

Recent Advances in Metal-Free Peptide Stapling Strategies

Wanglin Zhan, Hongliang Duan, and Chengxi Li*

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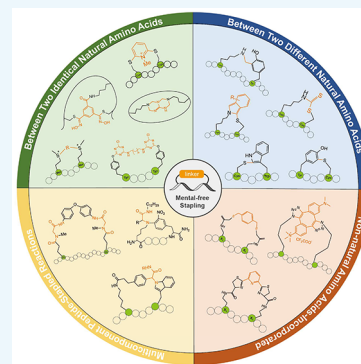
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ABSTRACT: Protein–protein interactions (PPIs) pose challenges for intervention through small molecule drugs, protein drugs, and linear peptides due to inherent limitations such as inappropriate size, poor stability, and limited membrane penetrance. The emergence of stapled α -helical peptides presents a promising avenue as potential competitors for inhibiting PPIs, demonstrating enhanced structural stability and increased tolerance to proteolytic enzymes. This review aims to provide an overview of metal-free stapling strategies involving two identical natural amino acids, two different natural amino acids, non-natural amino acids, and multicomponent reactions. The primary objective is to delineate comprehensive peptide stapling approaches and foster innovative ideation among readers by accentuating methodologies published within the past five years and elucidating evolving trends in stapled peptides.



KEYWORDS: peptide stapling, peptide cyclization, metal-free, membrane penetrance, rigid structure

1. INTRODUCTION

The regulation of various life processes in living organisms is achieved through protein–protein interactions (PPIs). Due to the expansive interface of PPIs, it is challenging for small-molecule drugs to efficiently and selectively target and intercept the interaction.¹ Therefore, biologics such as proteins and peptides, with heightened protein binding affinity, have garnered attention as effective pharmaceuticals for PPI inhibitors. However, protein drugs encounter difficulty in passing through the cell membrane successfully and act on the extracellular target exclusively, without direct access to intracellular targets. The size of short-chain peptides falls between small molecules and large proteins, and their conformational structure is simpler.² Nevertheless, their limited structural stability in aqueous solution, subpar protease tolerance in vivo, and poor membrane penetrance restrict their application in the field of medical treatment. Consequently, researchers are pursuing a new type of drug exhibiting ideal membrane penetration ability and stability.

Cyclic peptides are anticipated to emerge as potential competitors for PPI inhibitors, which exhibit more stable structures and enhanced proteolytic enzyme tolerance compared to linear peptides,³ and demonstrate more effective interactions with targets. Depending on the sites of cyclization, the strategies for forming cyclic peptides can be categorized into head-to-tail, head-to-side chain, side chain-to-tail, and side chain-to-side chain cyclization. The latter plays a key role in achieving secondary conformations (α -helices or β -sheets) necessary for creating “stapled peptides”.⁴ The α -helices are

commonly situated on the interfaces of PPIs, so it is possible to reasonably design α -helical peptides that disturb such PPIs.⁵

In recent years, alongside the numerous classical cyclization strategies, significant progress has been made in new stapling strategies such as bicyclization, photoisomerization, reversible cyclization, ultra-rapid cyclization, etc., some of which even realize the stapling and further late-stage functionalization simultaneously. With the idea of green chemistry becoming more deeply ingrained in the consciousness of people, the metal-free cyclization is gaining increasing popularity. This review will focus on metal-free stapling strategies based on two identical natural amino acids, two different natural amino acids, non-natural amino acids, and multi-component reactions. Therefore, strategies involving the metal catalysis such as C–H activation are not included. Particularly, emphasis will be placed on the progress made over the last five years.

2. TWO IDENTICAL NATURAL AMINO ACIDS INCORPORATED STAPLING STRATEGIES

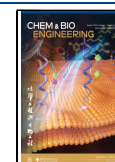
2.1. Cys-Cys Stapling. Natural amino acids containing modifiable active functional groups such as amines, hydroxyls, thiols, carboxyls, etc., can be directly used as binding sites in peptide stapling. Moreover, they are easily obtainable

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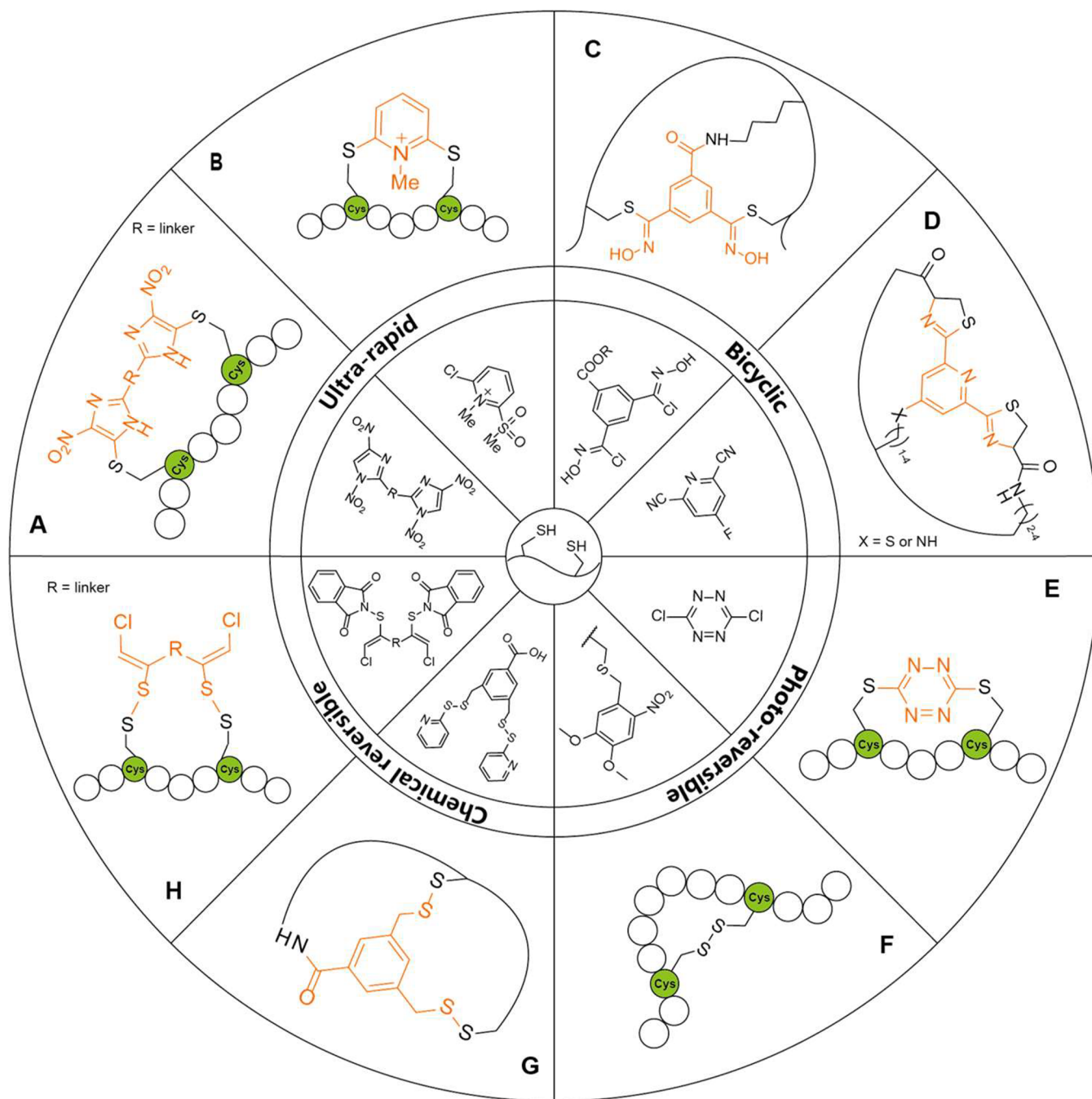
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Scheme 1. Late-Stage Cys-Cys Stapling Strategies



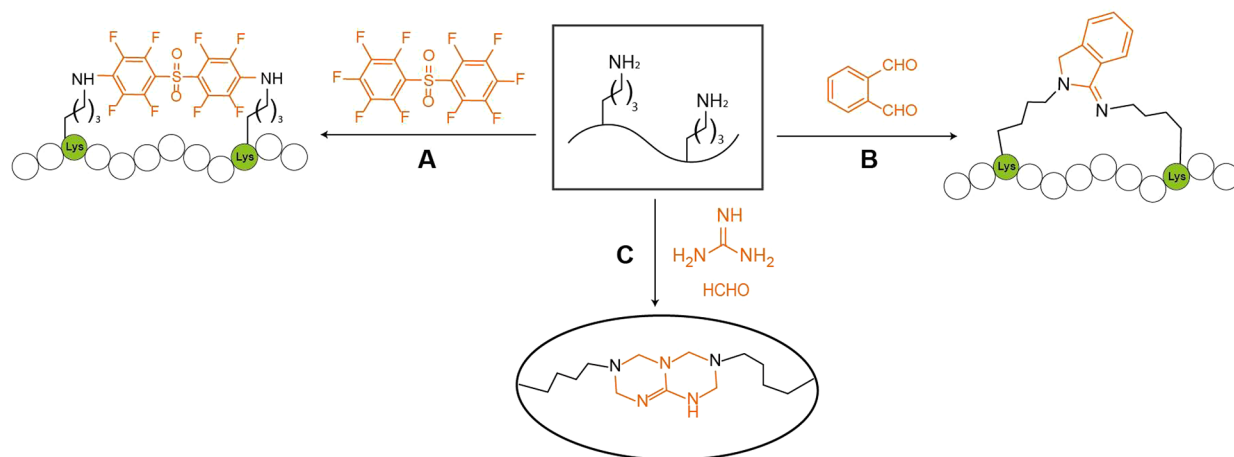
commercially, which avoids the need for complicated chemical or biological synthesis. Among 20 common natural amino acids, the sulfhydryl group of cysteine (Cys) exhibits great nucleophilicity, while the frequency of cysteine in peptide and protein sequences of vertebrates is only 3.3%.² The utilization of amino acids with low abundance as anchoring residues is intended to improve specificity and reduce unwanted side reactions. Furthermore, to functionalize the stapled peptides, a variety of groups such as fluorophores and biotins are designed to modify the cross-linkers.

The modification of cysteine is mainly dependent on electrophilic reagents, such as halides of alkanes or aromatics (S_NAr), alkenes, maleimides (Michael acceptors), etc. In the initial phase, numerous alkylation reagents have been gradually

developed, including alkyl bromides/iodides and allyl bromides. Wang et al. reported a thiol-ene reaction between the sulfhydryl groups of cysteine residues and a diene on an unprotected peptide to achieve cyclization and helicity.⁶ This approach complemented the classical ring-closing metathesis (RCM) method without the use of unnatural amino acids or metal-based catalysts.

