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Macrocyclization of Native Peptides through (Thio)urea Crosslinking of Two Amines

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Abstract

Cyclopeptides have emerged as promising candidates for developing bioactive molecules and drugs. However, owing to the scarcity of efficient cyclization strategies for natural peptides, the development of novel and diverse cyclopeptides remains a formidable challenge. In this study, a variety of novel macrocyclic peptides featuring (thio)urea bridges were developed through the site-selective Lys-Lys crosslinking of native peptides, employing CDI/TCDI as carbonylation reagents. In particular, the dual thiourea-bridged macrocyclic peptides were further synthesized by using a one-pot two-step multicomponent macrocyclization. These strategies enabled the chemoselective macrocyclization of peptides bearing diverse nucleophilic residue into novel (thio)urea-bridged macrocyclic analogs, as exemplified by the successful transformation of bioactive peptides including RGD, Anoplin, and HHC-36. Biological evaluation demonstrated that the cyclopeptides, particularly the thiourea- and bis-thiourea-bridged analogs, exhibit a greater than 10-fold enhancement in target binding affinity, potent antitumor activity, as well as improved membrane permeability and stability. Molecular docking elucidates the structural origin of the enhanced bioactivity, revealing their superior target engagement through reinforced molecular interactions.

Introduction

Cyclopeptides have garnered significant research attention in recent years for their potential in the development of bioactive molecules and drugs due to the enhanced stability, cell permeability, and affinity toward protein surfaces involved in protein-protein interactions (PPIs).¹⁻⁴ With the continuous expansion of the therapeutic application landscape, ranging from antibacterial and antitumor treatments to neurodegenerative diseases and immunomodulation, the development of diverse and versatile cyclopeptides is increasingly critical.⁵⁻⁸ Nevertheless, the presence of diverse reactive functional groups, such as thiol, guanidine, acids, alcohols, amines, together with the requirements for rapid kinetics and mild reaction conditions, poses significant

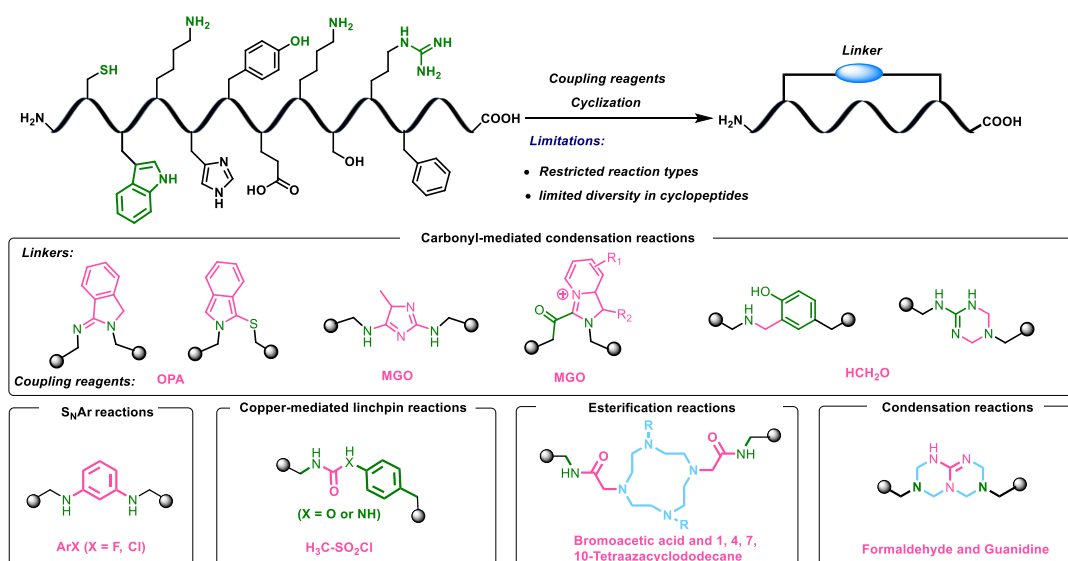
challenges for cyclizing native peptides. Over the past decades, numerous cyclization strategies have been developed to address these challenges by pre-installing uniquely reactive functional handles, such as azides, alkynes, or alkenes, into the peptide backbone.⁹⁻¹⁶ However, such approaches often reduce cyclization efficiency and introduce the synthetic limitations associated with unnatural amino acids.

Crosslinking two nucleophilic side chains within natural peptides provides a robust and versatile strategy for direct cyclization.¹⁷⁻²² This method obviates the need for unnatural residues and has therefore attracted considerable attention. Nevertheless, the limited number of nucleophilic residues, coupled with the need for high selectivity, poses a substantial challenge for peptide side-chain coupling. Recent advances have established diverse strategies for inter-residue cross-linking through the reactions of nucleophilic sites with coupling reagents, including carbonyl-mediated condensation reactions,²³⁻²⁹ S_NAr reactions,^{8, 30-32} esterification³³ and so on.³⁴⁻³⁸ (Fig. 1A) Despite significant progress, it remains critically challenging to achieve diverse and selective cyclization of natural peptides at their limited nucleophilic residues, which is key to constructing structurally novel and diverse cyclopeptides.

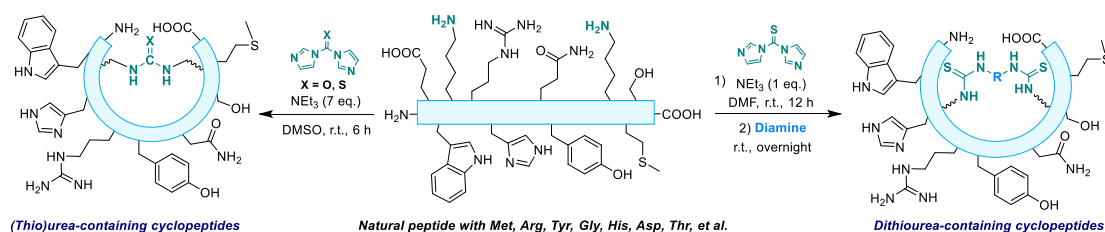
Urea and thiourea motifs are privileged scaffolds in medicinal chemistry. Their capability for hydrogen bonding donor/acceptor, conformational flexibility, and metabolic stability facilitates robust interactions with biomacromolecules, making them key to molecular recognition and drug-target binding.³⁹⁻⁴¹ These core scaffolds constitute fundamental frameworks for diverse bioactive compounds and therapeutic agents possessing a broad range of pharmacological activities, such as anti-tumor, anti-inflammatory, anti-viral and antimicrobial activities.⁴²⁻⁴⁷ One important study that we have recently become interested in is the cyclization modification of natural peptides and their activity studies. Inspired by the unique properties and broad bioactivities of the urea group, we propose incorporating the urea moiety into cyclopeptides to improve the physicochemical properties and physiological activities of natural peptides. It is important to note that, despite the established role of urea and thiourea as privileged motifs in small molecules, their strategic incorporation into macrocyclic peptide architectures has been scarce, with few examples reported in the past decade.

In this work, we report a straightforward cyclization of natural peptides *via* CDI/TCDI-mediated crosslinking between two amino groups, enabling efficient access to novel macrocyclic peptides featuring (thio)urea linkages (Fig. 1B). Furthermore, the direct multicomponent macrocyclization of natural peptides was achieved using a one-pot, two-step protocol, generating macrocyclic peptides featuring with dual thiourea linkages. Notably, these strategies exhibit excellent chemoselectivity and broad compatibility for the macrocyclization of native bioactive peptides containing multifunctional nucleophilic residues, such as Arg, Tyr, Asp, Thr, Met, His, *et al.*. Biological evaluation confirmed that the (thio)urea-bridged macrocycles display significantly enhanced cytotoxicity against multiple cancer cells, with concomitant improvements in membrane permeability and proteolytic stability.

