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ARTICLE

Intermolecular Sulfur Atom Transfer Cascade Enabled Late-Stage Introduction of Sulfilimines into Peptides†

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Zeyuan He,[a] Yuyang Liu,[a] Guangjun Bao,[a] Yiping Li,[a] Xiufang Zhao,[a] Quan Zuo,[b] Kai Li,[a] Wangsheng Sun*[a] and Rui Wang[a,b]

Sulfilimine, a privileged class of −S(IV)=N− functional group found in nature, have been exploited as valuable building blocks in organic synthesis and as pharmacophores in drug discovery, and have aroused significant interests for chemical community. Nevertheless, the strategies for late-stage introduction of sulfilimines into peptides and proteins have still met with limited success. Herein, we have developed a method of introducing biological sulfilimines fragment into peptides by an intermolecular sulfur atom transfer cascade reaction, utilizing hydroxylamine condensed with their acid moieties and varied diaryl disulfides. It provides a convenient, efficient, metal-free and widely applicable method for late-stage peptide modification and functionalization at their acid sites both in the homogeneous phase and on-resins in SPPS. Moreover, the modified peptides with sulfilimines have been demonstrated as a cleavable linker for peptide conjugates under reducible conditions, providing unique opportunities in peptide therapeutics development and drug discovery.

Introduction

Thanks to the significance of peptides as therapeutics,[1] platform for targeted therapeutics,[2] and chemical biology probes,[3] conjugations of peptides with functional molecules and conventional drugs have drawn tremendous interests during the past two decades, leading to an arsenal of peptide bioconjugation and modification.[4] Despite the fruitful advances, the development of novel peptide bioconjugation and modification is still urgently needed. Sulfilimine, a privileged class of −S(IV)=N− functional group, that was initially discovered approximately a century ago, have been exploited as valuable building blocks in organic synthesis,[5] and as pharmacophores in drug discovery.[6] More interestingly and significantly, it was identified by Hudson et al. in 2009 as a unique crosslink that covalently binds hydroxylysine-211 and methionine-93 in the collagen IV network, which is a highly conserved major component of basement membranes and associated with the occurrence of several biofunctions and diseases.[7] Later, the same group disclosed the Br-dependent peroxidase catalyzed biochemical formation of sulfilimines between methionine-93 and hydrox-ylysine-211 within collagen IV, an event critical for basement membrane assembly and tissue development in animals (Fig. 1a).[8] Inspired by this discovery, Tang et al. developed sulfilimine-based molecular probes for imaging native HOBr in live cells and Zebrafish.[9]

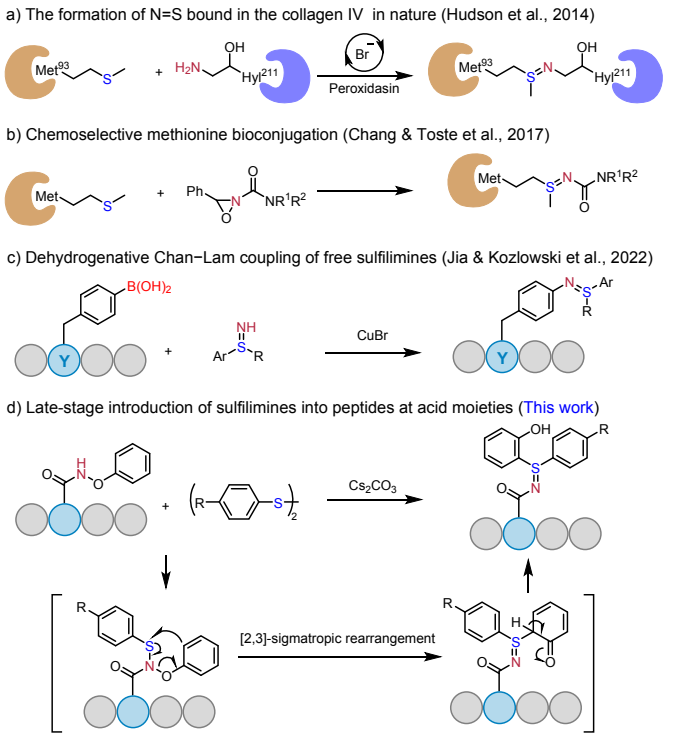


Fig. 1. Strategies for the Introduction of Sulfilimines into Peptides and Proteins.

