ORGANIC CHEMISTRY

Controlled reversible methionine-selective sulfimidation of peptides

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Site-selective chemical peptide manipulation is an effective strategy to understand and regulate structure and function. However, methionine-selective modification remains one of the most difficult challenges in peptide chemistry, with notable limited strategies. In this study, we report a general reversible modification strategy at methionine sites that uses the ruthenium-catalyzed sulfimidation of peptides. This method provides a convenient and effective strategy for late-stage peptide functionalization. The N═S bonds of the conjugates are reduced in the presence of glutathione, resulting the traceless releasing of corresponding peptides and amides. Practical applications are then demonstrated using precise reversible modifications of bioactive peptides, the stapling and linearization of peptides, peptide-drug conjugates, and split-and-pool synthesis. This on/off strategy through methionine-selective and reversible sulfimidation provides a unique tool for peptide chemistry and peptide-based drug discovery.

INTRODUCTION

Peptides play important roles in the regulation of the functional activities of various systems, organs, tissues, and cells in the body, as well as in life activities. Peptides have long been used as therapeutic agents and continue to serve as valuable platforms for drug discoveries. In 2020, there were over 100 approved naturally occurring or synthetic peptides for treatment or diagnosis applications (*1*). However, the primary challenge for peptide drug development is poor pharmacokinetics (*2*, *3*). Peptides that have been reasonably modified can not only maintain high biological activity, but this also endows them with new excellent properties, such as improved physicochemical properties, increased stability, reduced immunogenicity, extended half-life, and reduced toxic side effects. Modified peptides or cyclic peptides typically exhibit better pharmacokinetics and bioavailability compared to natural peptides (*4*–*6*). In addition, peptides can also be effectively used as drug carriers in peptide-drug conjugates (PDCs) (*7*, *8*). Substantial progress has been made in late-stage peptide modification (*9*–*14*), macrocyclization (*15*–*23*), and drug bioconjugation in recent years.

Methionine (Met), one of the two sulfur-containing amino acids in proteins (alongside cysteine), is notably hydrophobic and highly sensitive to redox conditions. These unique properties enable Met to play pivotal roles in various cell-regulatory processes (*24*). The notably low abundance (~2%) and distinct redox-sensitive properties of Met confer its unique chemical reactivity that differs significantly from other natural amino acids, rendering it an attractive target for precise manipulation of peptides and proteins. However, to date, only a handful of effective methods for peptide modification at the Met residue have been described (*24*). These include sulfidation or sulfonation through oxidation (*25*, *26*), sulfonium salts formed using alkyl halides (*27*–*29*) or iodonium salts that contain diazonium (*30*), alkylation via α-thiol radicals (*31*), and sulfimidation using oxaziridines (*32*, *33*) or chloramine-T reagents (*34*) (Fig. 1A). Despite elegant progress, there remains an urgent need for a methodology to develop peptide manipulation at the Met residue. The limited nucleophilicity of Met poses a central challenge for achieving selective modification as competing nucleophilic residues (e.g., cysteine, lysine, serine, and tyrosine) may dominate undesired side reactions. Nevertheless, Met's characteristic sulfide moiety confers distinct chemical reactivity distinct from canonical nucleophiles. Building on our prior works in peptide functionalization (*35*–*42*), particularly our recent finding of glutathione (GSH)–mediated cleavage of peptide sulfilimines into corresponding amides and thioethers (*40*), we seek to exploit the redox sensitivity of Met to achieve Met-selective modification. In this study, we report a Ru(II)-catalyzed nitrene transfer reaction for Met-selective reversible peptide modification and its application to peptide stapling, split-and-pool synthesis, and PDCs (Fig. 1B).

RESULTS AND DISCUSSION

Reaction development

Inspired by the sulfur imidations between nitrene and sulfide (*43*), we hypothesized that the use of the nitrene transfer reaction could be a viable method for reversible Met modification due to the flexibility in introducing various functionalities on *N*-acyl nitrene (Fig. 1B). We thus initiated our study using *N*-acyloxyamides as *N*-acyl nitrene precursors as the foundation for our hypothesis. This was inspired by the Ru(II)-catalyzed nitrene transfer reaction between *N*-acyloxyamides and aryl sulfides (*44*). However, several challenges needed to be addressed. The multiple highly reactive functional groups in peptides may compete with reaction substrates and passivate catalysts. This would result in poor peptide modification selectivity and low conversion rates. In addition, the compatibility of catalytic systems in heterogeneous solid-phase synthesis also needed to be considered. With these in mind, we tested and optimized the reaction conditions and conducted compatibility studies based on the reaction between *N*-Boc-Met methyl ester (**S1**) and *N*-acetoxybenzamide (**2j**) (tables S1 to S6 of the Supplementary