However, compared to the flexible alkane, a rigid aromatic ring is more conducive to stabilizing the helical conformation of peptide chains, and thus it appears more commonly in diverse linkers. Timmerman et al. used symmetrically substituted bromomethylbenzene as a linker to complete the rapid cyclization of sulfhydryl groups in less than 15 min at room temperature.⁷ At the same time, the multi-substituted

Scheme 2. Representative Examples of Lys-Lys Stapling Strategies



bromomethylbenzene can realize stapling at three sites to form bicyclization, which is suitable for linear peptides ranging from 2 to 30 amino acids long with the side chains unprotected. On this basis, Lin and coworkers developed bis(aryl)methylene bromides as *i, i+7* Cys cross-linkers.⁸ The rigidity and hydrophobicity of the biaryl group and the increased helicity effectively improve the membrane penetration of the peptides. Pentelute and coworkers modified an unprotected cysteine mercaptan with perfluorinated aromatic compounds (S_NAr) at room temperature in polar organic media, exhibiting great selectivity.⁹ Compared with linear peptides, stapled peptides show stronger protease tolerance and cell permeability. These improvements in biophysical properties are likely to be attributed to the lipophilicity and rigidity of the perfluorinated aromatic linkers. Derda et al. used sulfonic acid groups to couple two perfluorobenzenes as linkers, with a reaction rate of up to $180 \text{ M}^{-1} \text{ s}^{-1}$, good stability at $\text{pH} = 7.4$, and excellent specificity and biocompatibility with polyprotein complexes.¹⁰

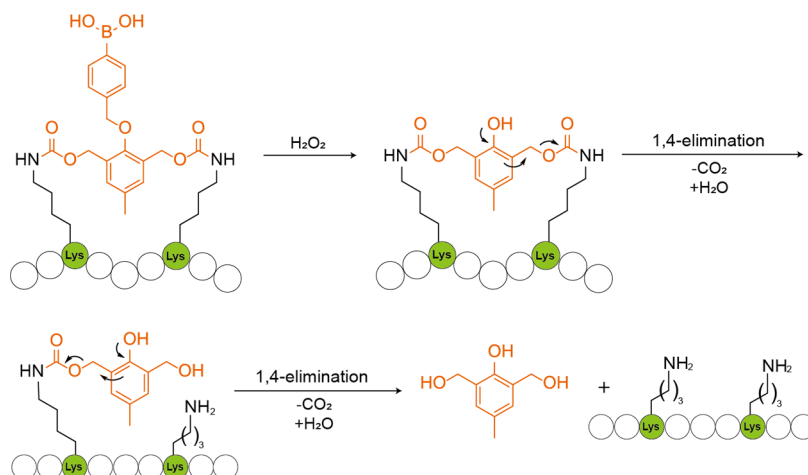
In recent years, an increasing number of researchers have dedicated themselves to developing aromatic ring coupling reagents with multiple functions, high reaction rates, and good stability. For example, a class of *N*-phenyl-divinyl sulfonamides, which can be inserted into disulfide bonds of peptides has been reported, and functional groups such as fluorophores and drug molecules can be also introduced into *N*-phenyl-divinyl sulfonamides via the hydroxyl group on the phenyl ring.¹¹ Wang and coworkers reported a highly chemically selective and bifunctional bioconjugation reagent 1,4-dinitroimidazoles (1,4-DNIMs) for peptide macrocyclization (Scheme 1A).¹² The conjugation between 1,4-DNIMs and cysteine thiols is achieved in seconds via the *cine*-substitution mechanism. The nucleophilic addition of thiol groups occurs on C-5 of 1,4-dinitroimidazoles with N1-nitro as the leaving group, and the obtained cysteine-(4-nitroimidazole) bond is significantly more stable than the commonly used maleimide conjugate. Lipka et al. used a variety of sulfone-activated pyridinium salts in a $25 \text{ }^\circ\text{C}$, $\text{pH} 7.0$ aqueous buffer to promote electrophilic cysteine arylation at extremely high rate constants ranging from 9800 to $320,000 \text{ M}^{-1} \cdot \text{s}^{-1}$,¹³ which exhibited excellent selectivity and allowed for ultra-rapid stoichiometric bioconjugation of micromolar cysteines (Scheme 1B). Gao et al. reported that the chlorooxime-cysteine conjugation had an apparent k_2 of $306 \pm 4 \text{ M}^{-1} \text{ s}^{-1}$,¹⁴ and utilized this rapid conjugation for Cys-Cys crosslinking by synthesizing bis-chlorooxime derivatives.

One year later, they applied this strategy to construct bicyclic peptides with a Cys-Cys-Lys, Lys-Lys-Cys, or Cys-Cys-*N*-terminus stapling pattern, which proceeded rapidly under physiological conditions (Scheme 1C).¹⁵ With stronger conformational rigidity and metabolic stability compared with linear or simple disulfide cyclic peptides, bicyclic peptides are demonstrated to have enormous potential for inhibiting PPIs. Another example is complex bicyclic peptides with high yield prepared by Nitsche et al. through a selective reaction of 1,2-aminothiols and 2,6-dicyanopyridine at the *N*-terminus of the peptide chain,¹⁶ which can be automated using standard solid-phase peptide synthesis. The bicyclic peptides exhibit conformational preorganization, plasma stability, and high target affinity (Scheme 1D).

Upon external stimuli such as light, heat, or chemical reactions, some cross-linking agents can release peptide chains to relieve the helical conformation and thus control the peptide activity. For example, Kumita et al. designed a short peptide crosslinked by a photoisomeric fragment of azobenzene.¹⁷ With appropriate wavelength and intensity of light, the isomerization of azobenzene crosslinkers from *trans*- to *cis*-form can be reversibly driven to control the conformation of the peptide. Zhang et al. increased the water solubility of the peptide by introducing sulfonic acid groups to azobenzene.¹⁸ However, this method cannot achieve a complete 100% transformation of azobenzene conformation. Brown et al. bridged two cysteines with dichlorotetrazine to construct cyclic peptides via solid-phase peptide synthesis.¹⁹ The restricted conformation is released upon the irradiation (355 or 410 nm) of *S,S*-tetrazine chromophore, generating two thiocyanates and molecular nitrogen on the ps time scale and realizing the local conformational recombination of peptides (Scheme 1E). Wan and coworkers introduced a light-mediated strategy based on 2-nitroveratryl (*oNv*), which served as a photocaging group and an oxidant after photolysis.²⁰ The irradiation of *oNv*-caged thiols with UV light releases free thiols, which are oxidized by side product nitrosoarene (Scheme 1F).

The chemical stimulus that unravels the ring structure mainly refers to the upregulated glutathione (GSH) or reactive oxygen species (ROS) within the tissues or cells. Pei et al. reported a reversible bicyclization strategy, which involved forming two disulfide bonds to transform the CPP-cargo fusion into a bicyclic structure.²¹ Outside the cell, highly restricted bicyclic peptides possess good membrane penetration and

Scheme 3. Reversible Lys-Lys Stapling via Dual 1,4-Elimination



proteolytic stability to make them pass through the cell membrane. After entering the cytosol, the S–S bonds are reduced by intracellular GSH and release the linear peptides with biological activity (Scheme 1G). Jiang et al. utilized a dual difunctional reaction between dialkynyls and phthalimidosulfonyl chloride to construct a reversible Cys-Cys linker.²² The difunctional reagent with dual leaving groups (Cl[−] and PhthN[−]) was easily obtainable. Additionally, the cleavage of disulfide bonds by GSH was achieved in vitro (Scheme 1H).

2.2. Lys-Lys Stapling. The nucleophilic lysine (Lys) is abundant in peptides and proteins, the frequency of which in vertebrates is 7.2%,² and it has good reactivity to achieve alkylation and arylation. Inouye and coworkers introduced a series of crosslinking agents, featuring acetylenic cores and oxyethylene spacers of different lengths.²³ These agents demonstrated a superior balance of rigidity and flexibility compared to traditional crosslinking agents, which are solely composed of alkyl or oxyethylene frameworks. Pentelute et al. reported a mild macrocyclization methodology by S_NAr on Lys residues of unprotected peptides.²⁴ The N-arylation of Lys is achieved via the perfluorosulfone (Scheme 2A) obtained by oxidation of the perfluorosulfide. An electron-withdrawing sulfonyl group introduced at the *para* position of the aromatic hydrocarbon causes the negative charge in the Meisenheimer complex to delocalize, thus effectively promoting S_NAr.

Positively charged arginines and lysines in antimicrobial peptide sequences can enhance membrane permeability, but lysine N-arylation can reduce the positive charge. To circumvent the effect of arylation on the charge, Zhang et al. stapled two lysine residues on the hydrophilic face of amphiphilic antimicrobial peptides through the N-alkylation reaction,²⁵ offering a chemical tool for the development of cationic antimicrobial peptides (CAMPs)-based antibiotics, which exhibit potent high resistance to proteolysis and antimicrobial activity.

In an initial study of the classic *ortho*-phthalaldehyde (OPA)-amine-thiol condensation reaction, the Chen group observed that under mild aqueous conditions, OPA could readily condense with two primary alkyl amines in the absence of thiols, yielding a type of isoindoline-1-imine compounds.²⁶ Based on the intramolecular OPA-2amine reaction, a methodology with high efficiency and selectivity was developed to staple two amino groups of the unprotected peptides. The macrocyclization reactions of the selected substrates could be

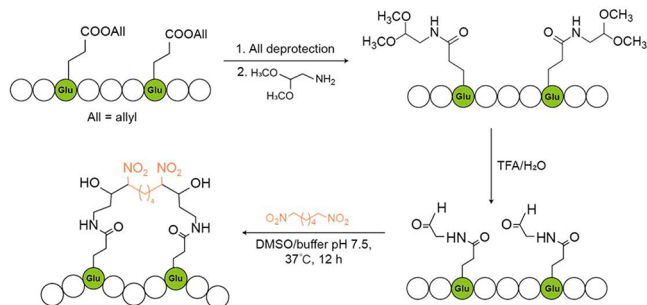
completed within 10 seconds at 5 mM concentration, and within 2 min at 50 μM concentration (Scheme 2B). In the same year, Chen et al. constructed complex macrocyclic peptides with novel topologies through three-component condensation reactions of primary amine, formaldehyde, and guanidine.²⁷ The macrocyclization reaction is feasible in both organic and aqueous media and demonstrates high selectivity and effectiveness (Scheme 2C).

The reversible peptide stapling has also come true on the Lysine-based cyclization. For example, the Wan group introduced a methodology comprising the Lys-Lys stapling and the decyclization facilitated by dual 1,4-elimination.²⁸ They achieved temporary cyclization of a peptide inhibitor targeting the lysine-specific demethylase 1 (LSD1) to bolster its stability and cell membrane permeability. Once inside the cell, the linear peptide with biological activity is released under a reductive environment (Scheme 3).