These elegant seminal works have aroused scientists to artificially build sulfilimines between biomolecules, particularly peptides and proteins, and small functional molecules. Chang, Toste, and co-workers developed a methionine selective bio-conjugation by oxidizing methionines to corresponding sulfilimines with oxaziridines,[10] which was demonstrated

[a] Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences & Research Unit of Peptide Science, Chinese Academy of Medical Sciences, 2019RU066, Lanzhou University, Lanzhou 730000, China. E-mail: sunws@lzu.edu.cn
[b] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China.
† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

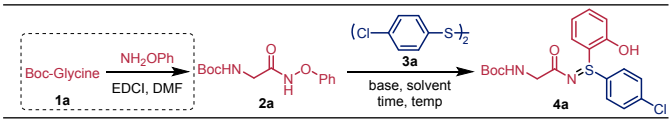
successful in cyclization of peptides, protein functionalization, antibody-drug conjugates (ADCs), and chemoproteomic identification of functional methionines in cells (Fig. 1b).[10,11] These studies indicate that N=S bonds have significant potential in drug conjugates. More recently, Kozlowski, Jia, and co-workers have developed a biocompatible Chan–Lam coupling reaction, which can introduce *N*-Ar diaryl sulfilimines into peptides and proteins at the site of an exogenous inserted arylboronic acid (Fig. 1c).[12] Nevertheless, a novel and mild alternative that beyond the above methods to constructing sulfilimines between peptides and functional small molecules, which could be of great potential for PDCs construction, is still appealing to the chemical community.

Very recently, we disclosed the late-stage introduction of oxime ethers into peptides using *N*-alkoxylpeptidylamides that were prepared in situ from amino acids[13] and C–S bond formation between tryptophans and thiophenols enabled site-selective functionalization of peptides.[14] As a continuation of our ongoing interests in peptide bioconjugation and medicinal chemistry,[13-15] and inspired by the intermolecular sulfur atom transfer cascade reaction between aryloxyamides and thiols that reported by Xiong et al.,[16] we sought to explore the reaction between thiols and *N*-phenoxylpeptidylamides that could be prepared in situ from acid moiety of peptides to furnish late-stage incorporation of sulfilimines with diverse functional groups into peptides at the position of interest via an intermolecular sulfur atom transfer cascade reaction, and thereby provide a novel alternative for peptide modification and PDC construction (Fig. 1d).

Result and discussion

Our investigation was initiated by using *N*-phenoxylglycinamide **2a** that was prepared by condensation of *N*-Boc-glycine **1a** and *O*-phenylhydroxylamine, and 4,4'-dichlorodiphenyl disulfide **3a** as model substrates in the presence of CsOAc in DMSO at room temperature for 10 h. To our delight, glycyl sulfilimine **4a** was obtained smoothly, albeit with moderate yield (Table 1, entry 1). Encouraged by this result, a series of reaction conditions including reaction media, base, temperature, and other parameters (for a complete list of the conditions screened, see the section 'Optimization studies' in the Supplementary Information) were screened to verify an optimal reaction condition. It revealed that base was crucial for the reaction (Table 1, entries 1-4 and Table S1-3) and 0.5 equiv Cs_2CO_3 gave the optimal results (Table 1, entry 3). The reaction could be promoted at 37 ℃ with significantly shortened reaction time in high isolated yield (Table 1, entry 5, defined as Method A). Interestingly, when simple 4-chlorothiophenol monomer **3a'** was used instead of 4,4'-dichlorodiphenyl disulfide **3a**, the reaction could proceed smoothly as well, providing glycyl sulfilimine **4a** in equivalent yield (Table 1, entry 5 vs 6), probably because simple thiophenols were easily oxidized to disulfides under air.[16b] Meanwhile, DMF, the most commonly used solvent in solid-phase peptide synthesis (SPPS), was also well compatible with this reaction (Table 1, entry 5 vs 7). To further simplify the reaction protocol, two-step-one-pot procedure was investigated. The reaction proceeded smoothly when **1a**, *O*-phenylhydroxylamine, EDCI and HOBt was stirred for 6 hours in DMF before addition of **3a** and Cs_2CO_3 (Table 1, entry 8, two-step-one-pot procedure defined as Method B). The condensation reaction between carboxyl group and *O*-phenylhydroxylamine could generate some by-products, which may explain why the yields of two-step-one-pot procedure are lower than the one-step reaction. Unluckily, it failed when **1a**, **3a**, *O*-phenylhydroxylamine, EDCI, HOBt and Cs_2CO_3 were all added together in solution (Table 1, entry 9, one-pot procedure).