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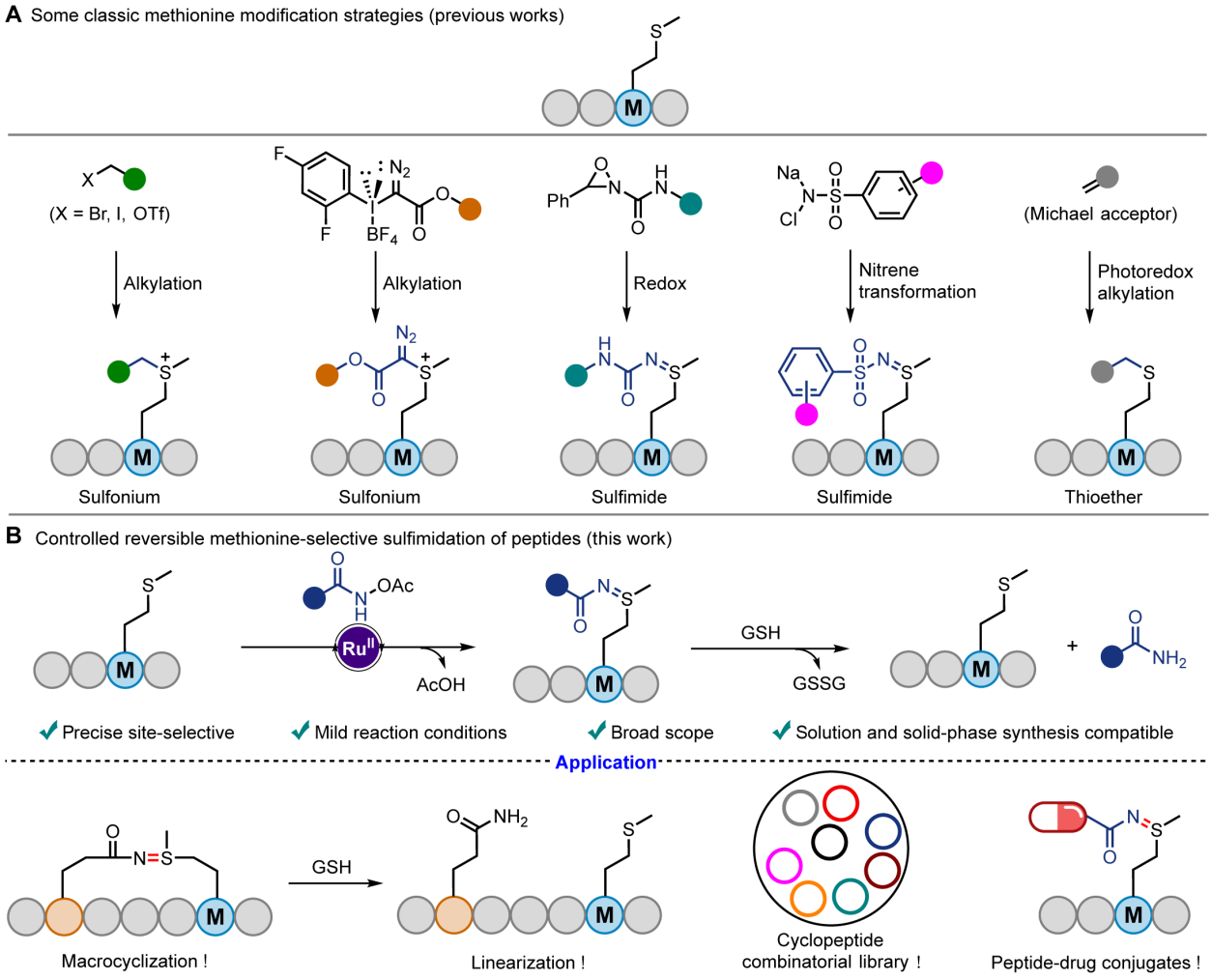

Fig. 1. Late-stage modification of Met-containing peptides. (**A**) Several existing strategies for Met-selective modification. (**B**) This work: Controlled reversible Met-selective sulfimidation of peptides.