A. Representative approaches of natural peptide cyclization via crosslinking two nucleophilic side chains



B. This work: Macrocyclization of native peptides for the synthesis of (thio)urea-containing cyclopeptides



Advantages:

- ♦ Diversified (thio)urea-containing cyclopeptides formation
- ♦ Enhanced stability and membrane permeability
- ♦ Compatible with various bioactive peptides
- ♦ In vitro bioactivity

Fig. 1. Macrocyclization of native peptides through (thio)urea crosslinking of two amines.

Results and Discussion

Optimization and scope of urea/thiourea bridged cyclopeptides

In the initial optimization studies, peptide **P1** (with sequence Ac-Lys-Leu-Val-Lys-OMe) was chosen as a model substrate for reaction conditions optimization, employing *N,N'*-carbonyldiimidazole (CDI, **2**) or *N,N'*-thiocarbonyldiimidazole (TCDI, **3**) as carbonylation reagent. In the presence of NEt₃, the reaction afforded the desired cyclopeptide in 88% yield by HPLC (Table S1, entry 1). Formation of the isocyanation product from the amino group constituted the major side reaction. This species was successfully scavenged by the addition of a primary alkylamine upon reaction completion. Screening of bases, including DIPEA, DBU, DABCO, TBD, and *t*-BuOK, identified NEt₃ as the most effective, giving higher conversion and isolated yield (Table S1, entries 2-6). Notably, the absence of NEt₃ also yielded the desired product, albeit with lower yield (Table S1, entry 7). Subsequently, the solvent was found to critically influence the reaction. Replacing DMSO with DMF slowed the cyclization, yet it remained efficient, giving a 48% yield (Table S1, entry 8). The use of MeCN led to low reactivity, yielding only 9% of **1a** (Table 1, entry 9). Furthermore, no desired

macrocyclic product was observed in either MeOH or PBS buffer, with recovery of the starting peptide instead (Table S1, entries 10-12). Finally, under higher reaction concentrations, the pathway leading to isocyanation was favored, which adversely affected the reaction efficiency and substantially reduced the yield of the target product (Table S1, entries 13-14).

Next, we performed this (thio)urea crosslinking on a range of peptides with varying amino acid compositions under optimized conditions. Notably, in addition to employing conventional CDI as a carbonyl source, we investigated the utilization of TCDI as an alternative sulfur-containing precursor. As expected, this approach enabled the efficient construction of both urea-based and thiourea-based cyclic peptides, respectively, demonstrating the potential for generating structural diversity (Fig. 2). Intriguingly, this (thio)urea crosslinking was successfully applied to noncanonical amino acids, which facilitated efficient crosslinking between the side chains of β -Ala and Dab to afford

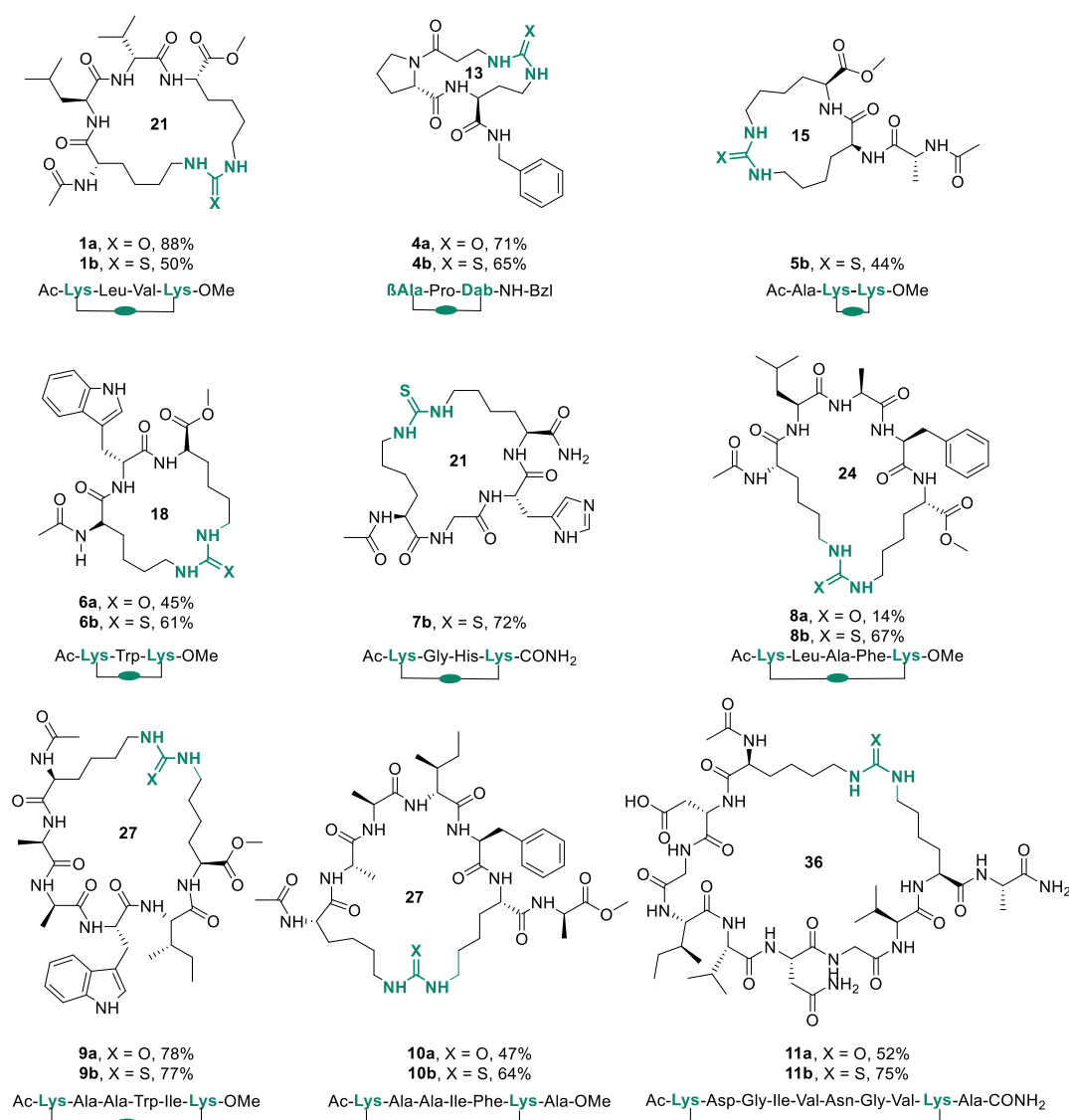


Fig. 2. (Thio)urea crosslinking of two amines at varied spacings. Conditions: linear peptides (0.01 mmol), **2** or **3** (1.5 equiv.), NEt₃ (7 equiv.), under air atmosphere at room temperature for 6 h.

the conformationally constrained 13-membered macrocyclic structures **4a** and **4b**. Subsequently, peptides with two Lys at varied spacings were examined for the (thio)urea crosslinking. Linear peptide **P5** bearing vicinal Lys and Lys did not yield any significant loss of efficiency and gave the desired products (**5b**) with 44% yield. Moreover, a series of structurally diverse linear peptides, featuring variable intervening spacer residues, such as (*i*, *i*+2) (**6a** and **6b**), (*i*, *i*+3) (**7b**), (*i*, *i*+4) (**8a** and **8b**) and (*i*, *i*+5) (**9a**, **9b**, **10a** and **10b**), underwent efficient cyclization under the standard conditions, providing the corresponding cyclic products in reasonable yields. Additionally, this (thio)urea crosslinking was also effective for the distally spaced (*i*, *i*+8) pair (**11a** and **11b**), demonstrating the robustness of this approach for accessing larger ring systems.