Table1 Optimization of the reaction conditions



entry	solvent	base	time (h)	temp. (℃)	yield[e] (%)
1[a]	DMSO	CsOAc (1 eq)	10	rt	61
2[a]	DMSO	Cs_2CO_3 (1 eq)	10	rt	83
3[a]	DMSO	Cs_2CO_3 (0.5 eq)	10	rt	90
4[a]	DMSO	–	10	rt	0
5[a]	DMSO	Cs_2CO_3 (0.5 eq)	6	37	90 (85)
6[a,d]	DMSO	Cs_2CO_3 (0.5 eq)	6	37	90
7[a]	DMF	Cs_2CO_3 (0.5 eq)	6	37	89
8[b]	DMF	Cs_2CO_3 (1.7 eq)	6	37	53
9[c]	DMF	Cs_2CO_3 (1.7 eq)	6	37	0

[a] **2a** (0.05 mmol), **3a** (0.05 mmol) and base in 1 mL solvent, [b] **1a** (0.1 mmol), NH_2OPh (0.1 mmol), EDCI (0.12mmol) and HOBt (0.12 mmol) in 2 mL DMF at 37 ℃ for 6 h, then **3a** (0.1 mmol) and Cs_2CO_3 (0.17 mmol) were added and stir for an additional 6 h. [c] **1a** (0.1 mmol), NH_2OPh (0.1 mmol), EDCI (0.12mmol), HOBt (0.12 mmol), **3a** (0.1 mmol) and Cs_2CO_3 (0.17 mmol) in 2 mL DMF at 37 ℃ for 6 h. [d] 0.1 mmol 4-chlorothiophenol **3a'** was used instead of **3a**. [e] Yield was determined by ^1H NMR spectroscopy using 1,3,5-trimethoxybenzene as an internal standard. Isolated yield after column chromatography was given in parenthesis.

With the optimum conditions in hand, we explored the scope of the reaction (Fig. 2). We first test the universality of the reaction by changing the *N*-phenoxypeptidylamide substrates scope. To our delight, all the substrates that started from different *N*-protected amino acids could be involved in the reaction through method A or method B. A wide variety of functional residues, including aromatic amino acid residues, aliphatic amino acid residues, and polar amino acid residues (**4a** - **4h**), were tolerated，albeit with that the presence of reactive side chains like hydroxyl and indole results in lower yield. Large scale (10 mmol) reactions can be carried out using method A or method B to obtain corresponding high and moderate yields (**4a**). In addition, the reaction can implement late-stage modification of oligopeptides, incorporating the special sulfilimines into oligopeptides at their C-terminal with good yields (**4i**, **4j**). Meanwhile, the late-stage introduction of sulfilimines into the side chain of amino acid or oligopeptide was demonstrated by one-pot reaction using Glu-containing oligopeptides (**4k – 4n**). Significantly, fully deprotected peptides worked well in reaction solution (**4n**), indicating that *O*-aryl hydroxamic acid derivatives have the potential to be a biorthogonal precursor for incorporation of a disulfide reagent.