Materials). The compatibility experiment results demonstrate that amino acids containing nucleophilic side chains, such as lysine, glutamic acid, arginine, glutamine, serine, tyrosine, and tryptophan, had good compatibilities with this reaction. Although this amino acid modification reaction shows incompatibility with free cysteine and histidine residues, successful modifications were achieved with cystine (the oxidized disulfide form of cysteine), Trt (triphenylmethyl)–protected cysteine, and some histidine-containing bioactive peptides (Fig. 2A and table S6). This differential reactivity profile suggests the reaction's potential for selective modification of unprotected nucleophilic residues while maintaining compatibility with protected cysteine derivatives and structurally constrained histidine moieties in peptide systems. We then used a more complex substrate, the neuropeptide substance P (**1a**, a 11-residue peptide containing a single Met), and we identified an optimized sulfimidation condition between substance P (**1a**) and *N*-acetoxybutyramide (**2a**) under the conditions of $RuCl_2(PPh_3)_3$ (10 mol %), *N*-hydroxybenzamide (20 mol %), and AgOAc (10 μmol/ml) in MeOH for two rounds [82% high-performance liquid chromatography (HPLC) yield] (table S7). According to our previous work (*41*), *N*-acyloxyamide substrates may be introduced directly through condensation reactions at the carboxyl sites of peptides, thereby achieving peptide stapling between Met and carboxyl sites.

Sulfimidation and cyclization

We first assessed the scope of the sulfimidation in the homogeneous phase under optimal conditions (Fig. 2A). The modular synthesis of the *N*-acetoxyamide substrates allows for the facile incorporation of different amide groups attached to the acetoxy motif, thereby enabling the delivery of a range of functional payloads to peptides. *N*-acetoxyamide substrates derived from a series of aliphatic acid, *N*-Cbz-hydroxylamine, and pepper propionic acid could be compatible with this reaction, and they were easily transferred to substance P (**3a** to **3g**) with 52 to 82% HPLC yields. Fluorescent groups, such as *N*-acetoxyamide derived from coumarin, were also readily transferred to substance P (**3h**). Notably, by using this strategy, PDCs **3i** (substance P–artesunate conjugate) and peptide-peptide conjugates **3j** were easily obtained with HPLC yields of 63 and 70%, respectively. This protocol worked well with Met-containing RGD cyclopeptide and provided the corresponding products in moderate yields (**4a** to **4d**). Cysteine protected by Trt groups were compatible