To further demonstrate the broad applicability of the protocol, a wide range of peptide substrates containing unprotected reactive residues were tested under standard conditions. As shown in **Fig. 3**, most desired cyclopeptides featuring urea or thiourea moieties were obtained in moderate to excellent yields. For example, linear peptide **P12** which incorporates both unprotected tyrosine residues and free carboxylic acids, successfully underwent lysine-lysine cyclization to form the well-defined macrocyclic structure in high yield. Furthermore, peptides containing a nucleophilic arginine residue

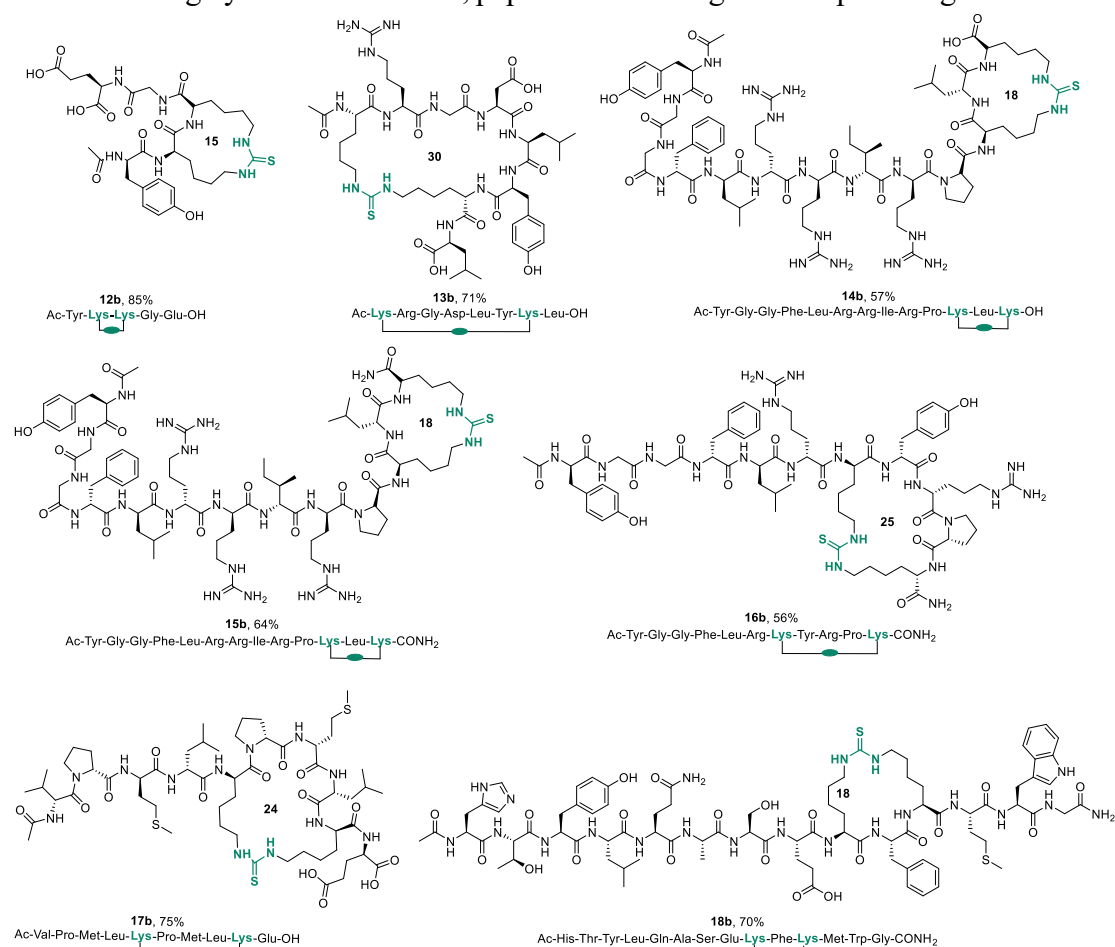


Fig. 3. (Thio)urea crosslinking of two amines containing unprotected reactive residues. Conditions: linear peptide (0.01 mmol), **3** (1.5 equiv.), NEt₃ (7 equiv.), under air atmosphere at room temperature for 6 h.

were also found to be applicable to this cyclization strategy. As evidenced by the cyclization of the RGD (Arg-Gly-Asp) peptide containing an arginine with an unprotected C-terminus (**P13**), the reaction afforded the target cyclic peptide in 71% yield. Furthermore, an amide group (**P15**) as well as histidine (**P17**) and tryptophan (**P18**) residues were all well tolerated under the reaction conditions, affording the corresponding cyclic peptides in good yields. Overall, except for Cys, all nucleophilic amino acids residues in unprotected form are compatible with this reaction system, as exemplified by Glu (**12b**, **17b**, **18b**, **22b**), Tyr (**12b**, **13b**, **14b**, **15b**, **16b**, **18b**), Arg (**13b**, **14b**, **15b**, **16b**, **22b**), Thr (**18b**, **22b**), Gln (**18b**, **22b**), Ser (**18b**), Met (**17b**, **18b**), His (**18b**), Pro (**14b**, **15b**, **16b**, **17b**) and Trp (**6a**, **6b**, **9a**, **9b**, **18b**). Excellent reactivity was maintained with substrates featuring either clusters of identical nucleophilic residues (**14b**, **15b**, **16b**) or combinations of different nucleophiles (Lys, Arg, His, Gln). Such broad compatibility is a key advantage for the site-selective modification of natural active peptides without compromising their bioactivity.

In addition, the (thio)urea crosslinking between the *N*-terminal amine and side-chain amine was also examined to expand the scope of the methodology (**Fig. 4**). Unfortunately, the cyclization reaction failed to proceed when the *N*-terminal amine was reacting with either an adjacent (**P19**) or a distally spaced (**P20**) lysine residue, instead generating lysine isocyanato derivatives. The observed selectivity presumably arises from the low nucleophilicity of the *N*-terminal amine or from sterically unfavorable conformations for macrocyclization. Interestingly, when peptide **P21**, bearing an isopropylidene group at *N*-terminal amine, was used as substrate, cyclization with side-chain residues successfully afforded the desired product (**21b**). Moreover, despite failed cyclization between the *N*-terminal amine and side-chain amine (**19b**, **20b**), the use of an *N*-terminal lysine residue enabled chemoselective amine-amine cyclization between side chains. This reaction delivered cyclopeptide featuring an