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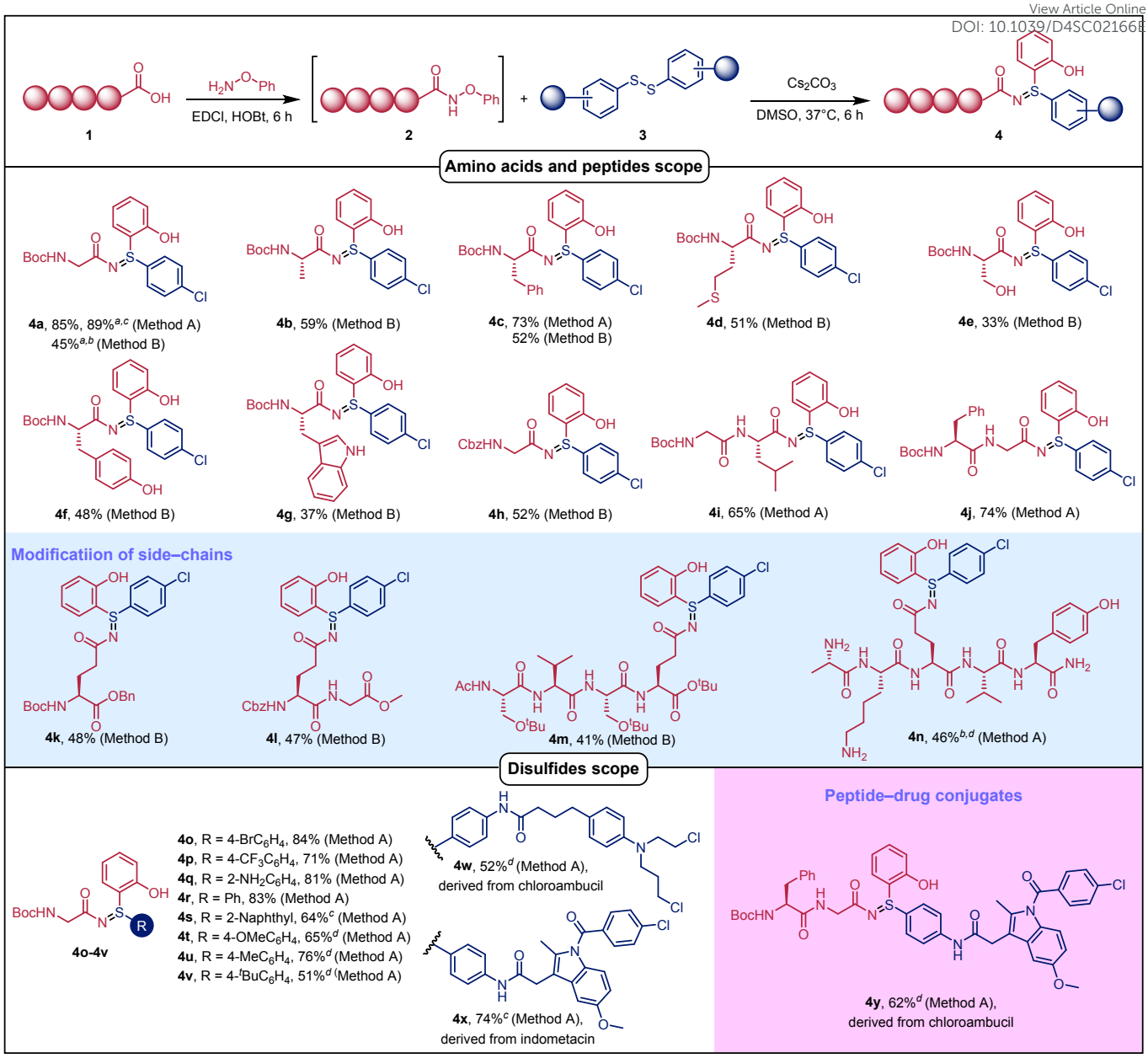

Fig. 2 Substrate Scope. Method A: 2 (0.05 mmol), 3 (0.05 mmol), and Cs_2CO_3 (0.025 mmol) in DMSO (1.0 mL) at 37 ℃ for 6 h. Method B: Corresponding aminoacids or peptides 1 (0.1 mmol), NH_2OPh (0.1 mmol), EDCI (0.12mmol) and HOBt (0.12 mmol) in 2 mL DMF at 37 ℃ for 6 h, then **3a** (0.1 mmol) and Cs_2CO_3 (0.17 mmol) were added and stir for an additional 6 h. Yields of isolated products are given. [a] 10 mmol scale. [b] 0.025 mmol scale. [c] 12 h. [d] 24h.