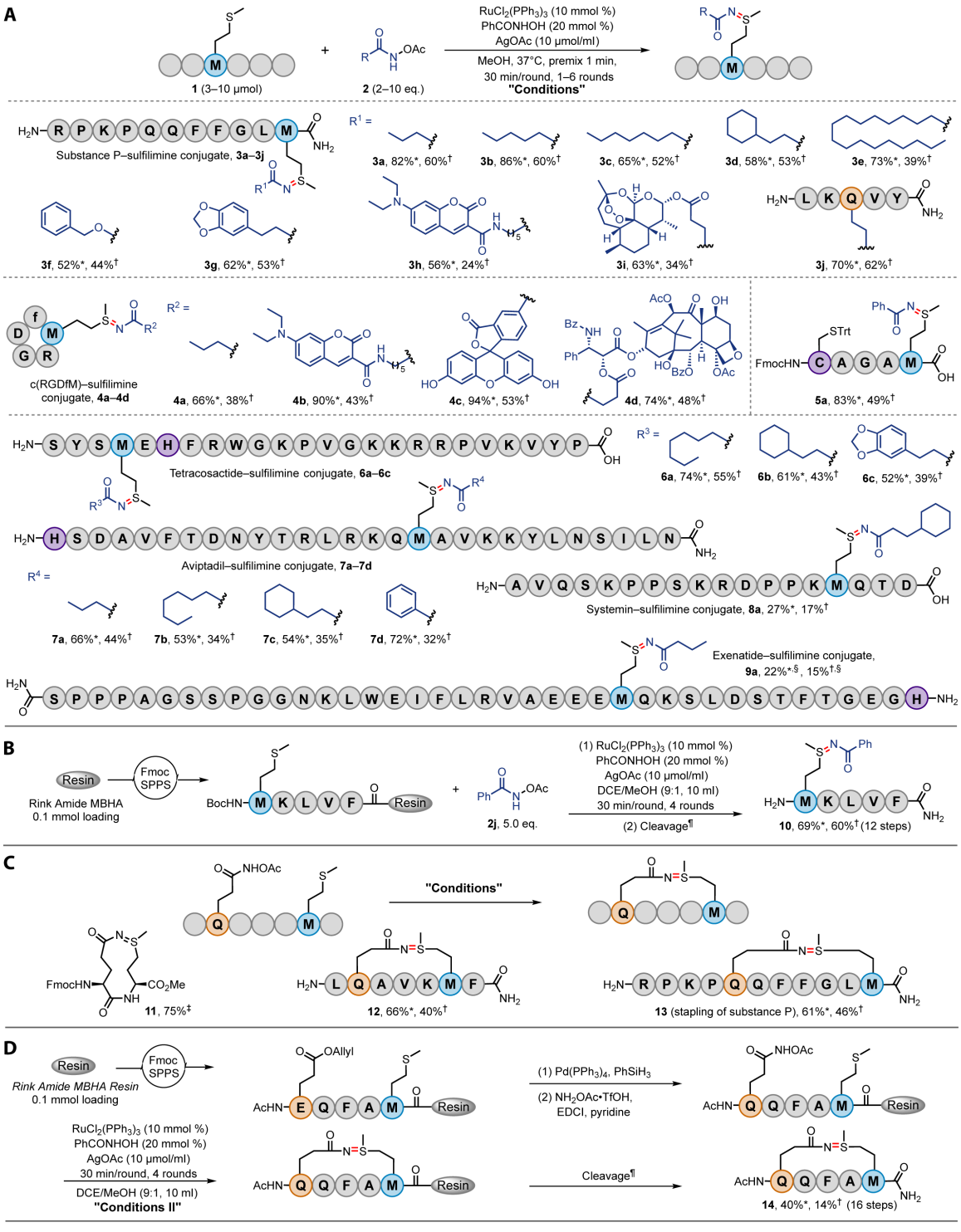

Fig. 2. Scope of Met-selective modification and stapling strategies. (**A**) Homogeneous-phase peptide modification. eq., equivalents. (**B**) Solid-phase peptide modification on resin. (**C**) Homogeneous-phase peptide stapling. (**D**) On-resin peptide stapling. *HPLC yields. †Isolated yield via semipreparative HPLC purification. ‡Isolated yield through column chromatography. §Reaction conducted in 50% (v/v) methanol/water cosolvent. ¶Cleavage condition: 95% TFA (trifluoroacetic acid), 2.5% TIPS (triisopropylsilane), 2.5% H_2O (v/v), 3 hours.

with this reaction (**5a**). Tetracosactide, a synthetic adrenocorticotropic hormone, was efficiently converted to sulfimide conjugates (**6a** to **6c**) with good yields (52 to 74% HPLC yields). Aviptadil, a vasoactive intestinal peptide, reacted with *N*-acetoxyamides to provide the desired products **7a** to **7d**. Systemin, an endogenous peptide produced in the cytoplasm of plants upon mechanical injury or insect invasion, was converted into the sulfimide conjugates **8a** with a 27% HPLC yield. Exenatide, a blockbuster drug for type 2 diabetes treatment, has poor solubility in MeOH. Therefore, 50% water was added as a solubilizer during the sulfimidation between exenatide and **2a** to lastly obtain a 22% HPLC yield of conjugate **9a**. Furthermore, the sulfimidation reaction was also conducted on the solid phase (Fig. 2B). Met was inserted at the position of interest during 9-fluorenyl methoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on the resin and then reacted with *N*-acetoxyamides in a solution of 1,2-Dichloroethane (DCE)/MeOH (9:1) for 2 hours (four rounds). The product was then separated from the resin and purified using semipreparative HPLC. The peptide, NH_2-Met-Lys-Leu-Val-Phe-$CONH_2$, was successfully converted to product **10** on the resin, with an isolated yield of 60% after 12 steps.

It is critical to develop strategies to construct peptide macrocyclization because peptide macrocyclization can help improve membrane permeability and peptide resistance to proteases (*6*, *16*, *17*). Therefore, we further advanced the method to construct structurally complex stapled peptides based on Met residues and *N*-acetoxyamide derived from the carboxyl site (Fig. 2C). Three peptides derived from Fmoc-Glu-Met-OMe, NH_2-Leu-Glu-Ala-Val-Lys-Met-Phe-$CONH_2$, and substance P were synthesized by coupling with NH_2OAc•TfOH. The cyclization between the Met residue and *N*-acetoxyamide proceeded efficiently and produced the desired sulfimidation-modified macrocyclic peptides in moderate-to-good yields (**11** to **13**). In this strategy, Met residues and Gln residues are directly stapled via N═S bonds without additional redundant groups. The macrocyclization of peptides can also be achieved through the SPPS protocol (Fig. 2D). Met and allyl ester–protected Glu were inserted at the position of interest during a routine Fmoc SPPS on the resin. The *N*-acetoxyamide substrate was obtained by coupling with NH_2OAc•TfOH after the removal of the allyl ester, and then the substrate was reacted under the same "conditions II," and the desired product was produced. Cyclopeptide **14** was obtained with an HPLC yield of 40% and an isolated yield of 14% after 16 steps. Unfortunately, the peptide stapling between the aspartic acid (Asp) and Met sites presented considerable challenges as the condensation reaction between the Asp carboxyl group and NH_2OAc•TfOH predominantly generated an aspartimide by-product (>80%) that resulted in a diminished yield of the desired condensation product (<10%) (fig. S3).