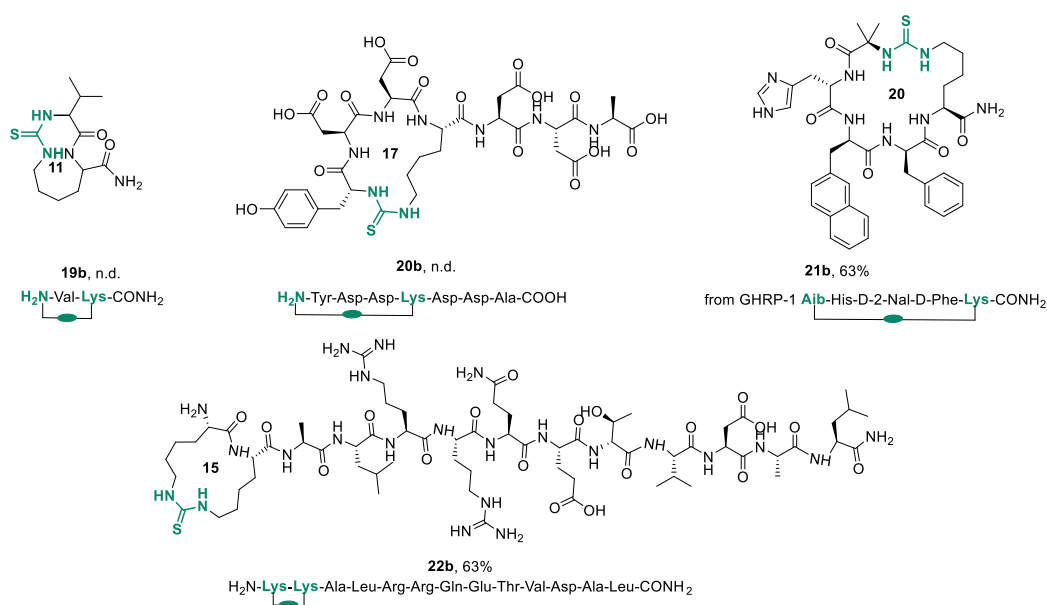


Fig. 4. (Thio)urea crosslinking between the *N*-terminal amine and side-chain amine. Conditions: linear peptide (0.01 mmol), **3** (1.5 equiv.), NEt₃ (7 equiv.), under air atmosphere at room temperature for 6 h.

exposed *N*-terminal amine and thiourea moieties (**22b**). This protocol enabled efficient peptide cyclization while preserving native *N*-terminal bioactivity, thereby bypassing the deprotection steps typically required in conventional macrocyclization strategies.

Given the significant influence of the linkage group on the biological activity, we sought to access bis-urea-bridged cyclic peptide analogs. Following optimization, the target bis-thiourea-bridged macrocyclic peptide was successfully obtained *via* a one-pot, two-step protocol, albeit in lower yield (Fig. 5). This result demonstrates the potential of our method for constructing challenging macrocyclic scaffolds that are difficult to access using conventional approaches. Notably, owing to the lower reactivity of CDI compared to TCDI, the reaction exclusively afforded the macrocyclic peptide featuring a bis-thiourea linkage.

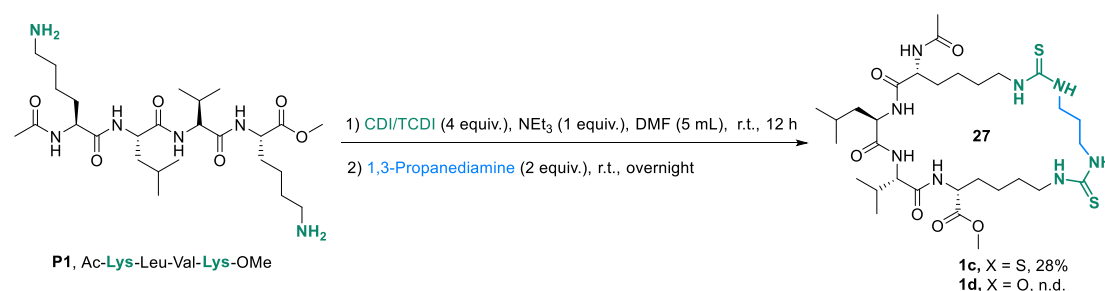


Fig. 5. Macrocyclization for constructing macrocyclic peptides featuring bis-thiourea linkage.

Next, the strategies were extended to the macrocyclization of natural antimicrobial peptides with extensive bioactivities (Fig. 7). Anoplin (GLLKRIKVLL-NH₂) is one of the shortest natural linear AMPs derived from the venom sac of the solitary wasp *Anoplius samariensis*, which exhibits broad-spectrum activity, as well as anti-fungal, anti-tumor, and anti-malarial activities.⁴⁸ Firstly, unprotected Anoplin was explored as starting substrate. However, the corresponding cyclopeptide product could only be observed by LCMS analysis. However, when *N*-terminal Fmoc-protected Anoplin was used as the substrate, cyclic peptides **23a** and **23b**, incorporating urea and thiourea moieties, were successfully obtained. The cyclization occurred concomitantly with spontaneous *N*-terminal deprotection. To unambiguously determine the structure, the product **23a** was definitively characterized by Edman degradation (see Figure. S1). Further, the desired bis-thiourea-bridged macrocyclic peptide **23c** was also successfully accessed *via* the multicomponent stapling approach with reasonable yield. Moreover, cyclization of the naturally occurring cationic antimicrobial peptide HHC-36 (KRWWKWRR-OH)⁴⁹ was also performed, generating the macrocyclic urea **24a** and thiourea **24b** derivatives.

Activity evaluation

To assess the efficacy of the cyclization strategies, we evaluated the stability of the macrocyclic peptides. Firstly, the chemical stability was evaluated in H₂O₂ (80 mM) conditions, and **P24**, **24a** and **24b** were chosen as representative peptides (Figure. S2-S3 and Fig. S8A). The results showed that **P24** degraded rapidly within 4 hours, generating a mixture of uncharacterized degradation products. Comparing with **P24**, no significant degradation was observed for either **24a** or **24b** under the same conditions, indicating their enhanced resistance to oxidative degradation. Analogously, the urea-

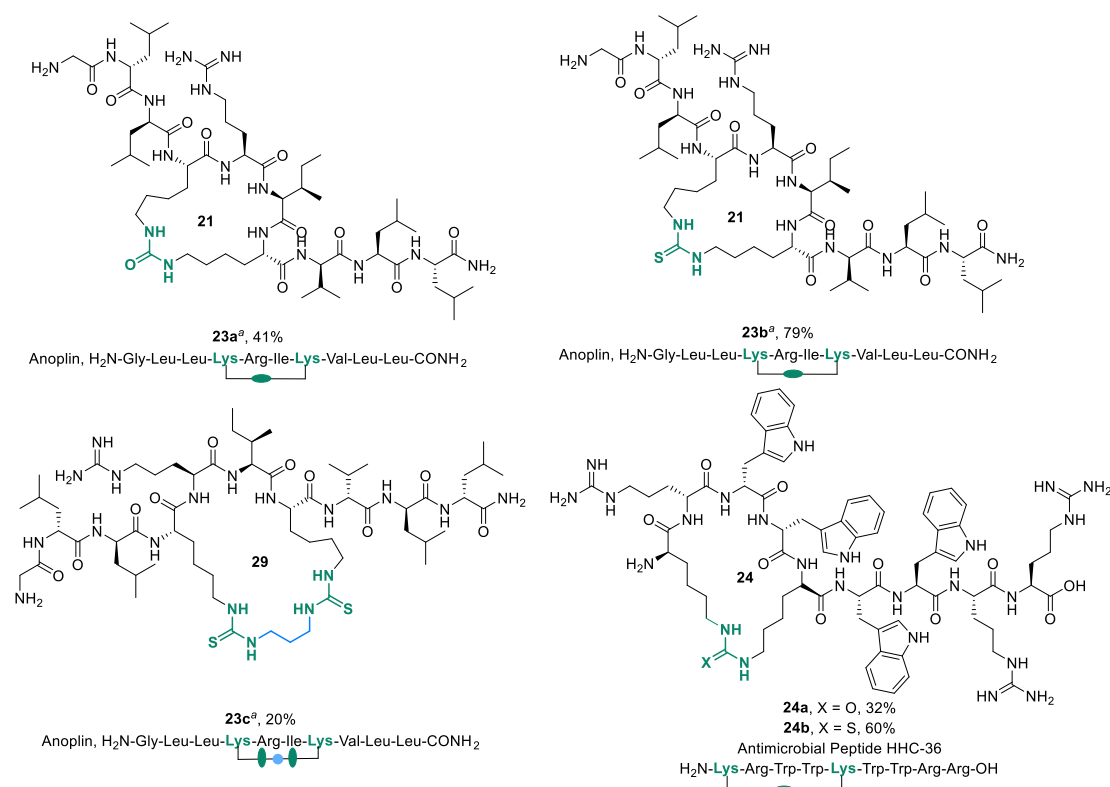


Fig. 7. Macrocyclization of bioactive peptides. Conditions: linear peptide (0.01 mmol), **2** or **3** (1.5 equiv.), NEt₃ (7 equiv.), under air atmosphere at room temperature for 6 h. Conditions of **23c**: linear peptide (0.01 mmol), **3** (4 equiv.), NEt₃ (1 equiv.), 1,3-propanediamine (2 equiv.), under air atmosphere at room temperature overnight. ^a *N*-terminal Fmoc-protected peptide was used.

