyrimidine based benzoxazole sulfides as templates to further diversify the modification reagents. Purine based benzoxazole sulfides with different functional groups could efficiently modify peptide **1a** in good yields (Fig. 2 and **3al–3an**). The arylation process proceeded more efficiently when replacing the purine skeleton with pyrazolopyrimidine. A variety of widely applied biorelevant groups, including alkyne, azide, poly ethylene glycol (PEG) polymer and affinity label, could be introduced into arylation reagents and gave the corresponding products in almost quantitative yields (Fig. 2 and **3ao–3av**). Notably, arylation reagents bearing amino acid fragments, drug molecules or fluorescent tags were also tolerated under the optimized conditions (Fig. 2, **3aw**, **3ax** and **3ay**), demonstrating

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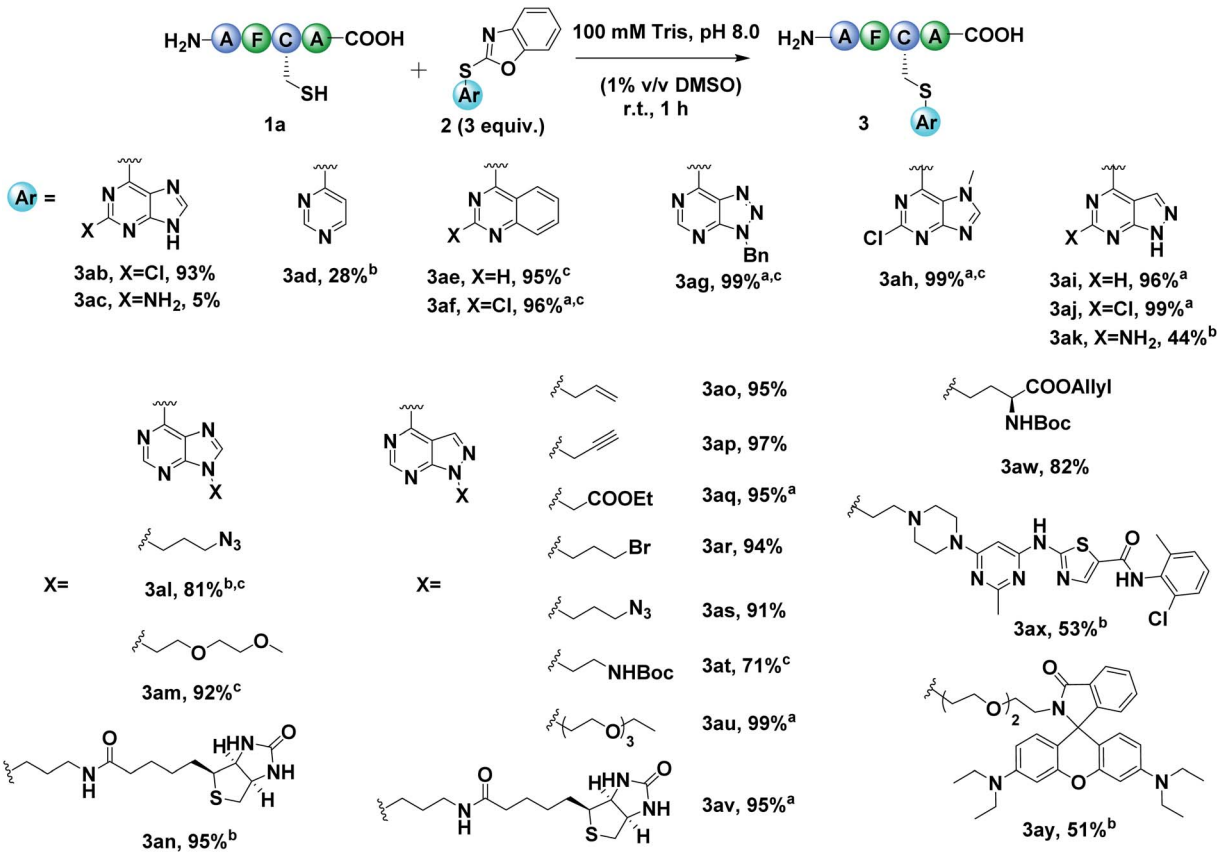