Deconjugation

Linkers determine the circulation time and stability of PDCs in vivo because they are the connecting bridge between drugs and peptides in PDCs. The ideal linker should remain stable in circulation to avoid premature drug release while being able to release the drug rapidly and efficiently once it reaches the focal tissue (*45*). We noted that sulfimide conjugates of substance P (e.g., **3j**) undergo decomposition in the presence of 1 mM GSH, a key reducing agent in vivo. This process led to cleavage of the N═S bond, regenerating the parent substance P (**1a**) and the corresponding amide compounds (**15**) with >95% conversion (Fig. 3A). Notably, this cleavage reaction also worked on the linearization of cyclopeptide **13** with a >99% conversion, providing a stimulus-responsive means of peptide linearization (Fig. 3B). To further investigate the stability of PDC **4d**, we conducted a time course study examining its degradation in different GSH concentrations and in fetal bovine serum (FBS) (Fig. 3C). Encouragingly, the results demonstrate that PDC **4d** undergoes GSH-mediated degradation, yielding the corresponding peptide **1b** and paclitaxel (PTX) derivative **16** (Fig. 3C). Rapid degradation was observed at a GSH concentration of 1 mM ($t_{1/2} \approx 5$ min; Fig. 3D and table S8), whereas slower degradation occurred at a lower GSH concentration of 10 μM ($t_{1/2} \approx 5$ hours; Fig. 3D and table S9). PDC **4d** decomposed into free paclitaxel and PTX derivatives **16** slowly in an FBS solution, with more than 53 and 37% existing in its original form after 12 and 24 hours, respectively ($t_{1/2} > 12$ hours; Fig. 3D and table S13). Expectedly, after a 24-hour incubation in Bis-Tris buffer (pH 6.5) solution, 88% of the PDC **4d** remained (Fig. 3D and table S14). The excellent stability of PDC **4d** in serum, combined with its GSH concentration–dependent drug release profile, demonstrate that the N═S bond can function as a reversible modification for peptide probe delivery. In addition, it may serve as a distinctive redox-cleavable linker for PDCs. This is supported by the fact that GSH concentrations in extracellular environments, such as plasma, typically range from 2 to 20 μM, whereas intracellular GSH levels are ~1 to 2 mM. Notably, in the tumor microenvironment, GSH concentrations can reach up to 10 mM, which is roughly 200- to 5000-fold higher than in the extracellular matrix and 4-fold higher than in normal cells (*46*, *47*).

Application in the split-and-pool SPPS

A mixture of eight peptides was then synthesized using split-and-pool SPPS to demonstrate the potential of our technology in combinatorial library synthesis (Fig. 4). A mass analysis showed that macrocyclization and linearization of the crude peptide mixture proceeded efficiently without any purification to form the product. The linearization process of macrocyclic peptides did not produce any by-products other than oxidized glutathione (GSSG), and the obtained linear peptides were returned to the parent peptides, maintaining structural stability. Linearization is an important step for application in tag-free combinatorial library synthesis because the sequencing of macrocyclic peptides is challenging. An effective method is to linearize the macrocyclic peptide prior to sequencing (*48*).

Application in PDCs

We analyzed the potential residual of Ru and Ag in purified PDC **4d** using inductively coupled plasma mass spectrometry (ICP-MS) to investigate the potential antitumor application of PDC **4d**. We found levels under 10 parts per billion (fig. S73) that are compatible with biological assays under physiological conditions. cRGD has been shown to have high affinity for the $\alpha_v\beta_3$ integrin receptors that are overexpressed on the endothelial cells of tumor angiogenic vessels as well as the U87MG cell line and A549 cell line (*49*–*53*). In contrast, the human embryonic kidney cell line, HEK-293, which expresses low levels of $\alpha_v\beta_3$ integrin, could serve as a negative control (*54*, *55*). Western blot analysis showed that integrin expression levels in the U87MG and A549 cell lines were 2.3- and 2.7-fold higher, respectively, than those in the HEK-293 cells (Fig. 5, B and C). The results of in vitro antitumor experiments (Fig. 5D) revealed that PDC **4d** exhibited median inhibitory concentration (IC_{50}) values of 62.0 ± 8.4, 120.0 ± 33.8, and 5.9 ± 0.7 nM against HEK-293, U87MG, and A549 cells, respectively. PTX displayed IC_{50} values of

