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Biocompatible strategies for peptide macrocyclisation

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Peptides are increasingly important drug candidates, offering numerous advantages over conventional small molecules. However, they face significant challenges related to stability, cellular uptake and overall bioavailability. While individual modifications may not address all these challenges, macrocyclisation stands out as a single modification capable of enhancing affinity, selectivity, proteolytic stability and membrane permeability. The recent successes of *in situ* peptide modifications during screening in combination with genetically encoded peptide libraries have increased the demand for peptide macrocyclisation reactions that can occur under biocompatible conditions. In this perspective, we aim to distinguish biocompatible conditions from those well-known examples that are fully bioorthogonal. We introduce key strategies for biocompatible peptide macrocyclisation and contextualise them within contemporary screening methods, providing an overview of available transformations.

Introduction

Macrocyclic peptides as therapeutics

The discovery of insulin therapy in the 1920s marked a major milestone in peptide therapeutics. Banting & Best were able to isolate insulin from the pancreatic isles of animals and used it for the treatment of type 1 diabetes.[1] Other examples of early peptide therapeutics include, for example, adrenocorticotropic hormone (ACTH) for the ACTH stimulation test,[2] salmon calcitonin for the treatment of Paget's disease and osteoporosis,[3] oxytocin for inducing childbirth,[4] vasopressin for the treatment of vasodilatory shock,[5] and octreotide for the treatment of acromegaly and neuroendocrine tumours.[6] Over time, an increasing number of peptide therapeutics successfully transitioned into the clinic. Between 2017 and 2023, approximately one in ten drugs approved by the FDA were peptides and peptidomimetics, with macrocyclic peptides accounting for nearly half of this category (Table S1† and Fig. 1).[7–12]

One reason why macrocyclic peptides have gathered such increasing attention within the pharmaceutical industries is due to their superior properties, frequently surpassing those of their linear counterparts.[13–19] Peptide macrocycles may have enhanced bioactivity and lower toxicity along with improved affinity for their targets. Unlike random-coil (linear) peptides, macrocycles are constrained by their ring structure, reducing the number of conformations which in turn reduces the

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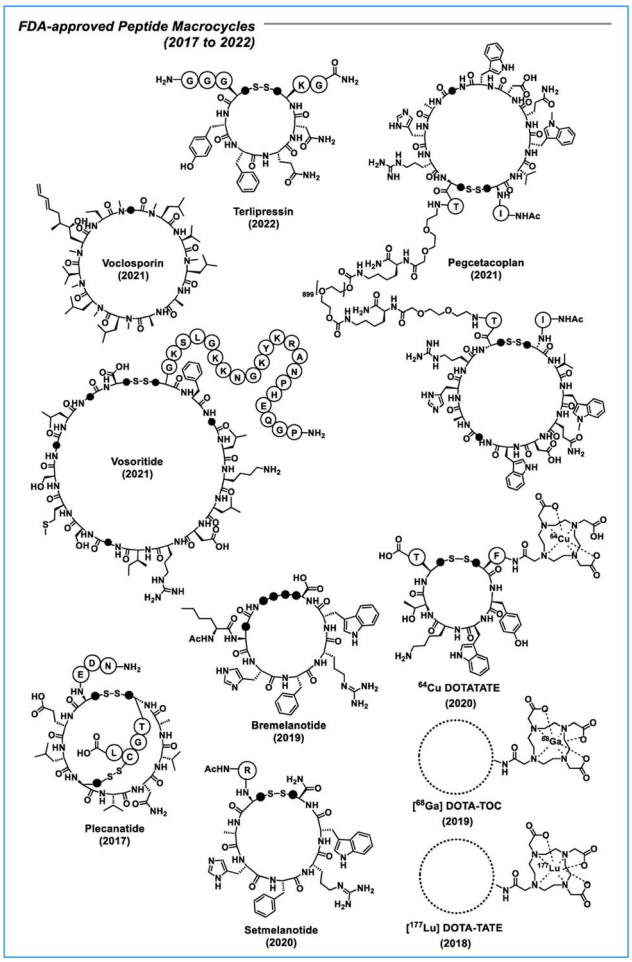

Fig. 1 Peptide macrocycles approved by the US Food and Drug Administration (FDA) between 2017 and 2022. Each • represents a CH_2 (methylene) group.

entropic penalty upon binding, thereby increasing selectivity and affinity at the target binding site. Additionally, macrocyclic peptides may exhibit greater resistance to proteolysis and enhanced cell-penetrating activity. In summary, simple macrocyclisation may lead to improved affinity, selectivity, stability and membrane permeability, which are four crucial parameters for successful drug candidates. Furthermore, macrocyclic peptides can be generated for virtually any drug target, thanks to the rapidly expanding toolbox for *de novo* discovery of macrocyclic ligands.