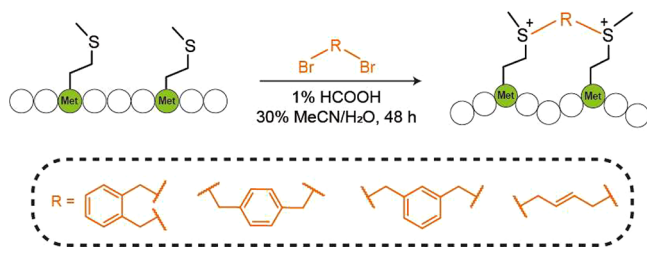
2.3. Glu-Glu Stapling. For the coupling between two glutamates (Glu), McDowell et al. initially utilized Boc-SPPS and connected them with suitable diaminoalkane as the linker, and the stapled peptide showed high helicity.²⁹ Pentelute et al. synthesized two γ-allyl esters-protected Glu residues on the resin, then selectively removed the protective groups, and formed two γ-hydrazide residues after hydrazination and cracking.³⁰ The C-terminal peptide hydrazine is oxidized by sodium nitrite to obtain the corresponding acyl azide, followed by Curtius rearrangement to produce isocyanates, which react with various external nucleophiles, such as hydrazine, aromatic thiols, and hydroxylamine. By stapling two glutamic acids rapidly through a reaction between nitroalkanes and aldehydes, Raj et al. demonstrated the efficiency and selectivity of nitroalkanes in bioconjugation reactions.³¹ These compounds can be easily incorporated into various proteins using chemical and biochemical methods (Scheme 4).

2.4. Met-Met Stapling. Methionine (Met) has a lower frequency (1.8%) relatively and weaker reactivity for stapling,² however, certain studies have explored the stapling of two Met residues. The Li group reported a reversible peptide stapling method via methionine bis-alkylation/dealkylation (Scheme 5), and the cyclic peptides could be reduced to release linear peptides in cellular environments.³² Two additional positive charges are generated during the dialkylation of Met, which can improve the cell membrane permeability of the stapling peptides. In addition, this method is compatible with all amino

Scheme 4. Glu-Glu Stapling Using Nitroalkanes as Linker



Scheme 5. Met-Met Stapling by Bis-alkylation



acids and different loop sizes. Subsequently, they formulated HBx-derived constrained peptides using this macrocyclization approach, which showed improved cell permeability and binding affinity.³³

2.5. Trp-Trp Stapling. Due to the low relative abundance of tryptophan (Trp) within protein structures (approximately 1%), Trp residues are suitable for modification, functionalization, and derivatization.² However, achieving activation of the aromatic scaffold of Trp under metal-free conditions is challenging. Mahalakshmi and Makwana utilized trifluoroacetic acid (TFA) to cleave the peptide from the resin and staple two

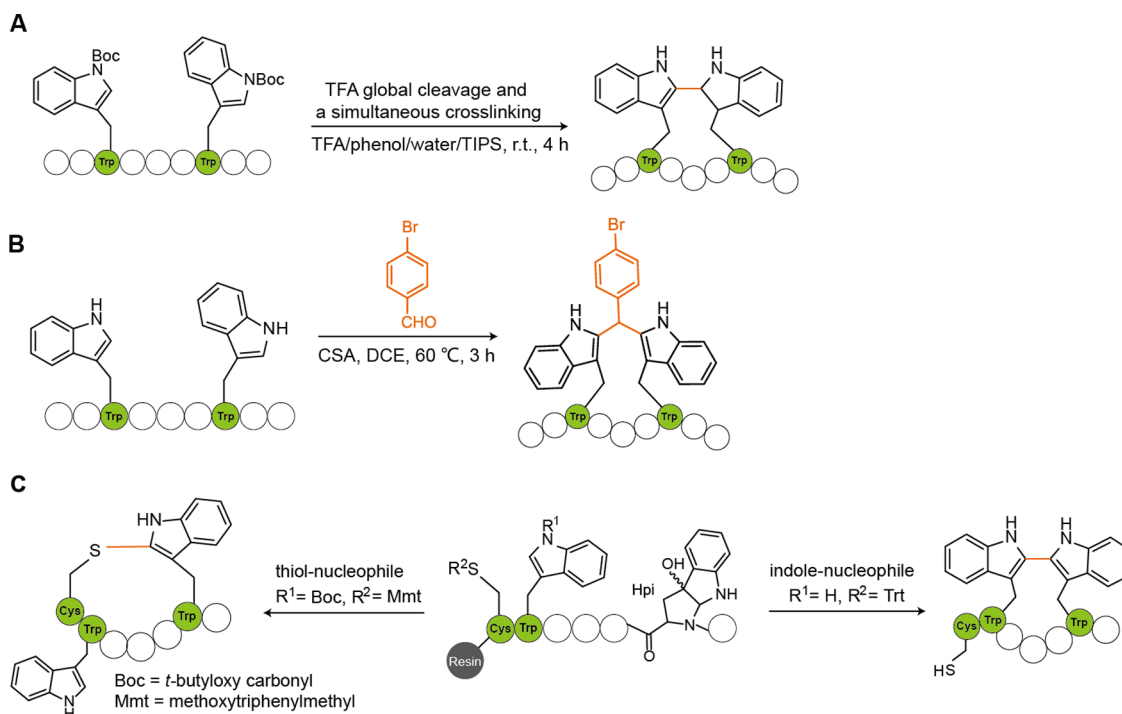
Trp residues of the peptide simultaneously (Scheme 6A).³⁴ Johannes et al. used an aldehyde via the acid-mediated concomitant condensation reaction to staple two Trp residues at the C2 position of the indole groups (Scheme 6B).³⁵ The Perrin lab recently disclosed a chemoselective stapling of the 5-hydroxypyrrroloindoline (Hpi) with either a cysteine-thiol or a tryptophan-indole to form a tryptathionine or 2,2'-bis-indole cross-link by using different protecting groups (Scheme 6C).³⁶

2.6. Tyr-Tyr Stapling. Zhang et al. developed a novel method for linking two tyrosines (Tyr) with 1,3,5-triazine-2-chloride and amines as cyclization reagents (Scheme 7).³⁷ By applying this method to staple the RGD peptide (an Arg-Gly-Asp peptide), the integrin-targeting ability and plasma stability of the peptide were significantly improved.

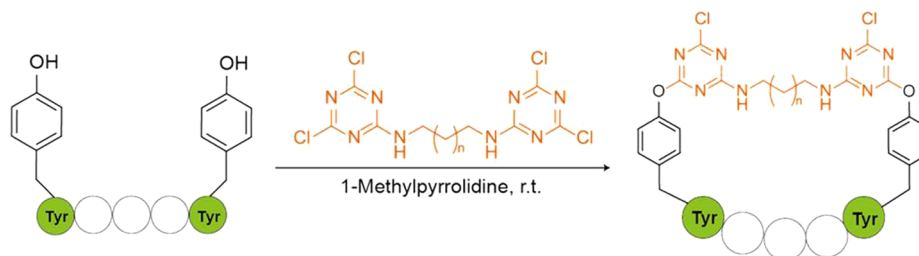
3. TWO DIFFERENT NATURAL AMINO ACIDS INCORPORATED STAPLING STRATEGIES

3.1. Lys-Asp Stapling. Coupling Glu or aspartic acid (Asp) with Lys represents one of the earliest stapling strategies through lactam formation. Although the natural abundance of Lys and Asp is high (7.2% and 5.9% respectively), different protective group strategies in peptide synthesis make specific cyclization possible.² Up to now, there have been three main strategies for Lys-Asp stapling: lactamization, coupling with linkers, and Ugi reaction. As early as 30 years ago, Felix et al. coupled Asp and Lys via lactamide bonds on Boc-SPPS,³⁸ and later prepared the cyclopeptides of double and three cycles.^{39–41} Yu and Taylor achieved the coupling of *i*, *i*+7 positions by dilactamization,⁴² and introduced the benzene ring into the linker to enhance rigidity and increase the helix stability of the peptides.⁴³ The comparison of multiple stapled peptides with linear peptides showed that lactam bonds could significantly enhance helix stability. The four-component Ugi reaction (Ugi-4CR) has been reported early for the head-to-tail cyclization of peptides, and the side chain-to-side chain

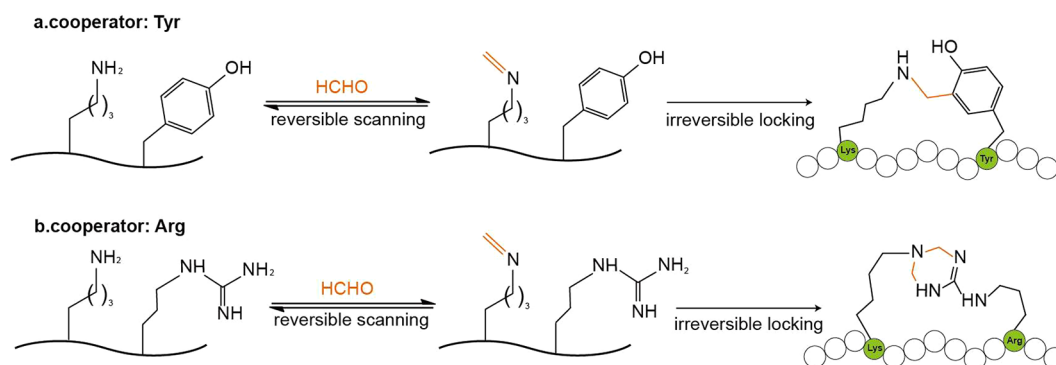
Scheme 6. Representative Examples of Trp-Trp Stapling Strategies



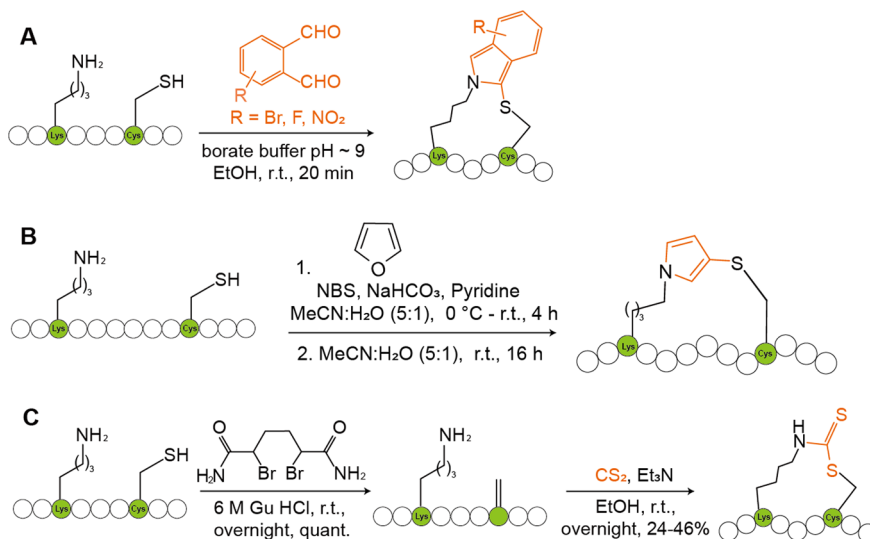
Scheme 7. Tyr-Tyr Stapling via Triazine



Scheme 8. Lys-Tyr/Arg Stapling with Formaldehyde



Scheme 9. Representative Examples of Cys-Lys Stapling Strategies



cyclization has followed closely on its heels.^{44–46} This multi-component reaction will be described in detail in Chapter 5.