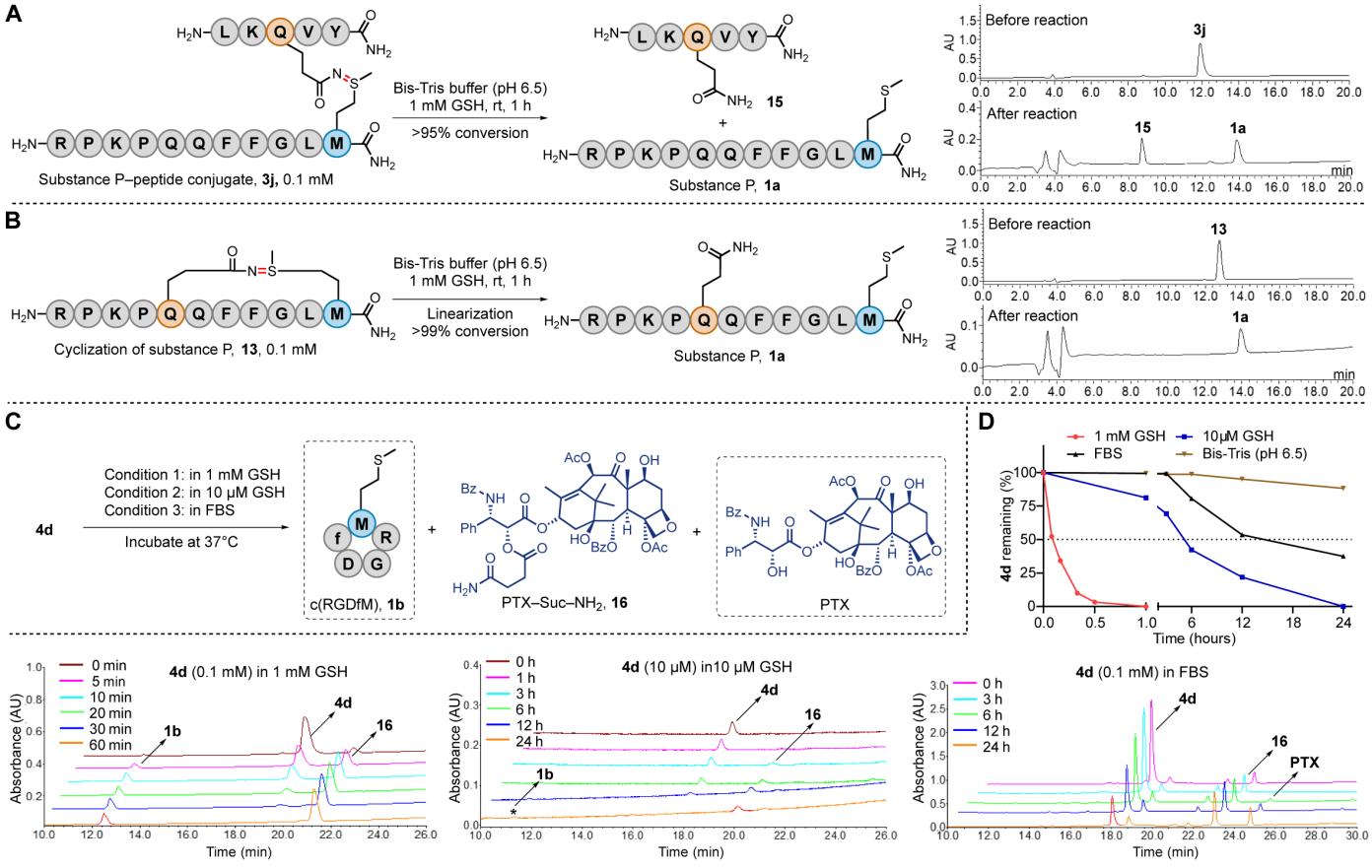

Fig. 3. GSH-responsive sulfimidation reversal and stability assessment of PDC 4d. (**A**) Deconjugation of the peptide conjugate **3j**. rt, room temperature; h, hour; AU, arbitrary units. (**B**) Structural linearization of cyclopeptide **13** via the cleavage of the N═S bond. (**C**) Main decomposition products of PDC **4d**. (**D**) Comparative stability profiles of PDC **4d** under different solution conditions.