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Types of macrocycles and examples from nature

Peptide macrocycles commonly exhibit distinct cyclisation patterns, which can be categorised into head-to-tail, side chain-to-side chain and termini-to-side chain (Fig. 2). Head-to-tail cyclic peptides are characterised by forming a closed loop that connects the C- and N-termini of the peptide. The points of cyclisation can usually not be determined in such a macrocycle. Prominent examples include cyclosporin A, gramicidin S and yunnanin A. While a homodetic bond refers specifically to such a loop formed through a head-to-tail macrolactam cyclisation,[20] all other macrocycles formed between two functional groups are considered heterodetic.[21] Side chain-to-side chain cyclisation involves connecting two side chain residues, as observed in somatostatin, oxytocin, salmon calcitonin and the crustacean cardioactive peptide. Moreover, termini-to-side chain cyclisation connects either the C- or N-terminus to one of the side chain residues, as observed, for example, in the antibiotic polymyxin B.[22,23] There are special cases where a peptide macrocycle contains multiple loops or is connected at multiple points within the peptide sequence, which are referred to as bicycles and tricycles (Fig. 2). Naturally occurring peptide macrocycles have been isolated from various organisms, including marine resources (invertebrates),[24,25] bacteria,[26–28] plants,[29] fungi[30] and mammals.[31,32] These peptides serve as hormones, growth factors or defensive mechanisms against competing microbes (*i.e.*, antibiotics and toxins). We recently reviewed contemporary methods to synthesise bicyclic peptides.[33]

The importance of biocompatible macrocyclisation strategies

The innovation of solid-phase peptide synthesis (SPPS) by Merrifield in the 1960s radically revolutionised the chemical synthesis of peptides *via* a facile, efficient and automated platform, enabling large scale production of bioactive peptides.[34,35] Chemical synthesis has long been the cornerstone for establishing structure–activity relationships well before genetically encoded peptide libraries gained prominence. It has been a fundamental tool in understanding the relationship between the chemical structure and the biological activity of peptides. The modification of peptides in the presence of their target protein has enabled the *in situ* generation and selection of peptide ligands.[36–38] Furthermore, peptide display techniques, such as phage or mRNA display,[39,40] have the capability to screen billions of peptides against drug targets. However, these screenings require chemical conditions that support the structural and functional integrity of biopolymers (*e.g.*, proteins, nucleic acids) involved.

Commonly used strategies for peptide macrocyclisation are highlighted in Fig. 3, covering a wide range of chemical linkages and reactions discussed in this perspective. Certain approaches require rather harsh and nonselective conditions, while others