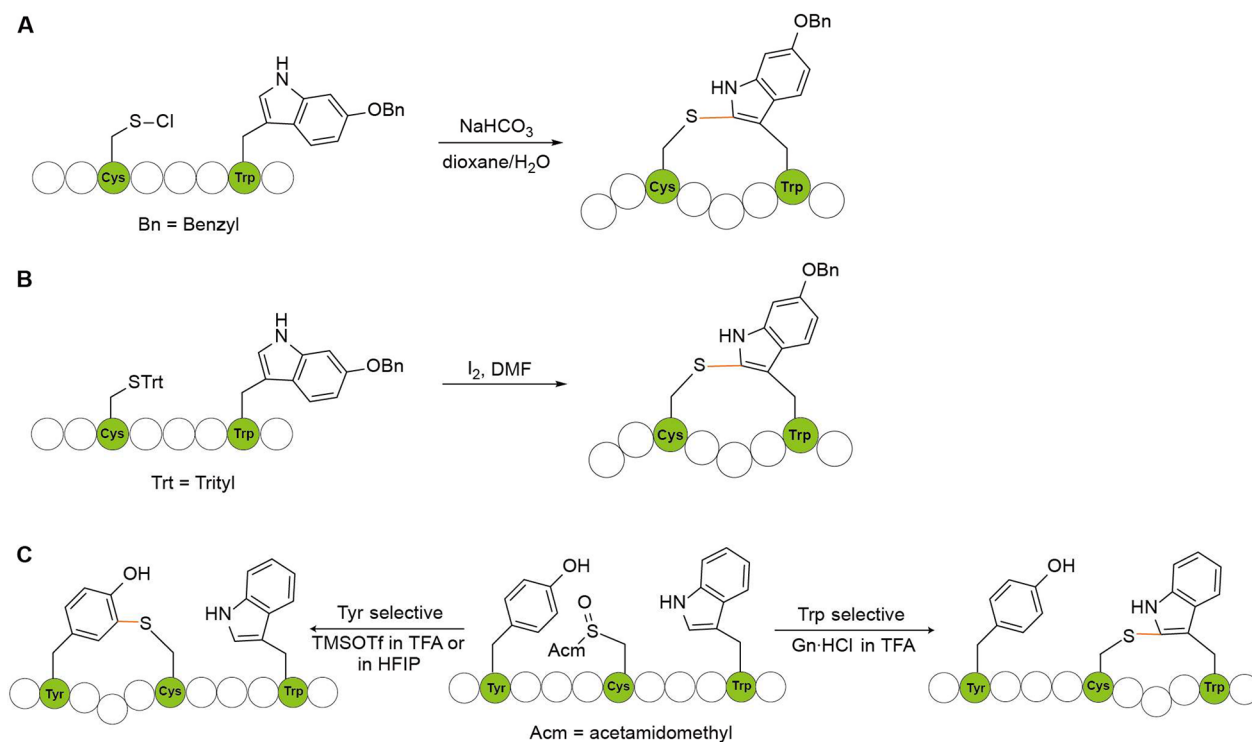
3.2. Lys-Tyr/Arg Stapling. Using an aldehyde to achieve intramolecular crosslinking of Lys with adjacent nucleophilic residues is a new method for stapling. Chen and his group stapled lysine residues and nearby tyrosine (Tyr) or arginine (Arg) residues with formaldehyde (Scheme 8).⁴⁷ The reactions are efficient and selective under mild conditions.

3.3. Cys-Lys Stapling. Brunel and Dawson presented a direct synthesis through thioether ligation between thiols and bromoacetyl groups, and the obtained stapled peptides showed greater homogeneity compared to the corresponding lactam peptides.⁴⁸ In 2019, Li's group and Perrin's group respectively employed the three-component reaction of OPA-amine-sulfhydryl to achieve Cys-Lys stapling (Scheme 9A).^{49,50}

Exogenous phthalaldehyde (OPA) can be linked to lysine and cysteine by imide formation, mercaptan addition, aromatization, and dehydration condensation with amino and mercaptan to form isoindole. Raj et al. reported another example of an aldehyde-mediated Cys-Lys stapling strategy through a Furan-Thiol-Amine (FuTine) reaction. The thiol and amine are linked through a furan-based electrophile to yield the pyrrole heterocycle (Scheme 9B).⁵¹

Lu and colleagues linked Lys (i) and Cys (i+4) of unprotected peptides based on the dithiocarbamate chemistry.⁵² The Cys residue is eliminated by oxidation, generating the corresponding dehydroalanine (DHA), which then reacts with the amino of Lys residue via the carbon disulfide (CS₂) (Scheme 9C). The Diels-Alder reaction has also been applied in Lys-Cys cyclization. Moellering et al. confirmed that Diels-

Scheme 10. Representative Examples of Cys-Trp/Tyr Stapling Strategies



Alder cyclization occurred rapidly with high yields in different diene-dienophile reactive pairs, and exhibited tunable stereochemical preferences in both solid-phase and aqueous solutions.⁵³ Waser et al. developed hypervalent iodine reagents for Cys-Cys or Cys-Lys stapling and produced various thioalkyne linkers.⁵⁴ Besides, the amidation of an activated ester or the cycloaddition onto the formed thioalkyne group could achieve the post-stapling modification.

3.4. Cys-Trp/Tyr Stapling. In nature, tryptathionine only exists among representatives of two death-cap toxins, amanitin, and phalloidin, which are produced by the death cap mushroom *Amanita phalloides*. Phallotoxin and amatoxin are toxic bicyclic cyclopeptides with a tryptathionine thioether bridge, and their synthesis has received wide attention.⁵⁵ A significant obstacle in synthesizing the bicyclic peptide structure of amanitin is the tryptathionine-based 6-hydroxy-tryptathionine-(*R*)-sulfoxide cross-link. There are several synthetic routes to staple the Cys and Trp. One way is utilizing the Savige-Fontana reaction^{56,57} by a 3a-fluoro-hexahydropyrrolo-[2,3-*b*]indoline (Fpi)-derivative or an *a*-hydroxy-pyrrolo[2,3-*b*]indole (Hpi)-derivative^{58–61} as the reactive Trp intermediate. Nevertheless, complex synthesis steps are needed to prepare suitable building blocks and intermediates in these methods. Another is utilizing an electrophilic reagent derived from cysteine, such as *S*-chlorocysteine,^{62,63} or *S*-iodocysteine,^{64–66} to achieve electrophilic substitution of the indole.

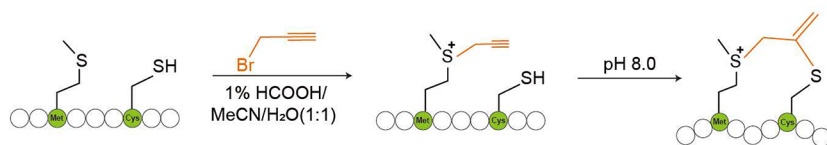
In 2018, the Perrin lab reported the first total synthesis of α -amanitin and conquered the major challenge of constructing the 6-hydroxy-tryptathionine sulfoxide bridge via a Savige-Fontana-type reaction.⁵⁹ To avoid harmful oxidants in the synthesis of Hpi/Fpi, Müller et al. developed a facile scalable synthetic route towards α -amanitin via a photochemical synthesis of the tryptathion precursor, solid-phase synthesis, and macrolactamization.⁶¹ In 2019, Süßmuth et al. presented

the first total synthesis of the toxin phalloidin based on Molecular-dynamics simulations (MD) calculations, and an enantioselective synthesis of (2*S*,4*R*)-4,5-dihydroxy-L-leucine (DHLeu).⁶⁵ Subsequently, they adopted a [5+1+2]-synthesis strategy, in which the thioether bond was performed, and achieved total synthesis of α -amanitin in the solution phase (Scheme 10A).⁶³ The iodine-mediated macrocyclization of Cys-/Trp-containing peptides was reported in a later study, leading to the total synthesis of α -amanitin and analogs (Scheme 10B).⁶⁶

The Akira Otaka group investigated the applicability of a novel umpolung electrophile: *S*-*p*-methoxybenzyl Cys sulfoxide (Cys(MBzl)(O)).⁶⁷ In the reaction between Cys(MBzl)(O) and Trp to form Cys-Trp thioether bonds, strong acids such as trifluoromethanesulfonic acid (TFMSA) or methanesulfonic acid (MSA) are required in the presence of guanidine hydrochloride (Gn-HCl) to activate sulfoxides, generating electrophilic Cys derivatives that bind to indole through aromatic electrophilic substitution. In a further research, they reported that *S*-Acetamidomethyl cysteine sulfoxide (Cys(Acm)(O)) could selectively make C-H sulfonylation of Tyr or Trp under suited acidic conditions (Scheme 10C).⁶⁸ The amide of the Acm group and the sulfoxide will achieve protonation by the acidic trimethylsilyl trifluoromethanesulfonate (TMSOTf), resulting in the formation of a dicationic species which selectively reacts with Tyr, whereas the *S*-chlorocysteine allows Trp-selective sulfonylation in the presence of Gn-HCl.

3.5. Cys-Met Stapling. After developing Met-Met linkage, Li et al. reported a macrocyclization method between methionine and cysteine via the reaction with di-alkylating reagents, such as 1,3-bis(bromomethyl)benzene.⁶⁹ Inspired by the specific reactivity of the sulfonium center, they developed another thiol-yne type reaction triggered by the sulfonium center.⁷⁰ After the propargylation of thioethers, the obtained

Scheme 11. Cys-Met Stapling through a Facile Thiol–Yne-Type Reaction



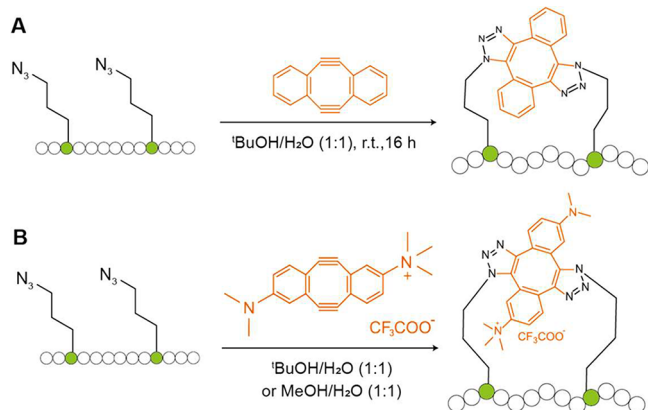
sulfonium can easily react with thiols at room temperature in an aqueous medium. The reaction was applied to staple adjacent Met and Cys in unprotected peptides, constructing cyclic peptides with better glutathione resistance and membrane permeability (Scheme 11).

4. NON-NATURAL AMINO ACIDS INCORPORATED STAPLING STRATEGIES

The utilization of natural amino acids as anchoring points is restricted due to the requirement for selective orthogonal protection. Hence, diverse non-natural amino acids-involved stapling strategies have been designed. Among these strategies, the RCM and click orthogonal reactions are the most extensively employed. Since RCM involves metal-catalyzed activation of C–H bonds, it is not within the scope of this paper. The Cu^I-catalyzed azide–alkyne 1,3-dipolar Huisgen cyclo-addition (CuAAC) is a typical representative of the click reaction, which requires the formation of *ω*-azido- and *ω*-yl- α -amino acids to form triazole ligation with metal Cu^I. However, through the exploration and optimization of reaction conditions, an eco-friendly route of metal-free catalysis has been discovered, which will be elaborated upon in the subsequent section.