modified cyclized peptides exhibited comparable stability under alkaline conditions (pH 10.0) (Figure. S4-S5 and Fig. S8B). To further evaluate the application potential of **24a** and **24b** under physiological conditions, we explored their stability in the presence of trypsin. In contrast to the **P24**, **24a** and **24b** underwent no significant digestion within 4 hours, exhibiting markedly enhanced trypsin resistance (Figure. S6-S7 and Fig. S8C). Collectively, these findings show a significant enhancement in peptide stability achieved through the macrocyclic strategy.

Furthermore, to investigate the cell membrane permeability of the cyclopeptides, we designed FITC-labeled cyclic derivatives **25a-25c** (Fig. 8A) and evaluated their cellular uptake efficiency in A549-Luc cells (Fig. 8B and 8C). Following 6 hours of co-incubation with A549-Luc cells, the cellular uptake of FITC-labelled peptides (**P25**, **25a**, **25b**, **25c**) was assessed by confocal laser scanning microscopy (CLSM) imaging. In contrast to **P25**, which showed no intracellular fluorescence, all cyclic analogues including those with urea, thiourea, and bis-thiourea linkages demonstrated significant signal enhancement, indicating superior cell-penetrating efficiency (Fig. S19). Among these, **25b**, incorporating a single thiourea linkage, demonstrated the most pronounced fluorescence intensity enhancement. Flow cytometry quantification further confirmed the superior cellular uptake of all cyclic derivatives over the linear peptide. Strikingly, mono-thiourea analogue **25b** exhibited the most pronounced enhancement, which may be attributable to the optimal balance of lipophilicity and hydrogen-bonding capacity

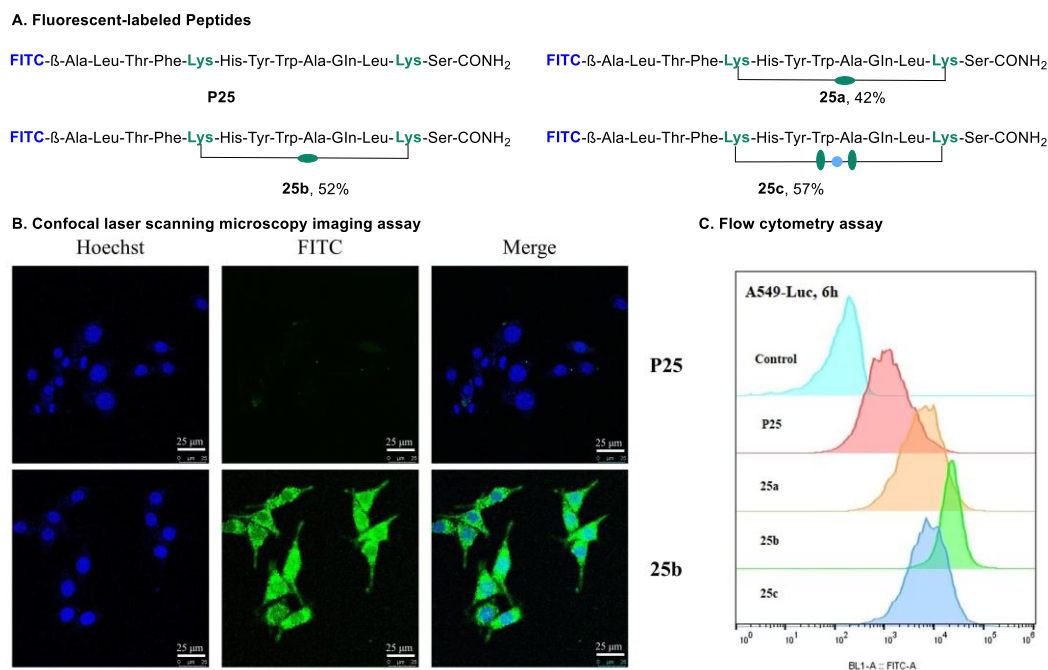


Fig. 8. Fluorescently labeled peptides. A. The structure of fluorescently labeled cyclic peptides **P25**, **25a**, **25b**, **25c**. B. CLSM imaging of **P25** and **25b** in A549-Luc cells for 6 h, scale bar represents 25 μ m. C. Mean fluorescence intensity of **P25**, **25a**, **25b**, **25c** and its cyclized analogs in A549-Luc cells for 6 h.

conferred by its linkage. In contrast, the urea-bridged analogue **25a** and the bis-thiourea analogue **25c** showed more moderate improvements. The restrained uptake of **25a** may result from its inherent polarity, while the unique structure of **25c** might confer excessive conformational restraint or hydrophobic bulk that slightly compromises.

Next, we investigated the biological activities of cyclopeptides to further evaluate the efficacy of the (thio)urea-bridged cyclization strategies. As a representative of an important class of integrin inhibitors,^{50, 51} RGD cyclopeptide **13b** and its linear precursor **P13** were evaluated for their binding affinity to α v β 6 integrin using a competitive ELISA (**Fig. 9**). The results demonstrated that **13b** ($IC_{50} = 0.059 \mu$ M)

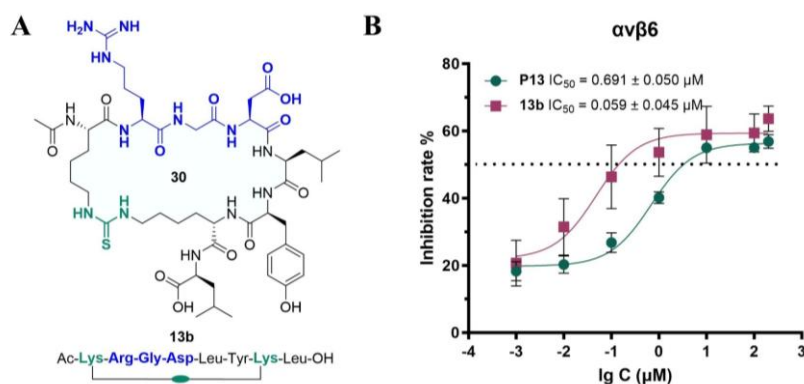


Fig. 9. The integrin binding assay. A. The chemical structure of **13b**; B. IC_{50} values of **P13** and **13b** against the α v β 6.

exhibited a more than 10-fold enhancement in binding affinity compared to its linear

precursor **P13** (IC₅₀ = 0.691 μ M).