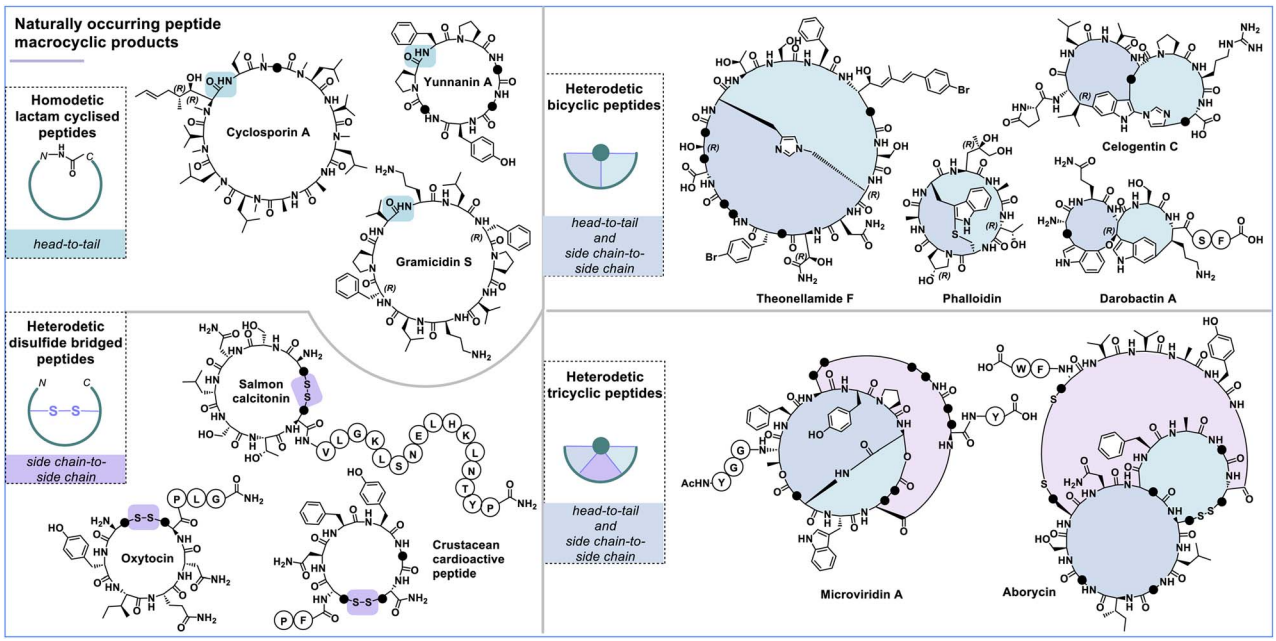


Fig. 2 Examples of peptide macrocycles found in nature categorised by their cycle type and chemical linkage. Each • represents a CH_2 (methylene) group.

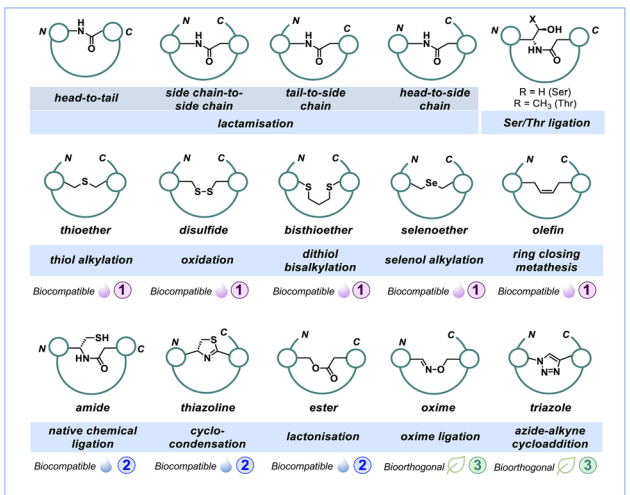
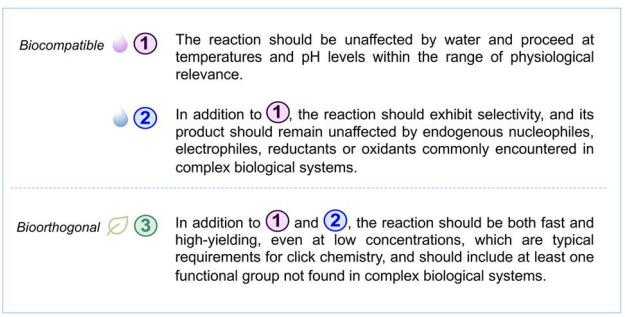


Fig. 3 Various types of cyclic peptides and selected synthetic strategies for cyclisation.

can proceed under very benign and selective conditions, even within a complex cellular environment. The latter chemical transformations are usually considered bioorthogonal, with the [3 + 2]-cycloaddition reaction between azide and alkyne motifs being the most prominent example.[41] The importance of this click reaction and bioorthogonal chemistry more generally was recently recognised by The Nobel Prize in chemistry awarded to Bertozzi, Meldal and Sharpless.[42]

While bioorthogonal chemistry plays a crucial role in numerous applications,[43,44] not every reaction used to modify or cyclise peptides needs to be fully bioorthogonal. Inspired by Zhou & co-workers from the Chemical Abstracts Service (CAS),[43] who recently reported criteria for bioorthogonal reactions, we defined the following characteristics for biocompatible and bioorthogonal reaction used throughout this perspective:



In fact, fulfilling only the first criterion listed above – a reaction that occurs in water at physiological pH and temperature – deems a reaction already biocompatible, as defined in this perspective article. Furthermore, it is highly desirable for a biocompatible reaction to meet the second criterion as closely as possible. Peptide display techniques often require robust reactions with minimal side products. Selectivity for certain amino acids in peptides and proteins, or even complete orthogonality to common endogenous nucleophiles and electrophiles, can be of great benefit. When screenings are performed in cells, considering toxicity and redox stability becomes an additional important factor. While reaction rate at low concentrations is a crucial factor for transformations used in bioconjugation, it is of lesser importance for macrocyclisation. Intramolecular reactions typically proceed faster than intermolecular reactions, and they are independent of concentration. In fact, lower concentrations can be desirable in macrocyclisation to suppress the formation of oligomers.