4.1. SPAAC. Strain-promoted azide–alkyne cycloaddition (SPAAC) can be extensively applied in biological systems as a substitute for CuAAC due to the metal-free stapling technique. Its core lies in the utilization of a high-energy, strained cyclooctyne molecule as the driving force for the reaction. In 2015, SPAAC was reported by Spring and coworkers as a novel stapling technique based on a Sondheimer dialkyne (Scheme 12A).⁷¹ This in situ approach

Scheme 12. Improvement of Linker in SPAAC Stapling



can be performed directly in cell culture and allows screening for cell-active stapled peptides in a parallel high-throughput format. However, the biological application of the Sondheimer dialkyne is limited by poor water solubility and aqueous stability ($\tau_{1/2} \sim 10$ min at pH = 7.4).⁷² To improve the above inherent shortcomings of the dialkyne, *meta*-trimethylammo-

nium substituted Sondheimer dialkyne was developed with better water solubility, stability, and azide-reactivity (Scheme 12B).⁷³ In another experiment, Spring et al. synthesized a cross-linking reagent featuring a diarylethene (DAE) core and two strained cyclooctenes.⁷⁴ As a result of the additional strain imposed on the cyclooctyne rings by the fused thiophene rings, the cyclohexene ring exhibits a high degree of reactivity towards azides.

4.2. Sec-Sec Stapling. In comparison to cysteine, selenocysteine (Sec) demonstrates higher reactivity, making rapid Sec-Sec stapling of unprotected peptides under mild aqueous conditions possible. Sec has a higher side chain acidity than Cys and is more likely to react with electrophilic alkanes ($pK_{a(\text{Sec})} = 5.2\text{--}5.6$; $pK_{a(\text{Cys})} = 8.2$).⁷⁵ Fairlie et al. discussed the ability of linkers with diverse sizes, rigidity, and hydrophobicity to staple two Sec residues.⁷⁶

4.3. Mpc-Mpc Stapling. To modify the triple helix structure of collagen (Pro-Hyp-Gly)_n peptide, Renner et al. incorporated (2*S*,4*S*)-mercaptoproline (Mpc), an unnatural amino acid containing sulfhydryl, into the peptide sequences and linked it with a diiodo azobenzene derivative.⁷⁷ The stability of the triple helix structure is regulated by the light-controlled *cis-trans* isomer of the azobenzene-derived chromophore.

4.4. Others. Fluoroacetamide as an unnatural amino acid side chain is also used in stapling strategies (Scheme 13A).⁷⁸ With benzenedimethanethiol linkers, the fluorine-thiol displacement reaction (FTDR) could selectively proceed in the presence of intrinsic cysteines. The results showed that the cellular uptake capacity of peptide analogs stapled by FTDR was 3 to 9 times higher than that of peptide analogs stapled by hydrocarbons.

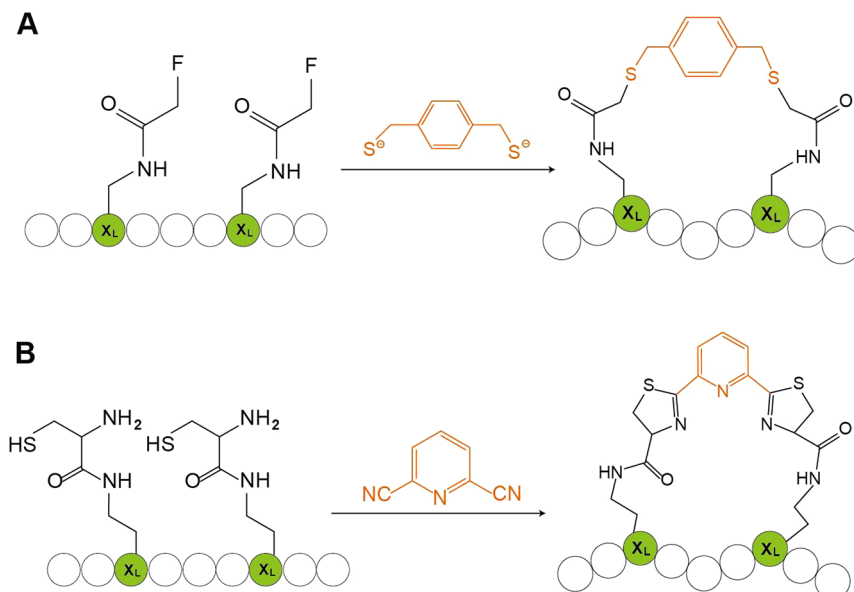
Nitsche and Morewood introduced a biocompatible stapling strategy utilizing the reagent 2,6-dicyanopyridine and a pseudo-cysteine amino acid (Scheme 13B).⁷⁹ This coupling reaction undergoes at physiological pH in aqueous solution and is orthogonal to side chains of natural amino acid, allowing it to be directly used for biochemical analysis.

5. MULTICOMPONENT PEPTIDE STAPLED REACTIONS

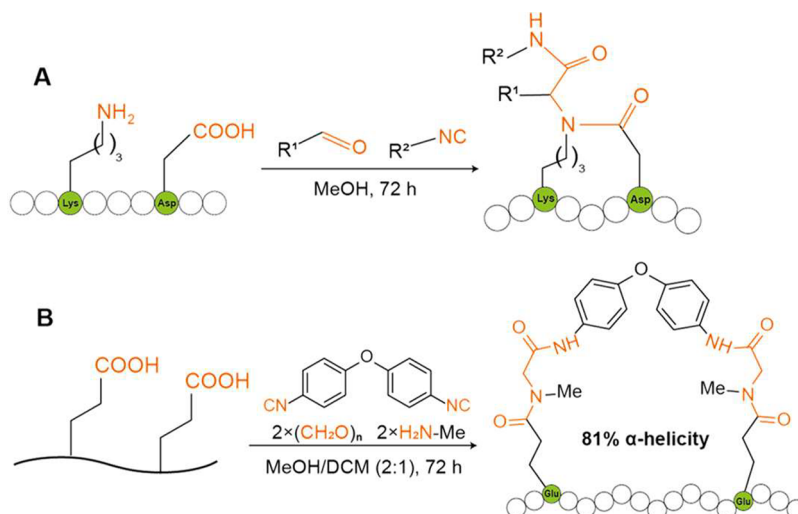
Multicomponent reactions (MCR) introduce conformational constraints in short and medium-sized peptide sequences while allowing for diverse skeletal modifications of additional components as needed during the cyclization process.⁴⁴ MCR strategies include three-components reactions such as Strecker,⁸⁰ Passerini,⁸¹ A³-coupling,⁸² Petasis,^{83,84} and the Ugi four-component reaction.^{45,85,86} The latter two, which are the most popular MCR strategies, will be discussed in this chapter.

5.1. Ugi-4CR. The Ugi four-component reaction (Ugi-4CR), featuring the condensation of a primary amine, an aldehyde, a carboxylic acid, and an isonitrile to produce an α -aminoacyl amide, has been widely applied in various fields of chemistry.⁸⁷ In 2015, Rivera demonstrated that Ugi-4CR can be applied for peptide side chains stapling, resulting in cyclic

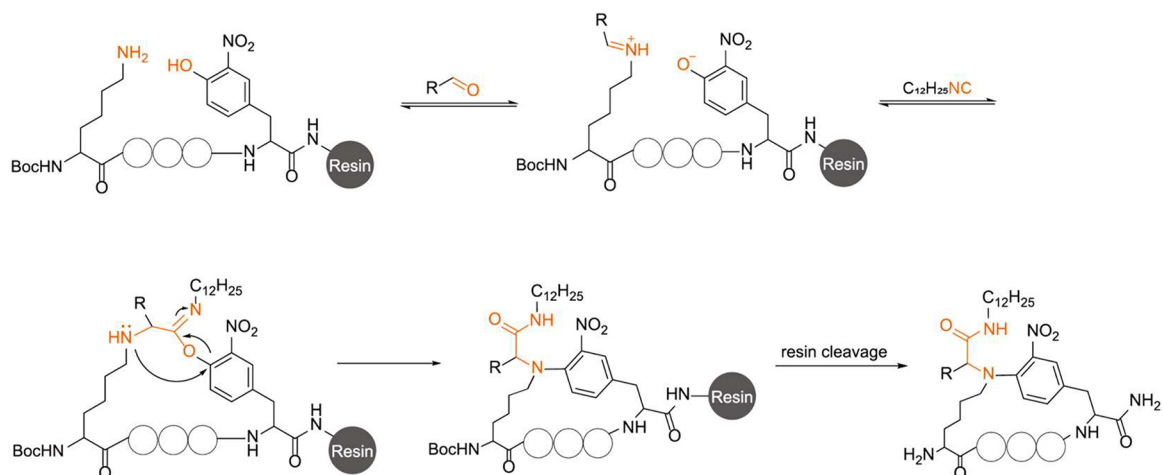
Scheme 13. Representative Non-natural Amino Acids-Involved Stapling Strategies



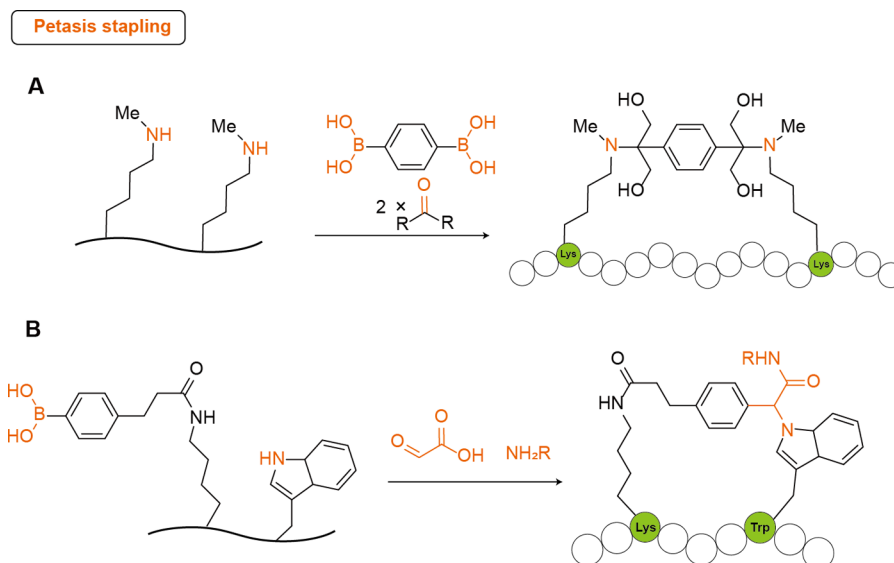
Scheme 14. Ugi Four-Component Reaction (Ugi-4CR) for Peptide Stapling