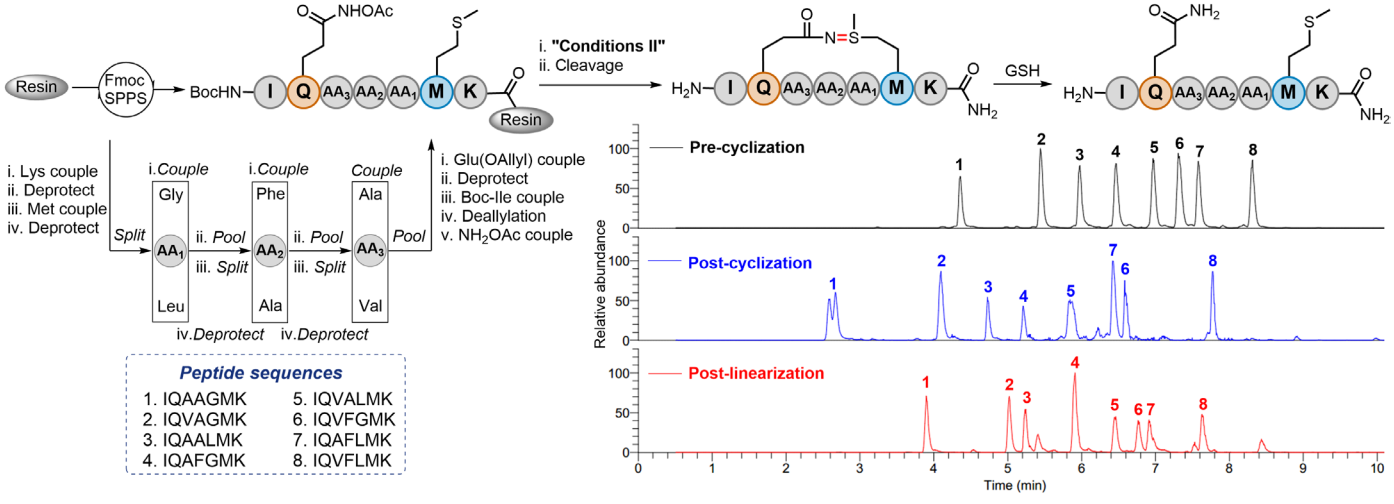

Fig. 4. Split-and-pool SPPS, macrocyclization, and linearization of an eight-peptide mixture without any purification. Mass-based search on the mass trace of the corresponding peptide.