Fig. 2 Substrate scope of cysteine arylation. Unless otherwise specified, all reactions were carried out with 1.0 μmol **1a** and 3 μmol **2** in 1 mL 100 mM Tris buffer (pH 8.0, 1% v/v DMSO) at room temperature for 1 h. Reported yields are LC-MS yields. [a]The reaction was analysed after 5 min. [b]The reaction was analysed after 5 h. [c]5% DMSO was added.

the potential applications of this method in branched peptide synthesis and cyclization, antibody–drug conjugates (ADCs) and physiological studies. It is worth mentioning that the unreacted modification reagents **2** could be easily removed after the reaction completion by simple filtration due to their poor solubility in water. The reaction kinetics was evaluated by time-course analysis and competition experiments, which showed that **2j** reacted with peptide **1a** faster than iodoacetamide and fluorinated arenes but slower than maleimide (Fig. S215 and Table S2†).

As benzoxazole sulfides **2** bearing pyrazolopyrimidine moieties presented satisfactory reaction efficiency and functionalization feasibility, we chose **2i** to further investigate the scope of cysteine containing peptides (Fig. 3). Tetrapeptide substrates with different functional groups were surveyed firstly. The results showed that the peptides with nucleophilic Lys (**3bi**), Arg (**3ci**) or Glu (**3di**) were well tolerated. In the presence of peptides with other nucleophilic amino acids, *e.g.*, His (**3ei**), Ser (**3fi**), Tyr (**3gi**) and Thr (**3hi**), no side reactions were detected and the desired products were obtained in almost quantitative yields, demonstrating the unique chemo-selectivity of the benzoxazole sulfides to Cys residues. The modification reactivity of benzoxazole sulfide **2i** on longer peptides was also examined. Hexapeptides, octapeptides and 14-mer peptide **2m** or **2n** with various amino acid residues all ran smoothly to afford the desired cysteine modified products in excellent yields. To further explore the potential utility of this method, different functional groups were incorporated into benzoxazole sulfides **2**. To our delight, pyrazolopyrimidine and purine based benzoxazole sulphides all reacted with octapeptide **1l** and afforded the desired cysteine modified products (Fig. 3, **3li**, **3ls**, **3lv**, **3la** and **3ll**). The control peptide **1l′**, which derived from **1l** by replacing Cys with Ala, didn't react with **2i** and no arylation product was detected, further demonstrating the excellent chemo-selectivity to Cys over other amino acids. The stability of the modified peptides **3li**, **3ls**, **3lv**, **3la** and **3ll** to acids, bases, oxidants and external thiols was tested (Fig. S150†). The results showed that all tested peptides were stable in AcOH buffer (pH 4.0), 5 mM K_2CO_3 solution and Tris buffer (pH 8.0) after 24 h. In 5 mM H_5IO_6 solution, approximately 50% of **3li**, **3ls** and **3la** remained, while **3lv** and **3ln** were completely oxidized after 24 h, demonstrating that the modified aryl sulfides were relatively stable compared to alkyl sulfides under oxidizing conditions.[10a] Most interestingly, modified peptides bearing pyrazolopyrimidine skeletons (**3li**, **3ls**, and **3lv**) could be attacked by external thiols and recovered to their native unmodified state, while purine skeleton based modified peptides (**3la**, **3ln**) could not, indicating the possibility of regenerating peptides/proteins