Scheme 15. Ugi-Smiles Using Phenol Instead of Carboxylic Acid



Scheme 16. Petasis Reaction for Peptide Stapling



peptides with a tertiary lactam bridge (Scheme 14A).⁴⁵ Afterwards, Rivera and coworkers completed the peptide macrocyclization based on the Ugi reaction on resin for the first time.⁸⁶ The isocyanide used in the Ugi-4CR stapling strategy allows for the modification of sugars, lipids, fluorescent labels, and other components. Besides, they explored the conformation of various stapling combinations, such as the diacid/diisocyanide, the diacid/diamine, and the diamine/diisocyanide combination, to find the applied range of double Ugi multicomponent stapling approach (Scheme 14B).⁸⁸ The research showed that the type of bifunctional combination, as well as the properties and flexibility of the linker, could affect the helicity of any structural conformation. Bernardes and coworkers combined the isonitrile-tetrazine (4+1) cycloaddition and the Ugi-4CR to produce pyrazole amide derivatives and used them as a tool for peptide modification and stapling.⁸⁷

Ugi-Smiles is a derivative reaction of Ugi-4CR, using 2-nitrophenol or 4-nitrophenol as an electron-deficient phenol instead of the carboxylic acid component in conventional Ugi-4CR, to condense with primary amines, aldehydes, and isocyanides to produce tertiary nitroaniline. The application of Ugi-Smiles to Lys-Tyr stapling can also introduce functional groups through isocyanides (Scheme 15).⁸⁹

5.2. Petasis Reaction. The Petasis reaction involving aldehydes, amines, and boric acids, exhibits good functional group tolerance and compatibility under physiological conditions. Rivera's group pioneered applying the Petasis reaction in peptide labeling and stapling (Scheme 16A).⁸³ This method not only produces stapled peptides with rigid aromatic linkages but also allows for the derivatization of peptides by incorporating sugars, fluorescent labels, steroids, lipids, etc.

Considering that the tryptophan residue is an amine-containing heterocycle, Krajcovicova and Spring combined the Petasis reaction with Trp modification and proposed a peptide stapling method suitable for solution and solid phase (Scheme 16B).⁹⁰ The cyclization exhibits strong functional group tolerance, suitable for peptides containing a C-terminal acid and an amide. Before this, the application of tryptophan in peptide stapling was mainly limited to transition metal-

catalyzed reactions, photochemical C–H activation, or benzaldehyde bridge condensation at the C2 position.⁹⁰

6. CONCLUSION

In conclusion, peptide stapling stands out as one of the most effective methods to control the conformation of peptides. This technique enables the adjustable constraint of the peptides' secondary structure, thereby significantly enhancing stability, membrane permeability, and resistance to enzymatic degradation. By coupling specific amino acid residues through appropriate orthogonal reactions and meticulously constructing linkers, peptide stability is achieved. Additionally, the incorporation of nonproteogenic amino acids into the peptide chains enhances the selectivity of cyclization reactions. Throughout this process, various conjugation strategies have emerged, offering substantial potential for investigating protein interactions, identifying drug targets, and developing novel drug candidates. These advancements demonstrate significant promise for widespread application in biomedical and chemical fields.

AUTHOR INFORMATION

Corresponding Author

Chengxi Li – College of Chemical and Biological Engineering, Zhejiang University, Hangzhou, Zhejiang 310030, China; Engineering Research Center of Functional Materials Intelligent Manufacturing of Zhejiang Province, ZJU-Hangzhou Global Scientific and Technological Innovation Center, Hangzhou 311215, China; orcid.org/0000-0003-3904-0299; Email: cxli@zju.edu.cn

Authors

Wanglin Zhan – College of Chemical and Biological Engineering, Zhejiang University, Hangzhou, Zhejiang 310030, China; Engineering Research Center of Functional Materials Intelligent Manufacturing of Zhejiang Province, ZJU-Hangzhou Global Scientific and Technological Innovation Center, Hangzhou 311215, China

Hongliang Duan – Faculty of Applied Sciences, Macao Polytechnic University, Macao 999078, China; orcid.org/0000-0002-9194-0115

Complete contact information is available at:
<https://pubs.acs.org/10.1021/cbe.3c00123>

Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Lazo, J. S.; Sharlow, E. R. Drugging Undruggable Molecular Cancer Targets. *Annu. Rev. Pharmacol. Toxicol.* **2016**, *56*, 23–40.
- (2) Li, X.; Chen, S.; Zhang, W.-D.; Hu, H.-G. Stapled Helical Peptides Bearing Different Anchoring Residues. *Chem. Rev.* **2020**, *120* (18), 10079–10144.
- (3) Rezaei, T.; Yu, B.; Millhauser, G. L.; Jacobson, M. P.; Lokey, R. S. Testing the Conformational Hypothesis of Passive Membrane Permeability Using Synthetic Cyclic Peptide Diastereomers. *J. Am. Chem. Soc.* **2006**, *128* (8), 2510–2511.
- (4) Moulahoum, H.; Ghorbanizamani, F.; Tok, K.; Zihnioglu, F. On the Cyclization of Non-cyclic Peptides for Biological Applications: Inspiration from Naturally Cyclic Peptides. *Chem. Select* **2023**, *8* (37), No. e202301335.
- (5) Vinogradov, A. A.; Yin, Y. Z.; Suga, H. Macrocytic Peptides as Drug Candidates: Recent Progress and Remaining Challenges. *J. Am. Chem. Soc.* **2019**, *141* (10), 4167–4181.
- (6) Wang, Y.; Chou, D. H.-C. A Thiol-Ene Coupling Approach to Native Peptide Stapling and Macrocyclization. *Angew. Chem. Int. Ed.* **2015**, *54* (37), 10931–10934.
- (7) Timmerman, P.; Beld, J.; Puijk, W. C.; Meloen, R. H. Rapid and Quantitative Cyclization of Multiple Peptide Loops onto Synthetic Scaffolds for Structural Mimicry of Protein Surfaces. *ChemBioChem.* **2005**, *6* (5), 821–824.
- (8) Muppidi, A.; Wang, Z.; Li, X.; Chen, J.; Lin, Q. Achieving Cell Penetration with Distance-Matching Cysteine Cross-Linkers: a Facile Route to Cell-Permeable Peptide Dual Inhibitors of Mdm2/Mdmx. *Chem. Commun.* **2011**, *47* (33), 9396–9398.
- (9) Spokoiny, A. M.; Zou, Y.; Ling, J. J.; Yu, H.; Lin, Y.-S.; Pentelute, B. L. A Perfluoroaryl-Cysteine S_NAr Chemistry Approach to Unprotected Peptide Stapling. *J. Am. Chem. Soc.* **2013**, *135* (16), 5946–5949.
- (10) Kalthor-Monfared, S.; Jafari, M. R.; Patterson, J. T.; Kitov, P. I.; Dwyer, J. J.; Nuss, J. M.; Derda, R. Rapid Biocompatible Macrocytic Cyclization of Peptides with Decafluoro-Diphenylsulfone. *Chem. Sci.* **2016**, *7* (6), 3785–3790.
- (11) Li, Z.; Huang, R.; Xu, H.; Chen, J.; Zhan, Y.; Zhou, X.; Chen, H.; Jiang, B. Divinylsulfonamides as Specific Linkers for Stapling Disulfide Bonds in Peptides. *Org. Lett.* **2017**, *19* (18), 4972–4975.
- (12) Luo, Q.; Tao, Y.; Sheng, W.; Lu, J.; Wang, H. Dinitroimidazoles as Bifunctional Bioconjugation Reagents for Protein Functionalization and Peptide Macrocytic Cyclization. *Nat. Commun.* **2019**, *10* (1), 142–150.
- (13) Lipka, B. M.; Honeycutt, D. S.; Bassett, G. M.; Kowal, T. N.; Adamczyk, M.; Cartnick, Z. C.; Betti, V. M.; Goldberg, J. M.; Wang, F. Ultra-rapid Electrophilic Cysteine Arylation. *J. Am. Chem. Soc.* **2023**, *145* (43), 23427–23432.
- (14) Chen, F.-J.; Zheng, M.; Nobile, V.; Gao, J. Fast and Cysteine-Specific Modification of Peptides, Proteins and Bacteriophage Using Chlorooximes. *Chem.—Eur. J.* **2022**, *28* (20), No. e202200058.
- (15) Chen, F.-J.; Pinnette, N.; Yang, F.; Gao, J. A Cysteine-Directed Proximity-Driven Crosslinking Method for Native Peptide Bicyclization. *Angew. Chem. Int. Ed.* **2023**, *62* (31), No. e202306813.
- (16) Ullrich, S.; George, J.; Coram, A.; Morewood, R.; Nitsche, C. Biocompatible and Selective Generation of Bicyclic Peptides. *Angew. Chem. Int. Ed.* **2022**, *61* (43), No. e202208400.
- (17) Kumita, J. R.; Smart, O. S.; Woolley, G. A. Photo-Control of Helix Content in a Short Peptide. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (8), 3803–3808.
- (18) Zhang, Z.; Burns, D. C.; Kumita, J. R.; Smart, O. S.; Woolley, G. A. A Water-Soluble Azobenzene Cross-Linker for Photocontrol of Peptide Conformation. *Bioconjugate Chem.* **2003**, *14* (4), 824–829.
- (19) Brown, S. P.; Smith, A. B. III, Peptide/Protein Stapling and Unstapling: Introduction of *s*-Tetrazine, Photochemical Release, and Regeneration of the Peptide/Protein. *J. Am. Chem. Soc.* **2015**, *137* (12), 4034–4037.
- (20) He, F.; Chai, Y.; Zeng, Z. Z.; Lu, F. L.; Chen, H. W.; Zhu, J. H.; Fang, Y. Y.; Cheng, K. G.; Miclet, E.; Alezra, V.; Wan, Y. Rapid Formation of Intramolecular Disulfide Bridges using Light: An Efficient Method to Control the Conformation and Function of Bioactive Peptides. *J. Am. Chem. Soc.* **2023**, *145* (41), 22639–22648.
- (21) Qian, Z. Q.; Rhodes, C. A.; McCroskey, L. C.; Wen, J.; Appiah-Kubi, G.; Wang, D. J.; Guttridge, D. C.; Pei, D. H. Enhancing the Cell Permeability and Metabolic Stability of Peptidyl Drugs by Reversible Bicyclization. *Angew. Chem. Int. Ed.* **2017**, *56* (6), 1525–1529.
- (22) Yu, Q.; Bai, L.; Jiang, X. Disulfide Click Reaction for Stapling of S-terminal Peptides. *Angew. Chem. Int. Ed.* **2023**, *62*, No. e202314379.
- (23) Fujimoto, K.; Oimoto, N.; Katsuno, K.; Inouye, M. Effective Stabilisation of α -Helical Structures in Short Peptides with Acetylenic Cross-Linking Agents. *Chem. Commun.* **2004**, No. 11, 1280–1281.
- (24) Lautrette, G.; Touti, F.; Lee, H. G.; Dai, P.; Pentelute, B. L. Nitrogen Arylation for Macrocytic Cyclization of Unprotected Peptides. *J. Am. Chem. Soc.* **2016**, *138* (27), 8340–8343.
- (25) Li, H.; Hu, Y.; Pu, Q.; He, T.; Zhang, Q.; Wu, W.; Xia, X.; Zhang, J. Novel Stapling by Lysine Tethering Provides Stable and Low Hemolytic Cationic Antimicrobial Peptides. *J. Med. Chem.* **2020**, *63* (8), 4081–4089.
- (26) Li, B.; Wang, L.; Chen, X. X.; Chu, X.; Tang, H.; Zhang, J.; He, G.; Li, L.; Chen, G. Extendable Stapling of Unprotected Peptides by Crosslinking Two Amines with *o*-Phthalaldehyde. *Nat. Commun.* **2022**, *13* (1), 311–319.
- (27) Li, B.; Wan, Z.; Zheng, H.; Cai, S.; Tian, H.-W.; Tang, H.; Chu, X.; He, G.; Guo, D.-S.; Xue, X.-S.; Chen, G. Construction of Complex Macromulticyclic Peptides via Stitching with Formaldehyde and Guanidine. *J. Am. Chem. Soc.* **2022**, *144* (22), 10080–10090.
- (28) Zeng, Z.; Zhu, J.; Deng, X.; Chen, H.; Jin, Y.; Miclet, E.; Alezra, V.; Wan, Y. Customized Reversible Stapling for Selective Delivery of Bioactive Peptides. *J. Am. Chem. Soc.* **2022**, *144* (51), 23614–23621.
- (29) Phelan, J. C.; Skelton, N. J.; Braisted, A. C.; McDowell, R. S. A General Method for Constraining Short Peptides to an Alpha-Helical Conformation. *J. Am. Chem. Soc.* **1997**, *119* (3), 455–460.
- (30) Vinogradov, A. A.; Choo, Z.-N.; Totaro, K. A.; Pentelute, B. L. Macrocytic Cyclization of Unprotected Peptide Isocyanates. *Org. Lett.* **2016**, *18* (6), 1226–1229.
- (31) Mahesh, S.; Adebomi, V.; Muneeswaran, Z. P.; Raj, M. Bioinspired Nitroalkylation for Selective Protein Modification and Peptide Stapling. *Angew. Chem. Int. Ed.* **2020**, *59* (7), 2793–2801.
- (32) Shi, X.; Zhao, R.; Jiang, Y.; Zhao, H.; Tian, Y.; Jiang, Y.; Li, J.; Qin, W.; Yin, F.; Li, Z. Reversible Stapling of Unprotected Peptides via Chemoselective Methionine Bis-Alkylation/Dealkylation. *Chem. Sci.* **2018**, *9* (12), 3227–3232.
- (33) Cai, X. D.; Zheng, W. H.; Shi, X. D.; Chen, L. J.; Liu, Z. H.; Li, Z. G. HBx-Derived Constrained Peptides Inhibit the Secretion of Hepatitis B Virus Antigens. *Mol. Pharmaceutics* **2018**, *15* (12), 5646–5652.
- (34) Makwana, K. M.; Mahalakshmi, R. Trp-Trp Cross-Linking: A Structure-Reactivity Relationship in the Formation and Design of Hyperstable Peptide β -Hairpin and α -Helix Scaffolds. *Org. Lett.* **2015**, *17* (10), 2498–2501.
- (35) Hui, E. Y. L.; Rout, B.; Tan, Y. S.; Verma, C. S.; Chan, K. P.; Johannes, C. W. An Intramolecular Tryptophan-Condensation Approach for Peptide Stapling. *Org. Biomol. Chem.* **2018**, *16* (3), 389–392.
- (36) Todorovic, M.; Blanc, A.; Wang, Z.; Lozada, J.; Froelich, J.; Zeisler, J.; Zhang, C.; Merckens, H.; Benard, F.; Perrin, D. M. S-