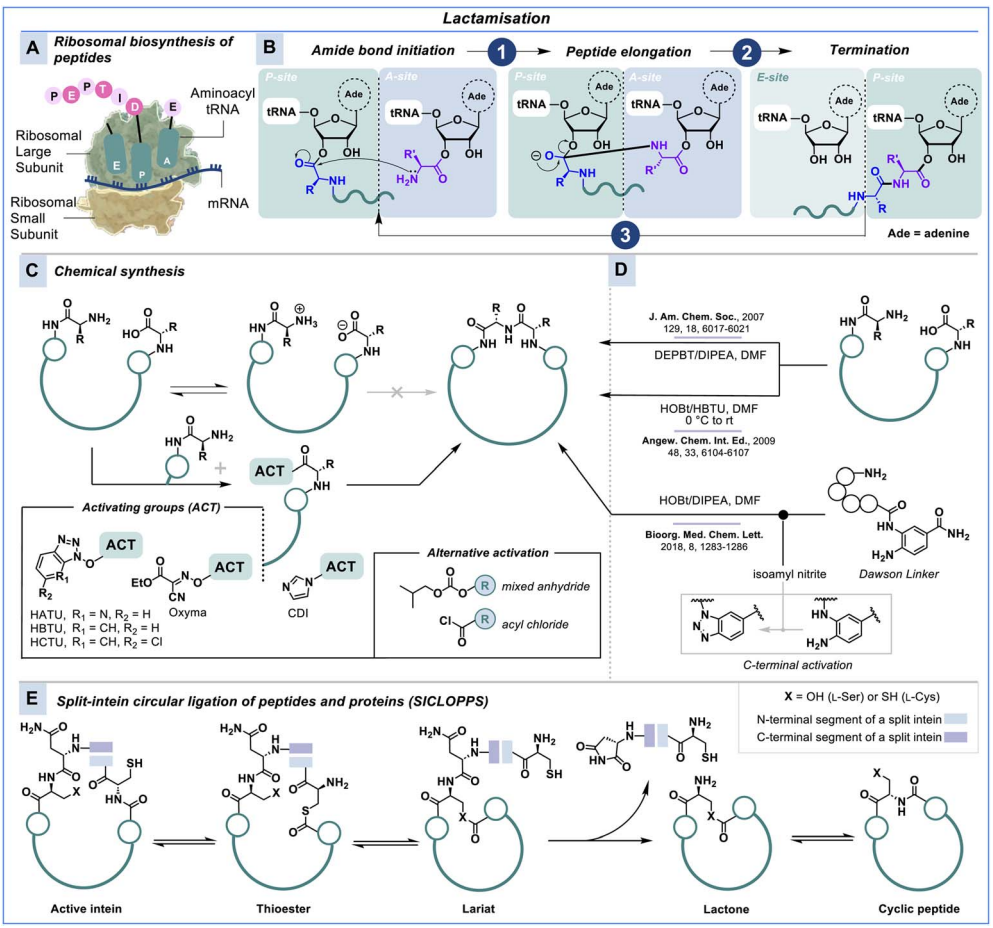
In this perspective article, our main objective is to highlight the most important strategies employed in peptide macrocyclisation with a particular focus on biocompatible methods. While some of these methods are routinely used for modifying peptides and proteins in biological environments, others hold potential for future applications. We will adhere to the definitions mentioned above, labelling reactions as biocompatible and bioorthogonal if they broadly meet the criteria. However, it is not our intention to clearly distinguish between biocompatible and bioorthogonal reactions, as current classifications are not definitive. Comprehensive reviews that consolidate current developments and challenges in this field are unavailable. Therefore, we have undertaken the task of reviewing conventional methods and highlighting emerging trends in biocompatible strategies for peptide macrocyclisation.