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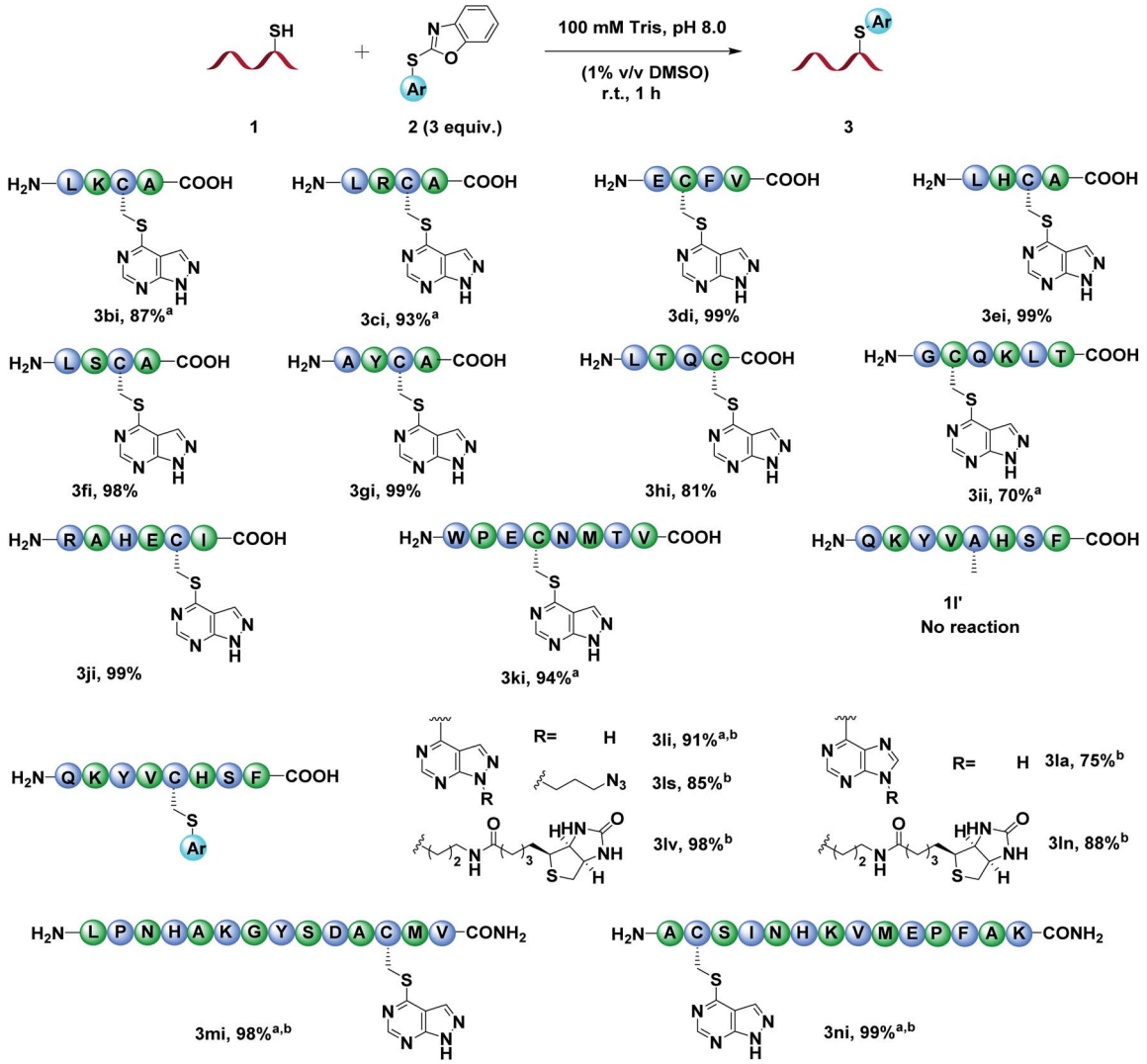

Fig. 3 Substrate scope of cysteine-containing peptides. Unless otherwise specified, all reactions were carried out with 1.0 μmol **1a** and 3 μmol **2** in 1 mL 100 mM Tris buffer (pH 8.0, 1% v/v DMSO) at room temperature for 1 h. Reported yields are LC-MS yields after 1 h. [a]The reaction was analysed after 5 min. [b]5% DMSO was added.

after modification by simply adjusting the skeleton structure of aryl thioethers (Fig. S150†).

To verify our hypothesis that the regenerating ability of the modified peptides could be adjusted by changing the skeleton of aryl thioethers, we performed the Cys arylation reaction using model peptide **1a** with different arylation reagents under the optimized conditions, followed by adding external thiols mercaptoethanol (βME) or glutathione (GSH) to the reaction mixture, and monitored the process by LC-MS. The results showed that modified peptides bearing different aryl skeletons exhibited distinct regenerating activity (Fig. 4A and Fig. S151†). The purine based products were relatively stable in βME or GSH (**3aa**, **3ab** and **3ah**), while quinazoline (**3ae**), triazole pyrimidine (**3ag**) and pyrazolopyrimidine (**3ai** and **3aj**) based modified peptides could be easily attacked by βME and recovered to the unmodified state. The introduction of an electron-withdrawing group into the arylation reagents led to an improved native peptide regeneration ability with βME but reduced ability with GSH, which might be attributed to the synchronously improved reactivity to thiols and steric hindrance of the modified peptides **3**, thereby accelerating the regeneration process with βME and preventing the native peptide regeneration with GSH (Fig. 4A and S151,† **3aa**, **3ab**, **3ah**–**3aj**). These results clearly demonstrated that a cleavable, tunable and Cys-specific peptide modification could be achieved by simply adjusting the skeletons and steric hindrance of our newly developed aryl thioethers. To demonstrate the generality of the cleavable and tunable Cys modification approach, we chose **2ad** and **2i** that showed different regeneration abilities to modify peptides, followed by adding βME to the reaction mixture and analysed by LC-MS. The results showed that the **2i** modified peptides **3** with a peptide length ranging from 4-mer to 14-mer all could be effective attacked by βME and recovered to the native state **1** with a recovery ratio over 90% after 1 h, while **2ad** modified peptides showed a weak regeneration ability with a recovery ratio of less than 14% (Fig. 4B).