Hydroxypyrrroloindoline Affords Tryptathionine and 2,2'-bis-Indole Peptide Staples: Application to Melanotan-II. *Chem.—Eur. J.* **2024**, *30*, No. e202304270.

(37) Zhang, Y.; Yin, R.; Jiang, H.; Wang, C.; Wang, X.; Wang, D.; Zhang, K.; Yu, R.; Li, X.; Jiang, T. Peptide Stapling through Site-Directed Conjugation of Triazine Moieties to the Tyrosine Residues of a Peptide. *Org. Lett.* **2023**, *25* (13), 2248–2252.

(38) Felix, A. M.; Heimer, E. P.; Wang, C.-T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toomey, V.; Fry, D.; Madison, V. S. Synthesis, Biological Activity and Conformational Analysis of Cyclic GRF Analogs. *Int. J. Pept. Protein Res.* **1988**, *32* (6), 441–454.

(39) Shepherd, N. E.; Abbenante, G.; Fairlie, D. P. Consecutive Cyclic Pentapeptide Modules form Short α -Helices that are Very Stable to Water and Denaturants. *Angew. Chem. Int. Ed.* **2004**, *43* (20), 2687–2690.

(40) Osapay, G.; Taylor, J. W. Multicyclic Polypeptide Model Compounds. 2. Synthesis and Conformational Properties of a Highly Alpha-Helical Uncosapeptide Constrained by Three Side-Chain to Side-Chain Lactam Bridges. *J. Am. Chem. Soc.* **1992**, *114* (18), 6966–6973.

(41) Osapay, G.; Taylor, J. W. Multicyclic Polypeptide Model Compounds. 1. Synthesis of a Tricyclic Amphiphilic Alpha-Helical Peptide Using an Oxime Resin, Segment-Condensation Approach. *J. Am. Chem. Soc.* **1990**, *112* (16), 6046–6051.

(42) Yu, C.; Taylor, J. W. A New Strategy Applied to the Synthesis of an α -Helical Bicyclic Peptide Constrained by Two Overlapping i, i+7 Side-Chain Bridges of Novel Design. *Tetrahedron Lett.* **1996**, *37* (11), 1731–1734.

(43) Yu, C.; Taylor, J. W. Synthesis and Study of Peptides with Semirigid i and i+7 Side-Chain Bridges Designed for α -Helix Stabilization. *Biorg. Med. Chem.* **1999**, *7* (1), 161–175.

(44) Reguera, L.; Rivera, D. G. Multicomponent Reaction Toolbox for Peptide Macrocyclization and Stapling. *Chem. Rev.* **2019**, *119* (17), 9836–9860.

(45) Vasco, A. V.; Pérez, C. S.; Morales, F. E.; Garay, H. E.; Vasilev, D.; Gavín, J. A.; Wessjohann, L. A.; Rivera, D. G. Macrocyclization of Peptide Side Chains by the Ugi Reaction: Achieving Peptide Folding and Exocyclic N-Functionalization in One Shot. *J. Org. Chem.* **2015**, *80* (13), 6697–6707.

(46) Morales, F. E.; Garay, H. E.; Muñoz, D. F.; Augusto, Y. E.; Otero-González, A. J.; Acosta, O. R.; Rivera, D. G. Aminocatalysis-Mediated on-Resin Ugi Reactions: Application in the Solid-Phase Synthesis of N-Substituted and Tetrazolo Lipopeptides and Peptidosteroids. *Org. Lett.* **2015**, *17* (11), 2728–2731.

(47) Li, B.; Tang, H.; Turlik, A.; Wan, Z.; Xue, X.-S.; Li, L.; Yang, X.; Li, J.; He, G.; Houk, K. N.; Chen, G. Cooperative Stapling of Native Peptides at Lysine and Tyrosine or Arginine with Formaldehyde. *Angew. Chem. Int. Ed.* **2021**, *60* (12), 6646–6652.

(48) Brunel, F. M.; Dawson, P. E. Synthesis of Constrained Helical Peptides by Thioether Ligation: Application to Analogs of Gp41. *Chem. Commun.* **2005**, No. 20, 2552–2554.

(49) Zhang, Y.; Zhang, Q.; Wong, C. T. T.; Li, X. Chemoselective Peptide Cyclization and Bicyclization Directly on Unprotected Peptides. *J. Am. Chem. Soc.* **2019**, *141* (31), 12274–12279.

(50) Todorovic, M.; Schwab, K. D.; Zeisler, J.; Zhang, C.; Bénard, F.; Perrin, D. M. Fluorescent Isoindole Crosslink (FLiCK) Chemistry: A Rapid, User-friendly Stapling Reaction. *Angew. Chem. Int. Ed.* **2019**, *58* (40), 14120–14124.

(51) Wang, Y.; Czabala, P.; Raj, M. Bioinspired one-pot furan-thiol-amine multicomponent reaction for making heterocycles and its applications. *Nat. Commun.* **2023**, *14* (1), 4086–4100.

(52) Li, X.; Tolbert, W. D.; Hu, H. G.; Gohain, N.; Zou, Y.; Niu, F.; He, W. X.; Yuan, W. R.; Su, J. C.; Pazgier, M.; Lu, W. Y. Dithiocarbamate-Inspired Side Chain Stapling Chemistry for Peptide Drug Design. *Chem. Sci.* **2019**, *10* (5), 1522–1530.

(53) Montgomery, J. E.; Donnelly, J. A.; Fanning, S. W.; Speltz, T. E.; Shangquan, X. H.; Coukos, J. S.; Greene, G. L.; Moellering, R. E.

Versatile Peptide Macrocyclization with Diels-Alder Cycloadditions. *J. Am. Chem. Soc.* **2019**, *141* (41), 16374–16381.

(54) Ceballos, J.; Grinhagena, E.; Sangouard, G.; Heinis, C.; Waser, J. Cys-Cys and Cys-Lys Stapling of Unprotected Peptides Enabled by Hypervalent Iodine Reagents. *Angew. Chem. Int. Ed.* **2021**, *60* (16), 9022–9031.

(55) Smolyar, I. V.; Yudin, A. K.; Nenajdenko, V. G. Heteroaryl Rings in Peptide Macrocycles. *Chem. Rev.* **2019**, *119* (17), 10032–10240.

(56) Savige, W. E.; Fontana, A. New method of linking tryptophan to cysteine sulphhydryl groups in peptides and proteins. *Chem. Commun.* **1976**, No. 15, 600–601.

(57) Savige, W. E.; Fontana, A. Novel Synthesis of 2-Thioether Derivatives of Tryptophan-Covalent Binding of Tryptophan to Cysteine Sulphydryl Groups in Peptides and Proteins. *Int. J. Pept. Protein Res.* **1980**, *15* (2), 102–112.

(58) May, J. P.; Fournier, P.; Pellicelli, J.; Patrick, B. O.; Perrin, D. M. High Yielding Synthesis of 3 α -Hydroxypyrrrolo[2,3-*b*]indoline Dipeptide Methyl Esters: Synthons for Expedient Introduction of the Hydroxypyrrroloindoline Moiety into Larger Peptide-Based Natural Products and for the Creation of Tryptathionine Bridges. *J. Org. Chem.* **2005**, *70* (21), 8424–8430.