Unfortunately, however, unprotected cysteine is incompatible with this reaction may be due to the high reactivity of thiol groups. Subsequently, we explored the substrate scope of diaryl disulfides. Various alkyl-, amino-, Cl-, Br- and CF_3-substituted phenyl or dinaphthyl disulfides could participate in the reaction smoothly to give the desired products in moderate to good yields (**4o – 4v**). Compared with electron withdrawing groups substitution such as 4-Cl, 4-Br and 4-CF_3 (71 - 89%), diaryl disulfides possessing electron donating substitutions such as 4-OMe, 4-Me, 4-^{t}Bu or 2-naphthyl provided lower yield (51 - 65%) and needed longer reaction time. It is particularly noticeable that drugs such as chlorambucil and indomethacin could be introduced as payload into amino acids (**4w**, **4x**) and oligopeptides (**4y**) with good yields.

We then turned our attention to examine the practicality of the reaction on-resin (Fig. 3). Allyl protected Glu(OAllyl)-OH or Asp(OAllyl)-OH was inserted at the position of interest during the routine Fmoc SPPS on-resin and thereby site-specific modification could be achieved with orthogonal protection of tert butyl ester and allyl ester. After the routine Fmoc-SPPS on-resin, the *N*-phenoxypeptidylamide substrates was obtained by coupling with *O*-phenylhydroxylamine after deallylation of Allyl. The substrate on-resin was reacted with diaryl disulfide substrate **3** in DMF or DMSO for 24 - 48 hours, followed by cleavage from the resin and purified by semi-preparative HPLC.

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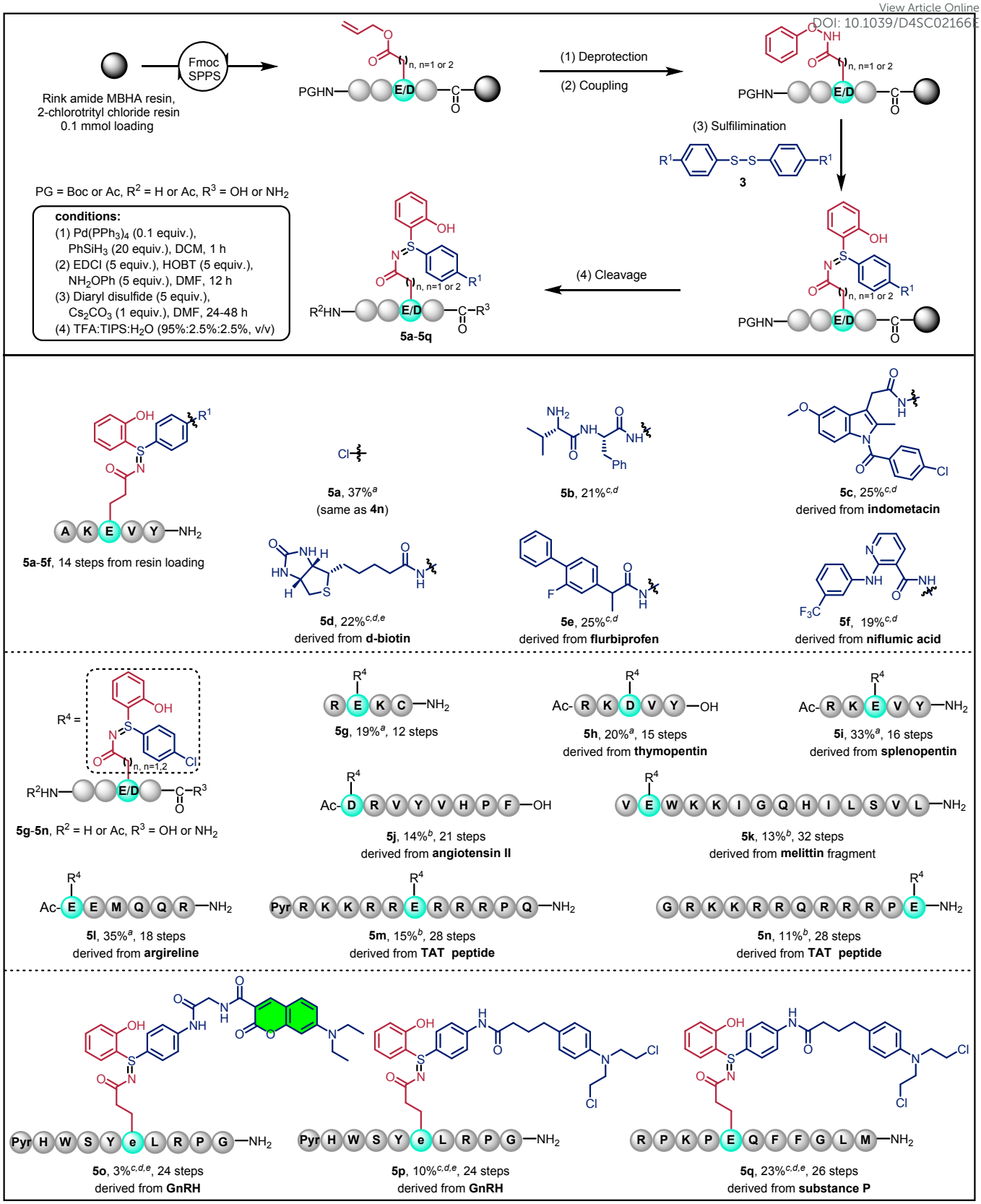