Then, the cytotoxicity of the selected bioactive cyclopeptides against several human cancer cell lines, such as A549-Luc (non-small cell lung carcinoma), Caco-2 (colorectal adenocarcinoma), and A498 (renal carcinoma) was evaluated using CCK-8 assay (Table 1). Overall, most of the selected cyclopeptides demonstrated pronouncedly improved anticancer activity relative to the linear analogues. Most notably, the bis-thiourea cyclized analogue **23c** exhibited superior potency compared to the parent compound **P23** across all three cell lines. This enhancement was particularly dramatic in the A549-Luc cell line, where its IC₅₀ values was 10.70 μ M. A significant potency boost was also observed in the Caco-2 cell (IC₅₀ = 18.04 μ M). Even in the A498 cell line, where all compounds showed reduced absolute potency, **23c** remained the most active, demonstrating its superiority over **23a** and **23b**. In addition, compared to the lead compound **P24**, which showed weak or no activity across all cell lines, analogue **24a** demonstrated marginal improvement in the A549-Luc and A498 cell lines. Notably, **24b** exhibited overwhelming superiority across all tested cell lines, with IC₅₀ values of 37.1 μ M (A549-Luc), 46.72 μ M (Caco-2), and 24.22 μ M (A498), which are substantially lower than those of both **P24** and **24a**. These results demonstrate that thiourea-cyclized peptides generally exhibit superior antitumor activity compared to their urea-cyclized analogs. This advantage is most pronounced in the bis-thiourea derivative (**23c**), which emerged as the most potent compound across all evaluations.

Table 1. Antiproliferation activity of macrocyclic peptides, and linear peptides

Cell lines	IC ₅₀ (μ M) $\pm$ SD						
	P23	23a	23b	23c	P24	24a	24b
A549-Luc	39.35 $\pm$ 3.14	166.4 $\pm$ 3.34	63.76 $\pm$ 3.53	10.70 $\pm$ 1.141	176.7 $\pm$ 19.13	123.5 $\pm$ 14.65	37.1 $\pm$ 1.84
Caco-2	56.11 $\pm$ 0.98	43.11 $\pm$ 0.63	> 200	18.04 $\pm$ 0.12	> 200	> 200	46.72 $\pm$ 2.64
A498	23.25 $\pm$ 7.69	161.0 $\pm$ 7.65	174.0 $\pm$ 11.80	59.97 $\pm$ 0.31	146.9 $\pm$ 4.84	135.3 $\pm$ 1.88	24.22 $\pm$ 10.24

Subsequently, the apoptotic impact of **23c** was assessed using the Annexin V-FITC/PI flow cytometry. A549-Luc and Caco-2 cells were co-incubated with **23c** and then stained with Annexin V-FITC and propidium iodide (PI). As detailed in Fig.10A-D, the untreated control group displayed a minimal percentage of apoptotic cells. Compared with the control group, after a 48h treatment with concentrations of 20, 50, 70 μ M for A549-Luc or 20, 35, 60 μ M for Caco-2 cells, the respective percentages of apoptotic cells in the A549-Luc cell population were 25.88%, 77.79%, and 86.80%, while the respective percentages of apoptotic cells in the Caco-2 cell population were 30.94%, 45.00%, and 74.40%. These results demonstrated **23c** showed dose-dependent induction of apoptosis.

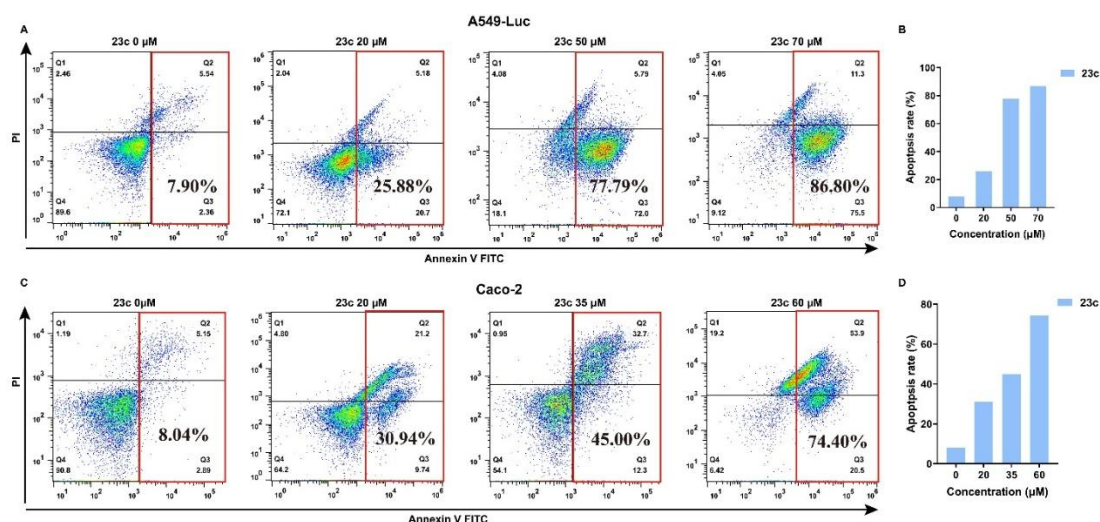


Fig. 10. Flow cytometry analysis of cell apoptosis in A549-Luc and Caco-2 cells treated with **23c**. A. The analysis of **23c** on A549-Luc cells apoptosis (48 h); B. Apoptosis rate at different concentrations of A549-Luc; C. The analysis of **23c** on Caco-2 cells apoptosis (48 h); D. Apoptosis rate at different concentrations of Caco-2.

Having established potent bioactivity profiles for the thiourea-bridged derivatives, we next sought to gain mechanistic insights into their potent efficacy at the molecular level. To this end, we performed molecular docking to visualize its potential binding mode with the target protein. Firstly, we performed molecular docking between integrin $\alpha\beta6$ and **13b**. The crystal structure of the integrin $\alpha\beta6$ protein (PDB ID: 4UM9) was used as the receptor model, and the lowest energy conformation was selected for analysis.⁵² The result showed that **13b** exhibited superior binding affinity to $\alpha\beta6$, which is consistent with the observed enhancement in antitumor activity (Table S2). Within a cutoff distance of 4.50 Å for potential interactions, **13b** was found to interact with $\alpha\beta6$ residues including Ile-216; Asp-218; Asp-219; Arg-248; Glu-316; Lys-338 (Fig. 11A). Furthermore, the hydrogen bond distances formed between **13b** and receptor (2.27 Å; 2.38 Å; 2.18 Å; 2.33 Å; 1.94 Å; 2.04 Å; 2.87 Å; 2.57 Å) were shorter than those formed by **P13** (2.20 Å; 1.99 Å; 2.61 Å; 1.98 Å; 2.25 Å). Subsequently, we conducted molecular docking between the target and **23c**. In order to obtain the possible targets, we first employed network pharmacology to systematically analyze potential targets for compound **P23** (see SI, Figure S21-S22)^{53, 54}. Due to the well-established roles in promoting tumor cell proliferation and inhibiting apoptosis, EGFR was selected from the PPI network as a candidate to evaluate compound binding affinity. Subsequently, we performed molecular docking between EGFR and the peptides **P23** and **23c**. The crystal structure of the EGFR (PDB ID: 9QXN) was retrieved from the Protein Data Bank and served as the receptor model for molecular docking.⁵⁵ Consistent with its superior antitumor efficacy, compound **23c** also demonstrated a stronger computational binding affinity for EGFR than the parent compound **P23**, with docking scores of -14.66 and -13.77, respectively (Table S2). Analysis of the binding modes indicated that both **P23** and **23c** are well-bound by the active site pocket (Fig. 11B). Within a cutoff distance of 4.50 Å for potential interactions, **23c** was found to interact with EGFR residues including Leu-718; Phe-795; Cys-797; Thr-854. The interaction is

stabilized by hydrogen bonds with distances of 2.05 Å; 2.09Å 2.28Å 2.74 Å. Notably, **23c** achieved a more optimal fit within the binding pocket than compound **P23**, which exhibited a relatively loose binding mode. This superior complementarity correlates with the more favorable docking energy computed for **23c**. Collectively, the molecular docking results provide a compelling computational rationale for the observed superior bioactivity of thiourea-bridged cyclopeptides, confirming that its enhanced bioactivity efficacy is likely mediated through stronger and more stable interactions with the target.