Strategies for peptide macrocyclisation

Macrolactam cyclisation

As outlined above, homodetic cyclic peptides are cyclised *via* an amide bond, resulting in a macrolactam ring. Ribosomes facilitate the biosynthesis of polypeptides (proteins) through a complex biomolecular machinery that forms amide bonds by linking α-amino acids as illustrated in Scheme 1A and B.[45,46] In this process of translation from RNA to proteins, nature utilises an ester bond between the terminal amino acid of the growing peptide chain and its corresponding tRNA for activation (P-site) in order to react with the amine of the incoming amino acid (A-site). While translation proceeds with fully unprotected amino acids under physiological conditions with a speed of 20 amino acids per second,[47] synthetic chemistry usually requires bulky protecting groups, additives, sensitising coupling reagents and excessive volumes of toxic organic solvents to generate peptide chains over many hours if not days.[48–51]

Given its significance in translation and the formation of naturally occurring cyclic peptides, amide bond formation is a widely employed and important method for constructing macrocyclic peptides;[52–55] however, most of these chemical strategies are bioincompatible (Scheme 1C). For example, Smith & co-workers used diphenylphosphoryl azide (DPPA) as coupling agent in DMF to form a tricyclic homodetic peptide.[56] Peptide macrocyclisation using DPPA also tolerates backbone modifications like *N*-methylation or thioamides, as demonstrated by Kessler, Chatterjee & co-workers.[57,58] The bicyclic



Scheme 1 Amide bond formation and macrolactam cyclisation. [A] Ribosomal translation from mRNA to proteins. [B] Addition of an amino acid to the growing peptide chain in the ribosome. [C] Chemical synthesis of peptides *via* activation of carboxylic acids. [D] Examples of synthetic methods for macrolactam cyclisation. [E] Split-intein circular ligation.

octapeptide celogentin C was synthesised using HBTU as the coupling agent in DMF,[59] and the antibiotic peptide lysobactin was accessed by treating the linear peptide with DEPBT in DMF.[60] The process of macrolactamisation is slow, as illustrated in these examples, taking from ten hours to three days to form the macrolactam ring. Alternatively, macrolactam cycles can be generated using the Dawson linker (Fmoc–Dbz) introduced in 2008.[61] Oishi & co-workers furnished a head-to-tail macrolactam cyclised peptide using this versatile linker.[62] Following C-terminal activation, head-to-tail macrocyclisation was achieved in only 2 h at room temperature in DMF (Scheme 1D).

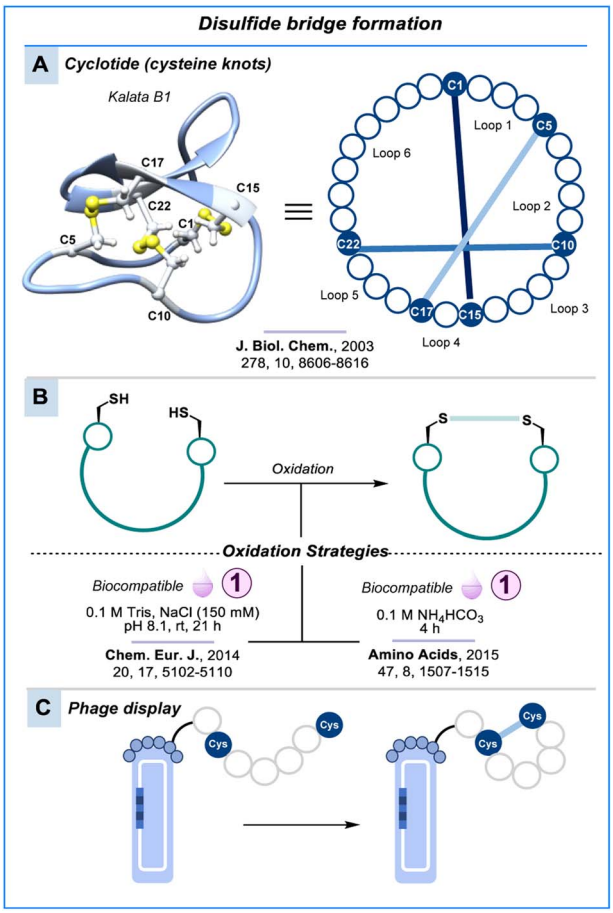
The lack of biocompatible synthetic approaches for macrolactam cyclisation is one of the main reasons why peptide display technologies like phage or mRNA display rely on non-canonical cyclisation methods, as discussed below. An alternative strategy for genetically encoded macrocyclic peptide libraries that supports pure head-to-tail macrocyclisation is the split-intein circular ligation of peptides and proteins (SICLOPPS), which uses protein splicing to generate cyclic peptide libraries (Scheme 1E).[40,63,64]

Disulfide bond formation

An alternative way to cyclise peptides commonly observed in nature is through the formation of disulfide bonds between cysteine residues. Examples include oxytocin, which forms a cyclic peptide from one disulfide bond (Fig. 1),[4] and cyclotides, which are macrolactam cyclic peptides that possess three internal disulfide bonds, making them a highly constrained class of peptides (Scheme 2A).[65,66] Disulfide bond formation is dependent on the spatial proximity of the two cysteine residues, the pH and the redox environment. Alkaline pH promotes disulfide formation due to increased cysteine nucleophilicity (pK_a = 8.5). Air is sufficient for oxidation to disulfides; however, since the intracellular environment is mostly reducing (*e.g.*, glutathione), most disulfide bonds are unstable in the cytosol.