Fig. 3 Late-stage sulfilimination of glutamate/aspartate-containing peptides on resin. Pyr, pyroglutamic acid; e, *D*-Glu. Isolated yield after semi preparative HPLC was given. [a] 24 h in step (3). [b] 36 h in step (3). [c] 48 h in step (3). [d] 5 equiv. Cs_2CO_3 was added. [e] DMSO instead of DMF in step (3).

To our delight, this protocol worked well with some bioactive molecule analogues and bioactive peptide analogues. **5a** was obtained in 37% isolated yield after 14 steps from resin loading compared to the 46% isolated yield of one-step reaction in

solution (**4n** in Fig. 2). Peptide-peptide conjugates (**5b**), peptide-indomethacin conjugates (**5c**), peptide-d-biotin conjugates (**5d**), peptide-flurbiprofen conjugates (**5e**) and peptide-niflumic acid conjugates (**5f**) were successfully obtained through this strategy after 14 steps. More importantly, product **5g** that containing cysteine can be synthesized through this solid-phase synthesis using a Trt-protected cysteine. Conjugate **5g** was slowly degraded in solution due to the unprotected thiol group, which is consistent with the subsequent experimental results. Bioactive peptide such as thymopentin (**5h**), splenopentin (**5i**), angiotensin II (**5j**), melittin fragment (**5k**), argireline (**5l**) and TAT cell penetrating peptides (**5m**, **5n**) were all applicable to this protocol. Conjugate **5o** only achieved 3% yield, possibly due to poor solubility of substrate coumarin (**3o**). Furthermore, the success of gonadotropin-releasing hormone (GnRH) derivatives and substance P conjugated with chlorambucil (**5p, 5q**), further demonstrated the potential application of this reaction to construct PDCs for cancer therapy.