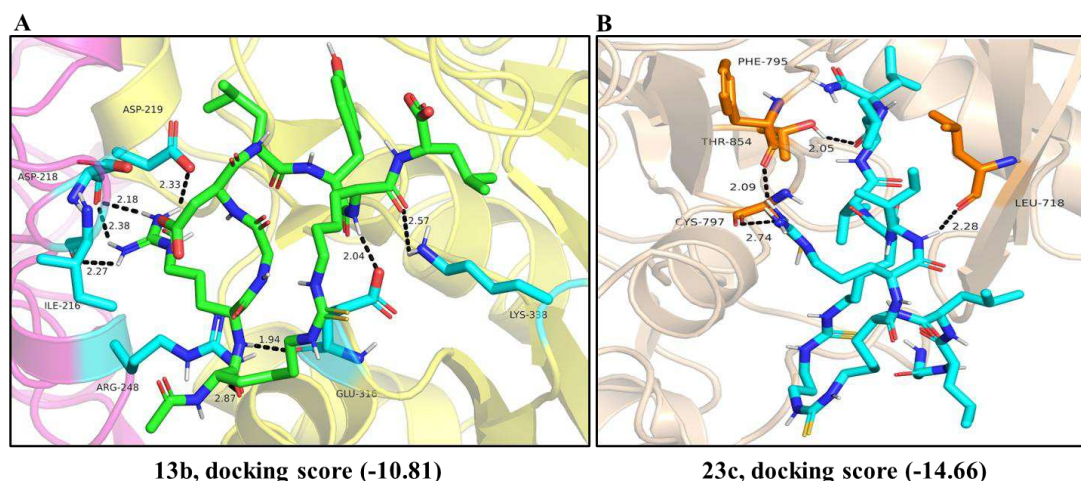


Fig. 11. Molecular docking analysis of **13b**, **23c**, hydrogen bonds are shown in black. A. The docking results between **13b** compound and the $\alpha\text{v}\beta 6$ protein. B. The docking results between **23c** compound and the EGFR protein.

Conclusion

In conclusion, a straightforward macrocyclization of diverse natural peptides through CDI/TCDI-mediated lysine-lysine crosslinking was achieved, generating an array of novel macrocycles incorporating (thio)urea scaffolds. More importantly, an unprecedented multicomponent macrocyclization of natural peptides through a one - pot two - step protocol was further realized for the synthesis of dual thiourea bridged macrocyclic peptides. In addition, the macrocyclization strategies feature broad substrate compatibility, amino acid residue compatibility and excellent chemoselectivity. Biological evaluation showed that these macrocyclic peptides exhibited significantly enhanced cytotoxicity across a panel of cancer cell lines, concomitant with improved membrane permeability and increased proteolytic stability. Molecular docking provided structural insights for these observations, confirming that their enhanced bioactivity efficacy is likely mediated through stronger and more stable interactions with the target.

References

1. Lu H, et al. Recent advances in the development of protein–protein interactions

- modulators: mechanisms and clinical trials. *Signal. Transduct. Target. Ther.* **5**, 213 (2020).
2. Muttenthaler M, King GF, Adams DJ, Alewood PF. Trends in peptide drug discovery. *Nat. Rev. Drug Discov.* **20**, 309-325 (2021).
 3. Zhang H, Chen S. Cyclic peptide drugs approved in the last two decades (2001–2021). *RSC Chem. Biol.* **3**, 18-31 (2022).
 4. Ji X, Nielsen AL, Heinis C. Cyclic Peptides for Drug Development. *Angew. Chem. Int. Ed.* **63**, e202308251 (2023).
 5. Henninot A, Collins JC, Nuss JM. The Current State of Peptide Drug Discovery: Back to the Future? *J. Med. Chem.* **61**, 1382-1414 (2017).
 6. Vinogradov AA, Yin Y, Suga H. Macrocyclic Peptides as Drug Candidates: Recent Progress and Remaining Challenges. *J. Am. Chem. Soc.* **141**, 4167-4181 (2019).
 7. Li CM, et al. Novel Peptide Therapeutic Approaches for Cancer Treatment. *Cells.* **10**, 2098 (2021).
 8. Xu L, Fan X, He Y, Xia X, Zhang J. Design, Synthesis, and Biological Evaluation of Lysine-Stapled Peptide Inhibitors of p53-MDM2/MDMX Interactions with Potent Antitumor Activity In Vivo. *J. Med. Chem.* **67**, 17893-17904 (2024).
 9. Islam MS, et al. Unprotected peptide macrocyclization and stapling via a fluorine-thiol displacement reaction. *Nat. Commun.* **13**, 350 (2022).
 10. Ullrich, S., George, J., Coram, A. E., Morewood, R. & Nitsche, C. Biocompatible and Selective Generation of Bicyclic Peptides. *Angew. Chem. Int. Ed.* **61**, e202208400 (2022).
 11. Vanjari R, Panda D, Mandal S, Vamiseti GB, Brik A. Gold(I)-Mediated Rapid Cyclization of Propargylated Peptides via Imine Formation. *J. Am. Chem. Soc.* **144**, 4966-4976 (2022).
 12. Liu X-Y, Cai W, Ronceray N, Radenovic A, Fierz B, Waser J. Synthesis of Fluorescent Cyclic Peptides via Gold(I)-Catalyzed Macrocyclization. *J. Am. Chem. Soc.* **145**, 26525-26531 (2023).
 13. Lamartina CW, Chartier CA, Hirano JM, Shah NH, Rovis T. Crafting Unnatural Peptide Macrocycles via Rh(III)-Catalyzed Carboamidation. *J. Am. Chem. Soc.* **146**, 20868-20877 (2024).
 14. Li B, Parker J, Tong J, Kodadek T. Synthesis of Membrane-Permeable Macrocyclic Peptides via Imidazopyridinium Grafting. *J. Am. Chem. Soc.* **146**, 14633-14644 (2024).
 15. Luo J, et al. Copper-Catalyzed Late-Stage Modification of Peptide-Bearing Unprotected Side Chains. *CCS Chem.* **7**, 1-10 (2025).
 16. Wang N, et al. Tyrosinase-Catalyzed Peptide Stapling Using Para-Amino Phenylalanine and Tyrosine Anchoring Residues. *Angew. Chem. Int. Ed.* **64**, e202420522 (2025).
 17. White CJ, Yudin AK. Contemporary strategies for peptide macrocyclization. *Nat. Chem.* **3**, 509-524 (2011).
 18. Lau YH, de Andrade P, Wu Y, Spring DR. Peptide stapling techniques based on