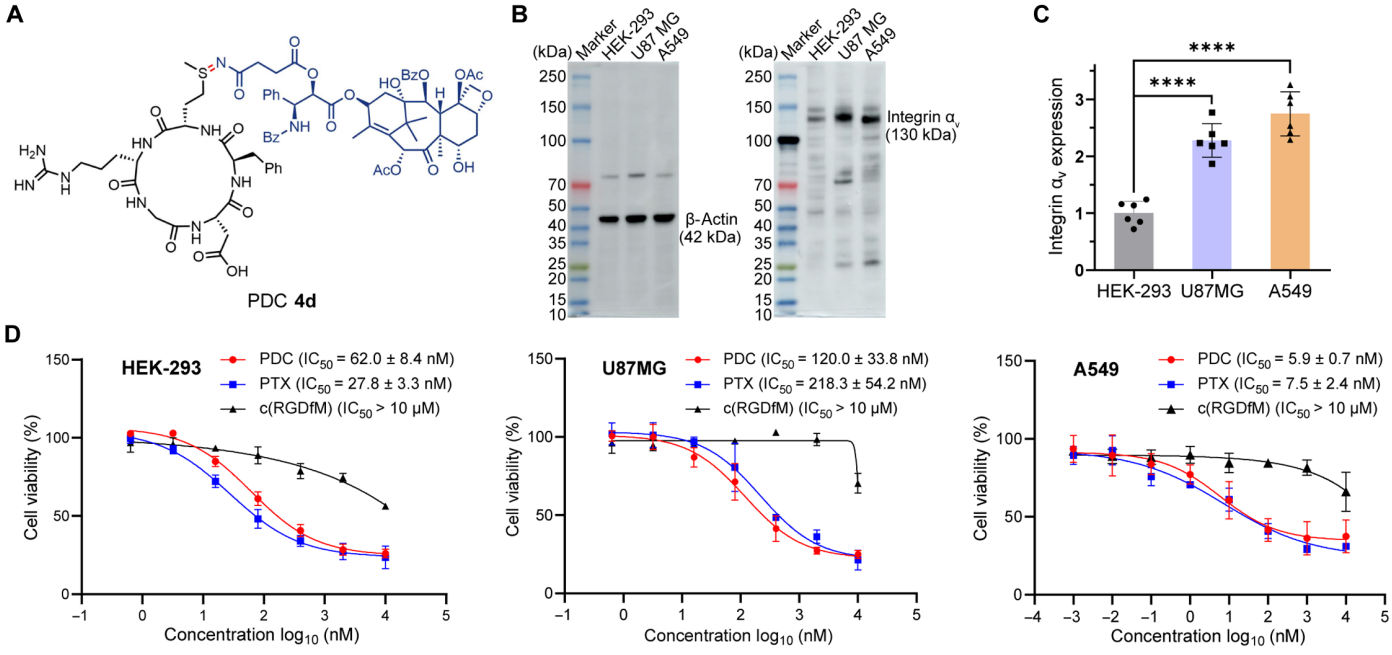

Fig. 5. Biological evaluation of PDC 4d. (**A**) Chemical structure of PDC **4d**. (**B**) Western blot analysis of integrin α_v expression in HEK-293, U87MG, and A549 cells. (**C**) Quantitative analysis of integrin α_v expression levels in HEK-293, U87MG, and A549 cells. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. ****$P < 0.0001$. (**D**) Cytotoxicity evaluation of PDC **4d**, c(RGDfM), and PTX in HEK-293, U87MG, and A549 cells, respectively. Data are shown as means ± SEM ($n = 3$ biological replicates).

27.8 ± 3.3, 218.3 ± 54.2, and 7.5 ± 2.4 nM for the same cell lines. Expectedly, the c(RGDfM) peptide showed IC_{50} values greater than 10 μM across all three cell types (HEK-293, U87MG, and A549). Compared to free PTX, PDC **4d** showed increased cytotoxicity against $\alpha_v\beta_3$-positive U87MG and A549 cells, whereas the cytotoxicity decreased against the negative control HEK-293 cells. In addition, although there are reports that RGD-targeting conjugates are taken up by cells through RGD-dependent endocytosis mechanisms (*56*), we cannot completely exclude the possibility of fluid-phase endocytosis (*57*), which may explain why the negative control still maintained a certain cytotoxicity degree.

In summary, we developed a controlled reversible strategy for the sulfimidation of peptides at Met-specific sites using a Ru(II)-catalyzed sulfimidation reaction. This strategy enabled the introduction of sulfilimines with various functional groups, such as lipid tags, drugs, natural products, and fluorescent dyes, into peptides both in homogeneous solution phase and on resins. Furthermore, this protocol provided a unique type of stapling through N═S bonds between Met and Gln sites that was linearized tracelessly in the presence of GSH. Additional applications of split-and-pool synthesis and PDCs have also been developed. We are optimistic that this reversible Met modification protocol will be a useful tool for labeling and fine-tuning Met residues of peptides for both biological and medicinal chemistry studies, which are ongoing in our laboratory.

MATERIALS AND METHODS

Materials

All commercially available reagents were used without further purification unless otherwise noted. Bioactive peptides such as aviptadil, tetracosactide, systemin, and exenatide were purchased from GL Biochem and used without further purification. c(RGDfM) was purchased from Hangzhou Allpeptide Biotechnology Co. Ltd. and used without further purification. All solvents in reagent grade or HPLC grade were used without purification. Dulbecco's modified Eagle's medium (DMEM) high glucose, RPMI 1640 medium and penicillin-streptomycin (P/S) were purchased from Hyclone and Solarbio. FBS was purchased from Gibco. Cell Counting Kit-8 (CCK-8) was purchased from Sunview Biotech. Integrin α_v polyclonal antibody (27096-1-AP) and β actin polyclonal antibody (20536-1-AP) were purchased from Proteintech; horseradish peroxidase–labeled Goat Anti-Rabbit IgG(H+L) (A0208) was purchased from Beyotime Biotechnology.

Cell lines

U87MG cell line (CL-0238) was purchased from Wuhan Pricella Biotechnology Co. Ltd. A549 (SCSP-503) and HEK-293 (SCSP-5209) cell lines were purchased from the China Center for Type Culture Collection (CCTCC).

Typical procedure for methionine-selective modification of bioactive peptides

In an oven-dried 10-ml vial with a magnetic stir bar was charged with substance P **1a** (1.0 equiv, 5 μmol, 6.7 mg), **2a** (5.0 equiv, 3.7 mg), AgOAc (10 μmol, 1.7 mg), and MeOH (0.5 mL), followed by adding a premixture of MeOH solution (0.5 ml) containing $RuCl_2(PPh_3)_3$ (0.5 μmol) and PhCONHOH (1.0 μmol). The vial was stirred at 37°C for 30 min (one round), followed by adding the abovementioned MeOH solution (0.5 ml) and for another 30 min (two rounds). The reaction was monitored by liquid chromatography–mass spectrometry. After 1 hour, the crude reaction mixture was directly purified by semipreparative HPLC, followed by lyophilization, and the desired

product **3a** was obtained as a lyophilized white powder (4.3 mg, 60% yield, >99.9% purity).

Supplementary Materials

This PDF file includes:

Supplementary Text
Figs. S1 to S74
Tables S1 to S15
NMR spectra
References

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