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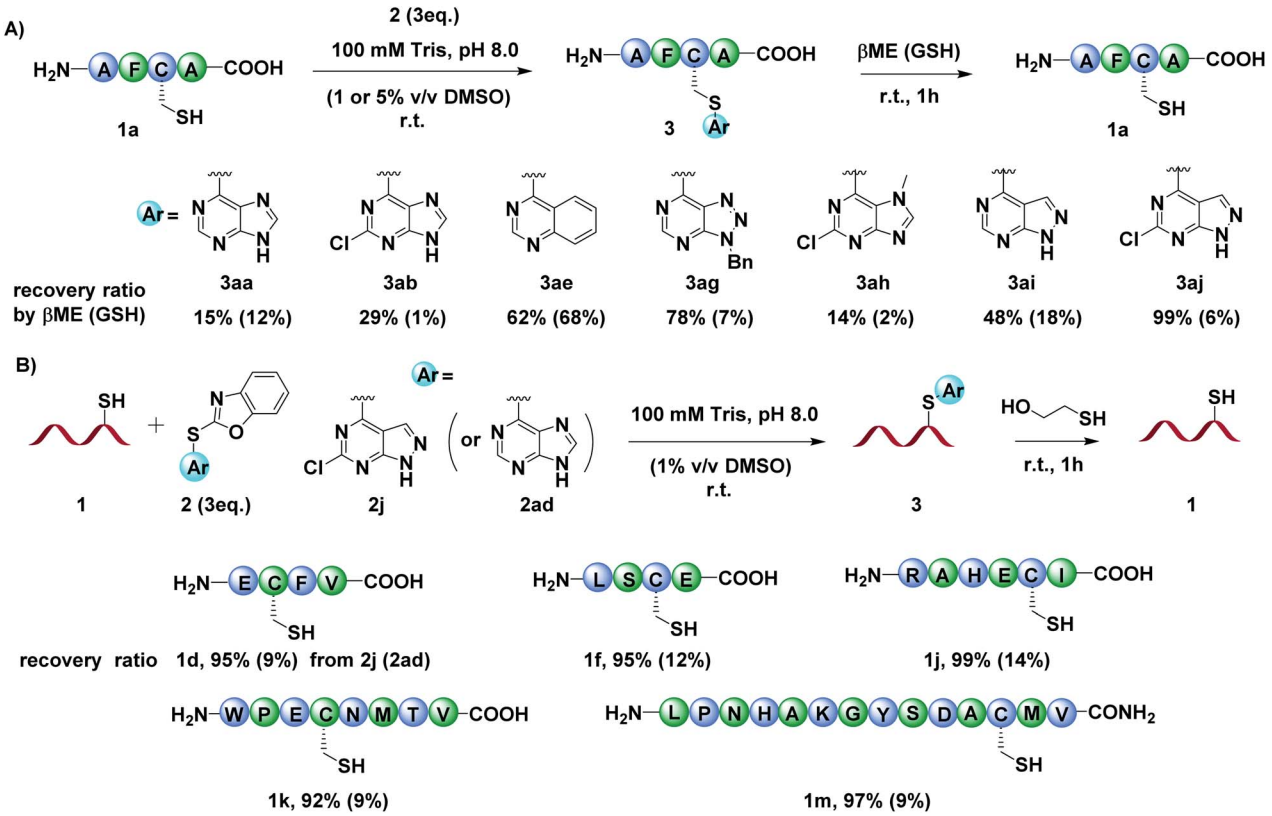

Fig. 4 (A) Regeneration activity evaluation of modified peptides. Reported yields are LC-MS yields of **1a** regenerated from **3** with βME after 1 h. (The yields in parentheses correspond to the yields of **1a** regenerated from **3** with GSH after 1 h). (B) Substrate scope of peptide regeneration. Reported yields are LC-MS yields of peptides **1** regenerated from **2j** and **2ad** modified peptides **3** with βME after 1 h.