- different macrocyclisation chemistries. *Chem. Soc. Rev.* **44**, 91-102 (2015).
19. Dong S, et al. Recent advances in chemical protein synthesis: method developments and biological applications. *Sci. China Chem.* **67**, 1060-1096 (2024).
 20. Fang P, Pang W-K, Xuan S, Chan W-L, Leung KC-F. Recent advances in peptide macrocyclization strategies. *Chem. Soc. Rev.* **53**, 11725-11771 (2024).
 21. Liu T, Peng Z, Lai M, Hu L, Zhao J. Inverse Peptide Synthesis Using Transient Protected Amino Acids. *J. Am. Chem. Soc.* **146**, 4270-4280 (2024).
 22. Zhan W, Duan H, Li C. Recent Advances in Metal-Free Peptide Stapling Strategies. *Chem. Bio. Eng.* **1**, 593-605 (2024).
 23. Todorovic M, Schwab KD, Zeisler J, Zhang C, Bénard F, Perrin DM. Fluorescent Isoindole Crosslink (FLICk) Chemistry: A Rapid, User-friendly Stapling Reaction. *Angew. Chem. Int. Ed.* **58**, 14120-14124 (2019).
 24. Zhang Y, Zhang Q, Wong CTT, Li X. Chemoselective Peptide Cyclization and Bicyclization Directly on Unprotected Peptides. *J. Am. Chem. Soc.* **141**, 12274-12279 (2019).
 25. Li B, et al. Cooperative Stapling of Native Peptides at Lysine and Tyrosine or Arginine with Formaldehyde. *Angew. Chem. Int. Ed.* **60**, 6646-6652 (2021).
 26. Li B, et al. Construction of Complex Macromulticyclic Peptides via Stitching with Formaldehyde and Guanidine. *J. Am. Chem. Soc.* **144**, 10080-10090 (2022).
 27. Li B, et al. Extendable stapling of unprotected peptides by crosslinking two amines with o-phthalaldehyde. *Nat. Commun.* **13**, 311 (2022).
 28. Galindo AV, Raj M. Solvent-Dependent Chemoselectivity Switch to Arg-Lys Imidazole Cross-Links. *Org. Lett.* **26**, 8356-8360 (2024).
 29. Chu X, et al. Formamidine as an Easy-On and Easy-Off Linker for Reversible Crosslinking of Two Alkyl Amines in Peptide Stapling and Conjugation. *Angew. Chem. Int. Ed.* **64**, e202422844 (2025).
 30. Spokoyny AM, Zou Y, Ling JJ, Yu H, Lin Y-S, Pentelute BL. A Perfluoroaryl-Cysteine SNAr Chemistry Approach to Unprotected Peptide Stapling. *J. Am. Chem. Soc.* **135**, 5946-5949 (2013).
 31. Lee HG, Lautrette G, Pentelute BL, Buchwald SL. Palladium-Mediated Arylation of Lysine in Unprotected Peptides. *Angew. Chem. Int. Ed.* **56**, 3177-3181 (2017).
 32. Yang P, et al. Teraryl Braces in Macrocycles: Synthesis and Conformational Landscape Remodeling of Peptides. *J. Am. Chem. Soc.* **145**, 13968-13978 (2023).
 33. Cheung TL, et al. Facile Peptide Macrocyclization and Multifunctionalization via Cyclen Installation. *Small Methods.* **8**, e2400006 (2024).
 34. Li X, et al. Dithiocarbamate-inspired side chain stapling chemistry for peptide drug design. *Chem. Sci.* **10**, 1522-1530 (2019).
 35. Xiao, Y., Zhou, H., Liu, H. & Li, X. Trifluoroacetic Acid Mediated Additive-Free Late-Stage Native Peptide Cyclization to Form Disulfide Mimetics via Thioketalization with Ketones. *Org. Lett.* **26**, 6512-6517 (2024).
 36. Voss S, Rademann J, Nitsche C. Peptide-Bismuth Bicycles: In Situ Access to

- Stable Constrained Peptides with Superior Bioactivity. *Angew. Chem. Int. Ed.* **61**, e202113857 (2021).
37. Zhang Y, et al. Peptide Stapling through Site-Directed Conjugation of Triazine Moieties to the Tyrosine Residues of a Peptide. *Org. Lett.* **25**, 2248-2252 (2023).
 38. Ding Y, et al. Selective Macrocyclization of Unprotected Peptides with an Ex Situ Gaseous Linchpin Reagent. *Angew. Chem. Int. Ed.* **63**, e202405344 (2024).
 39. Garuti, L., Roberti, M., Bottegoni, G. & Ferraro, M. Diaryl Urea: A Privileged Structure in Anticancer Agents. *Curr. Med. Chem.* **23**, 1528-1548 (2016).
 40. Ghosh AK, Brindisi M. Urea Derivatives in Modern Drug Discovery and Medicinal Chemistry. *J. Med. Chem.* **63**, 2751-2788 (2019).
 41. Ronchetti R, Moroni G, Carotti A, Gioiello A, Camaioni E. Recent advances in urea- and thiourea-containing compounds: focus on innovative approaches in medicinal chemistry and organic synthesis. *RSC Med. Chem.* **12**, 1046-1064 (2021).
 42. Han L-Q, et al. Urea-containing peptide boronic acids as potent proteasome inhibitors. *Eur. J. Med. Chem.* **125**, 925-939 (2017).
 43. Sabir S, et al. Novel Seleno- and Thio-Urea Containing Dihydropyrrol-2-One Analogues as Antibacterial Agents. *Antibiotics (Basel)*. **10**, 321 (2021).
 44. Yang Y, et al. Near quantitative synthesis of urea macrocycles enabled by bulky N-substituent. *Nat. Commun.* **12**, 1572 (2021).
 45. Listro R, et al. Urea-based anticancer agents. Exploring 100-years of research with an eye to the future. *Front. Chem.* **10**, 995351 (2022).
 46. Previti S, et al. Development of Urea-Bond-Containing Michael Acceptors as Antitrypanosomal Agents Targeting Rhodesain. *ACS Med. Chem. Lett.* **13**, 1083-1090 (2022).
 47. Wang, J. et al. Synchronous recognition of amines in oxidative carbonylation toward unsymmetrical ureas. *Science*. **386**, 776-782 (2024).
 48. Wu Y, et al. Stapled Wasp Venom-Derived Oncolytic Peptides with Side Chains Induce Rapid Membrane Lysis and Prolonged Immune Responses in Melanoma. *J. Med. Chem.* **64**, 5802-5815 (2021).
 49. Gao W, et al. Antibacterial properties of antimicrobial peptide HHC36 modified polyetheretherketone. *Front. Microbiol.* **14**, 1103956 (2023).
 50. Kapp, T. G. et al. A Comprehensive Evaluation of the Activity and Selectivity Profile of Ligands for RGD-binding Integrins. *Sci. Rep.* **7**, 39805 (2017).
 51. Di Leva, F. S. et al. From a Helix to a Small Cycle: Metadynamics-Inspired $\alpha\beta6$ Integrin Selective Ligands. *Angew. Chem. Int. Ed.* **57**, 14645-14649 (2018).
 52. Dong, X., Hudson, N. E., Lu, C. & Springer, T. A. Structural determinants of integrin β -subunit specificity for latent TGF- β . *Nat. Struct. Mol. Biol.* **21**, 1091-6 (2014).
 53. Lan, Y., Peng, Q., Fu, B. & Liu, H. Effective analysis of thyroid toxicity and mechanisms of acetyltributyl citrate using network toxicology, molecular docking, and machine learning strategies. *Toxicol.* **511**, 154029 (2025).
 54. He, Q. et al. Exploring the mechanism of curcumin in the treatment of colon

- cancer based on network pharmacology and molecular docking. *Front. Pharmacol.* **14**, 1102581 (2023).
55. Siegel, F. et al. Sevabertinib, a Reversible HER2 Inhibitor with Activity in Lung Cancer. *Cancer. Discov.* 10.1158/2159-8290.CD-25-0605 (2025).

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Data and materials availability: All data are available in the main text or the supplementary materials.

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