(59) Matinkhoo, K.; Pryyma, A.; Todorovic, M.; Patrick, B. O.; Perrin, D. M. Synthesis of the Death-Cap Mushroom Toxin α -Amanitin. *J. Am. Chem. Soc.* **2018**, *140* (21), 6513–6517.

(60) Pryyma, A.; Bu, Y. J.; Wai, Y.; Patrick, B. O.; Perrin, D. M. Synthesis and Activation of Bench-Stable 3 α -Fluoropyrrroloindolines as Latent Electrophiles for the Synthesis of C-2-Thiol-Substituted Tryptophans and C-3 α -Substituted Pyrroloindolines. *Org. Lett.* **2019**, *21* (20), 8234–8238.

(61) Lutz, C.; Simon, W.; Werner-Simon, S.; Pahl, A.; Müller, C. Total Synthesis of α - and β -Amanitin. *Angew. Chem. Int. Ed.* **2020**, *59* (28), 11390–11393.

(62) Anderson, M. O.; Shelat, A. A.; Guy, R. K. A Solid-Phase Approach to the Phallotoxins: Total Synthesis of [Ala⁷]-Phalloidin. *J. Org. Chem.* **2005**, *70* (12), 4578–4584.

(63) Siegert, M.-A. J.; Knittel, C. H.; Süßmuth, R. D. A Convergent Total Synthesis of the Death Cap Toxin α -Amanitin. *Angew. Chem. Int. Ed.* **2020**, *59* (14), 5500–5504.

(64) Schuresko, L. A.; Lokey, R. S. A Practical Solid-Phase Synthesis of Glu⁷-Phalloidin and Entry into Fluorescent F-Actin-Binding Reagents. *Angew. Chem. Int. Ed.* **2007**, *46* (19), 3547–3549.

(65) Yao, G.; Joswig, J.-O.; Keller, B. G.; Süßmuth, R. D. Total Synthesis of the Death Cap Toxin Phalloidin: Atropoisomer Selectivity Explained by Molecular-Dynamics Simulations. *Chem.—Eur. J.* **2019**, *25* (34), 8030–8034.

(66) Yao, G.; Knittel, C. H.; Kosol, S.; Wenz, M. T.; Keller, B. G.; Grub, H.; Braun, A. C.; Lutz, C.; Hechler, T.; Pahl, A.; Süßmuth, R. D. Iodine-Mediated Tryptathionine Formation Facilitates the Synthesis of Amanitins. *J. Am. Chem. Soc.* **2021**, *143* (35), 14322–14331.

(67) Kobayashi, D.; Kohmura, Y.; Sugiki, T.; Kuraoka, E.; Denda, M.; Fujiwara, T.; Otaka, A. Peptide Cyclization Mediated by Metal-Free S-Arylation: S-Protected Cysteine Sulfoxide as an Umpolung of the Cysteine Nucleophile. *Chem.—Eur. J.* **2021**, *27* (56), 14092–14099.

(68) Ohkawachi, K.; Anzaki, K.; Kobayashi, D.; Kyan, R.; Yasuda, T.; Denda, M.; Harada, N.; Shigenaga, A.; Inagaki, N.; Otaka, A. Residue-Selective C-H Sulfenylation Enabled by Acid-Activated S-Acetamidomethyl Cysteine Sulfoxide with Application to One-Pot Stapling and Lipidation Sequence. *Chem.—Eur. J.* **2023**, *29* (26), No. e2023007.

(69) Wang, D. Y.; Yu, M. Y.; Liu, N.; Lian, C. S.; Hou, Z. F.; Wang, R.; Zhao, R. T.; Li, W. J.; Jiang, Y. X.; Shi, X. D.; Li, S. M.; Yin, F.; Li, Z. G. A Sulfonium Tethered Peptide Ligand Rapidly and Selectively Modifies Protein Cysteine in Vicinity. *Chem. Sci.* **2019**, *10* (19), 4966–4972.

(70) Hou, Z. F.; Wang, D. Y.; Li, Y.; Zhao, R. T.; Wan, C.; Ma, Y.; Lian, C. S.; Yin, F.; Li, Z. G. A Sulfonium Triggered Thiol-yne

Reaction for Cysteine Modification. *J. Org. Chem.* **2020**, *85* (3), 1698–1705.

(71) Lau, Y. H.; Wu, Y. T.; Rossmann, M.; Tan, B. X.; de Andrade, P.; Tan, Y. S.; Verma, C.; McKenzie, G. J.; Venkitaraman, A. R.; Hyvönen, M.; Spring, D. R. Double Strain-Promoted Macrocyclization for the Rapid Selection of Cell-Active Stapled Peptides. *Angew. Chem. Int. Ed.* **2015**, *54* (51), 15410–15413.

(72) Sutton, D. A.; Yu, S. H.; Steet, R.; Popik, V. V. Cyclopropanone-Caged Sondheimer Diyne (Dibenzo[*a,e*]Cyclooctadiyne): A Photoactivatable Linchpin for Efficient SPAAC Crosslinking. *Chem. Commun.* **2016**, *52* (3), 553–556.

(73) Sharma, K.; Strizhak, A. V.; Fowler, E.; Wang, X. L.; Xu, W. S.; Jensen, C. H.; Wu, Y. T.; Sore, H. F.; Lau, Y. H.; Hyvönen, M.; Itzhaki, L. S.; Spring, D. R. Water-soluble, Stable and Azide-Reactive Strained Dialkynes for Biocompatible Double Strain-Promoted Click Chemistry. *Org. Biomol. Chem.* **2019**, *17* (34), 8014–8018.

(74) Strizhak, A. V.; Sharma, K.; Babii, O.; Afonin, S.; Ulrich, A. S.; Komarov, I. V.; Spring, D. R. Highly Reactive Bis-Cyclooctyne-Modified Diarylethene for SPAAC-Mediated Cross-Linking. *Org. Biomol. Chem.* **2018**, *16* (44), 8559–8564.

(75) Huber, R. E.; Criddle, R. S. Comparison of the Chemical Properties of Selenocysteine and Selenocystine with Their Sulfur Analogs. *Arch. Biochem. Biophys.* **1967**, *122* (1), 164–173.

(76) de Araujo, A. D.; Perry, S. R.; Fairlie, D. P. Chemically Diverse Helix-Constrained Peptides Using Selenocysteine Crosslinking. *Org. Lett.* **2018**, *20* (5), 1453–1456.

(77) Kusebauch, U.; Cadamuro, S. A.; Musiol, H.-J.; Lenz, M. O.; Wachtveitl, J.; Moroder, L.; Renner, C. Photocontrolled Folding and Unfolding of a Collagen Triple Helix. *Angew. Chem. Int. Ed.* **2006**, *45* (42), 7015–7018.

(78) Islam, M. S.; Junod, S. L.; Zhang, S.; Buuh, Z. Y.; Guan, Y. F.; Zhao, M.; Kaneria, K. H.; Kafley, P.; Cohen, C.; Maloney, R.; Lyu, Z. G.; Voelz, V. A.; Yang, W. D.; Wang, R. S. E. Unprotected Peptide Macrocyclization and Stapling via a Fluorine-Thiol Displacement Reaction. *Nat. Commun.* **2022**, *13* (1), 350–365.

(79) Morewood, R.; Nitsche, C. A Biocompatible Stapling Reaction for In Situ Generation of Constrained Peptides. *Chem. Sci.* **2021**, *12* (2), 669–674.

(80) Malins, L. R.; deGruyter, J. N.; Robbins, K. J.; Scola, P. M.; Eastgate, M. D.; Ghadiri, M. R.; Baran, P. S. Peptide Macrocyclization Inspired by Non-Ribosomal Imine Natural Products. *J. Am. Chem. Soc.* **2017**, *139* (14), 5233–5241.

(81) Morejón, M. C.; Laub, A.; Kaluderovic, G. N.; Puentes, A. R.; Hmedat, A. N.; Otero-González, A. J.; Rivera, D. G.; Wessjohann, L. A. A Multicomponent Macrocyclization Strategy to Natural Product-like Cyclic Lipopeptides: Synthesis and Anticancer Evaluation of Surfactin and Mycosubtilin Analogues. *Org. Biomol. Chem.* **2017**, *15* (17), 3628–3637.

(82) Zhang, J. Q.; Mulumba, M.; Ong, H.; Lubell, W. D. Diversity-Oriented Synthesis of Cyclic Azapeptides by A³-Macrocyclization Provides High-Affinity CD36-Modulating Peptidomimetics. *Angew. Chem. Int. Ed.* **2017**, *56* (22), 6284–6288.

(83) Ricardo, M. G.; Llanes, D.; Wessjohann, L. A.; Rivera, D. G. Introducing the Petasis Reaction for Late-Stage Multicomponent Diversification, Labeling, and Stapling of Peptides. *Angew. Chem. Int. Ed.* **2019**, *58* (9), 2700–2704.

(84) Yamaguchi, A.; Kaldas, S. J.; Appavoo, S. D.; Diaz, D. B.; Yudin, A. K. Conformationally Stable Peptide Macrocycles Assembled Using the Petasis Borono-Mannich Reaction. *Chem. Commun.* **2019**, *55* (71), 10567–10570.

(85) Frost, J. R.; Scully, C. C. G.; Yudin, A. K. Oxadiazole Grafts in Peptide Macrocycles. *Nat. Chem.* **2016**, *8* (12), 1105–1111.

(86) Vasco, A. V.; Méndez, Y.; Porzel, A.; Balbach, J.; Wessjohann, L. A.; Rivera, D. G. A Multicomponent Stapling Approach to Exocyclic Functionalized Helical Peptides: Adding Lipids, Sugars, PEGs, Labels, and Handles to the Lactam Bridge. *Bioconjugate Chem.* **2019**, *30* (1), 253–259.

(87) Méndez, Y.; Vasco, A. V.; Ivey, G.; Dias, A. L.; Gierth, P.; Sousa, B. B.; Navo, C. D.; Torres-Mozas, A.; Rodrigues, T.; Jiménez-

Osés, G.; Bernardes, G. J. L. Merging the Isonitrile-Tetrazine (4+1) Cycloaddition and the Ugi Four-Component Reaction into a Single Multicomponent Process. *Angew. Chem. Int. Ed.* **2023**, *62* (44), No. e202311186.

(88) Ricardo, M. G.; Vázquez-Mena, Y.; Iglesias-Morales, Y.; Wessjohann, L. A.; Rivera, D. G. On The Scope of the Double Ugi Multicomponent Stapling to Produce Helical Peptides. *Bioorg. Chem.* **2021**, *113*, 104987.

(89) Morejón, M. C.; Laub, A.; Westermann, B.; Rivera, D. G.; Wessjohann, L. A. Solution- and Solid-Phase Macrocyclization of Peptides by the Ugi-Smiles Multicomponent Reaction: Synthesis of N-Aryl-Bridged Cyclic Lipopeptides. *Org. Lett.* **2016**, *18* (16), 4096–4099.

(90) Krajcovicova, S.; Spring, D. R. Tryptophan in Multicomponent Petasis Reactions for Peptide Stapling and Late-Stage Functionalisation. *Angew. Chem. Int. Ed.* **2023**, *62* (34), No. e202307782.