Protein modification is vital to explore their structures and functions. To investigate the possibility of our method for protein modification, we tested the reactivity of the developed benzoxazole sulfide on bovine serum albumin (BSA). Benzoxazole sulfide **2v** equipped with an affinity label was incubated with BSA to afford the modified product with good efficiency under slightly modified reaction conditions (Fig. 5A, B). Besides, secondary labelling of the modified peptide was

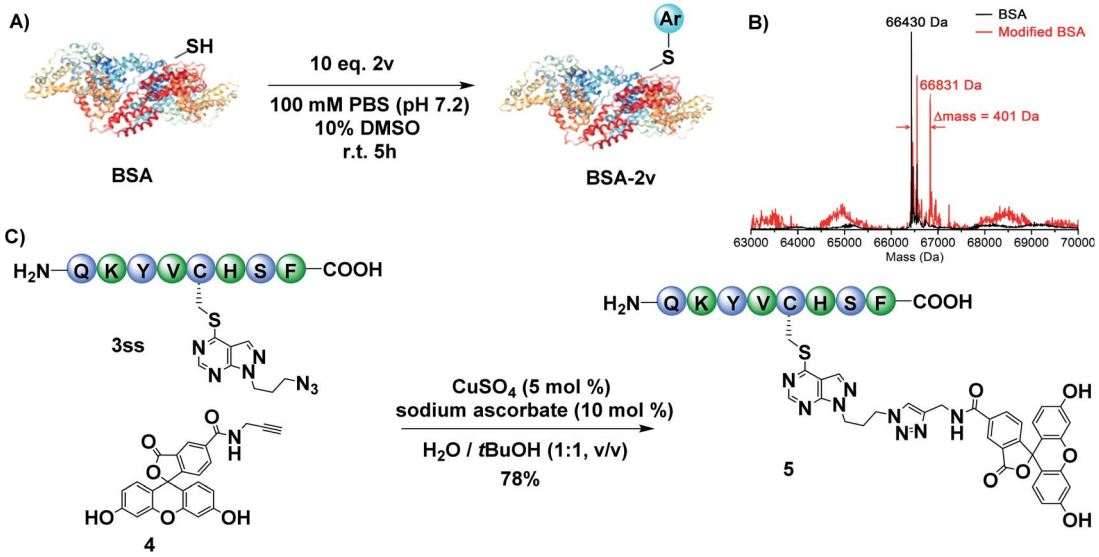

Fig. 5 (A) BSA modification with **2v**. (B) ESI-MS spectra of native BSA and modified BSA-**2v** proteins. (C) Secondary labeling of modified peptide **3ss**.

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also investigated. The modified product **3ls** bearing an azide handle could react with fluorescent tag **4** to afford the desired fluorescent product **5** *via* the azide–alkyne click reaction, suggesting the potential application of this method in biochemistry (Fig. 5C).

Conclusions

In conclusion, we developed a class of aryl thioethers for cysteine-containing peptide/protein modification with excellent chemoselectivity and efficiency *via* a S_NAr approach. This modification strategy has broad substrate scope and functional group compatibility under optimized reaction conditions. A variety of biorelevant groups could be well introduced into modification agents. By simply adjusting the skeleton of aryl thioethers, a cleavable and regeneration tunable modification for peptides/proteins could be achieved. Moreover, secondary labeling of modified peptides was also realized, indicating the broad application of our method in the peptide area.

Author contributions

Xiao-Feng Xiong conceived the study and was responsible for the funding acquisition, project administration and writing of the manuscript. Jian Li designed and performed the study, collected and analysed the data, and wrote the original draft. The other authors also assisted in analysing and validating the data and were involved in preparing the manuscript.

Conflicts of interest

There are no conflicts to declare.

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18 The LC-MS yields were determined as follows: %yield = $S_{\text{product}}/S_{\text{total}}$, where S_{product} is the peak area of the product and Stotal is the peak area of combined peptide-containing species (product, starting material and by-product).

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