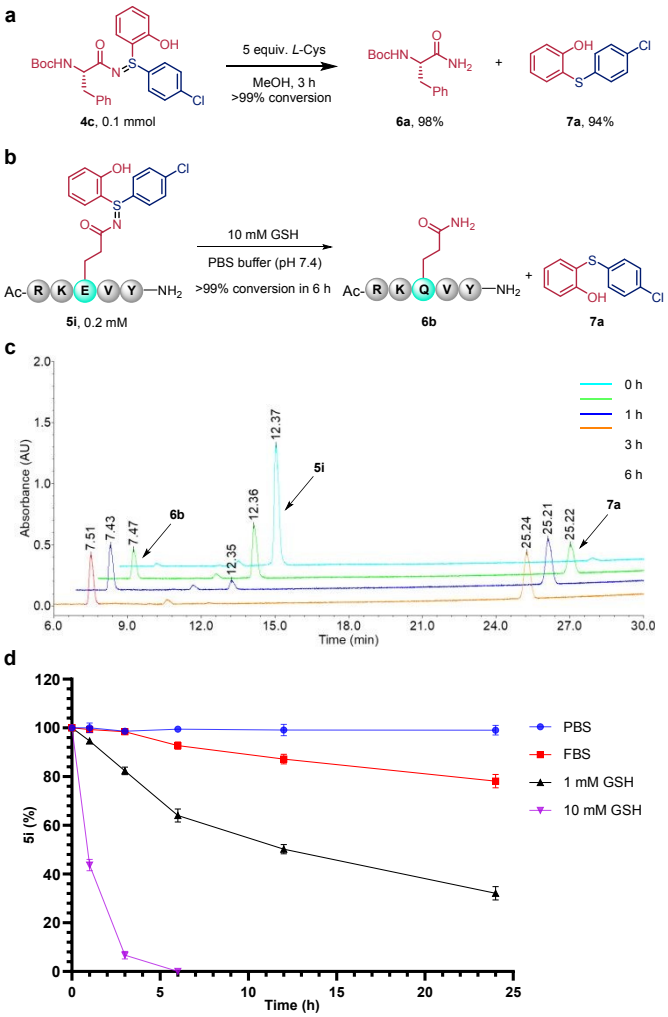

Fig. 4 Deconjugation and stability of modified products. (a) Deconjugation of products **4c**. The reaction was carried out with 0.1 mmol **4c**, 0.5 mmol *L*-Cys in 1 mL MeOH at room temperature for 3 h, isolated yield of **6a** (98%) and **7a** (94%) were given. (b) Deconjugation of products **5i**. **5i** (0.2mM) could be decomposed into corresponding peptidyl amides (**6c**) and diaryl thioether (**7a**) by 10 mM GSH in PBS buffer (pH 7.4) solution. (c) HPLC chromatogram schematic diagram indicated conversion of peptide **5i** in 10 mM GSH solution at different times. (d) Stability of **5i**. **5i** (0.2 mM) was incubated at 37 ℃ with PBS buffer (pH 7.4) solution, 95% FBS in PBS buffer solution (pH 7.4), 1 mM GSH in PBS buffer (pH 7.4) solution and 10 mM GSH in PBS buffer (pH 7.4) solution respectively.

As the connecting bridge between drugs and peptides in PDCs, linkers determine the circulation time and stability of PDCs *in vivo*.[17] To explore the possibility of this sulfilimine as a releasable PDC linker under reducible conditions,[5b,10a,10b] a series of experiments were then carried out. It was found that the sulfilimines could be decomposed into corresponding peptidyl amides and diaryl thioethers under reducible conditions (Fig. 4). Particularly interesting, for example, is that the product peptide conjugate **5i** could be deconjugated in a GSH concentration-dependent manner. Specifically, **5i** was decomposed slowly ($t_{1/2}$ ~ 12 h) in 1 mM GSH of PBS buffer (pH 7.4) solution and decomposed rapidly ($t_{1/2}$ < 1 h) in 10 mM GSH of PBS buffer (pH 7.4) solution, with completely decomposed within 6 hours (Fig. 4b). In addition, **5i** was stable in PBS solution and relatively stable in FBS (Fetal Bovine Serum) solution, with more than 79% existing in their original form after 24 hours (Fig. 4c and Fig. S19-S22). These features provide us with an opportunity to design GSH-sensitive PDCs and prodrugs for therapy.[18]

Conclusions

In summary, a late-stage introduction of sulfilimines into peptides at a carboxylic acid site has been achieved. This method enabled the introduction of sulfilimines with diverse functional groups, such as drugs, natural products, bioactive handles, and fluorescent tags into peptides both in the homogeneous phase and on-resins in SPPS under mild conditions. Moreover, sulfilimines was demonstrated as a cleavable linker for peptide conjugates under reducible conditions, enabling on-demand control of peptides functions. It might provide a novel tool kit for peptide chemical biology and prodrug discovery. Further applications of this strategy in these areas are currently under investigation in our laboratory.

Author Contributions

W.S. and R.W. conceptualized the project, supervised the work, designed the experiments, and assisted data analysis. Z.H., Y.L., G.B., Y.L., X.Z., Q.Z. and K.L. performed the experiments and analyzed the data. All authors discussed the results and commented on the manuscript. W.S. and Z.H. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

All data associated with this study are available in the article and ESI.

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Data Availability Statement

All data associated with this study are available in the article and ESI.