Despite their susceptibility to reducing conditions, including enzymes like reductases,[67,68] disulfide-bridged peptides have received widespread attention due to their diverse bioactivity, ability for cell penetration, proteolytic stability and resilience to some harsh conditions such as high temperature, acidic pH and chaotropic agents.[69]

Synthetic methods to form disulfides rely on the simple oxidation of cysteine residues. Often, these methods can proceed in buffer at near-neutral pH and room temperature, deeming them highly biocompatible (Scheme 2B). For example, oxidative folding of cysteine-rich peptides can be accomplished in Tris buffer at pH 8.0, as illustrated in the folding of the 63-mer snakin-1 peptide at room temperature over 21 h.[70] The oxidative folding of a CXCR4 antagonist peptide followed a similar approach using NH_4HCO_3 buffer.[71] Alternatively, using iodine as an oxidising agent, cysteine-rich peptides can rapidly cyclise within only a few minutes.[72–74] The liability of disulfide bonds to reducing conditions has motivated chemists to explore various bioisosteric crosslinks.[75–78]

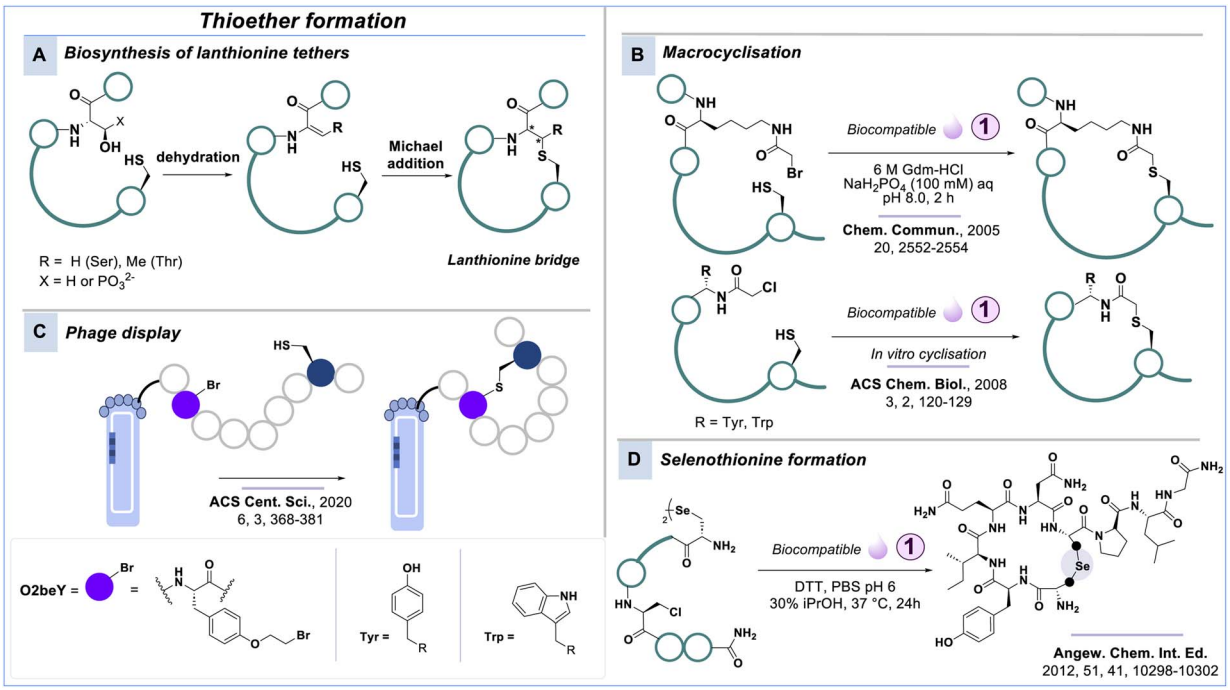


Scheme 2 Disulfide bridge formation. [A] The cyclotide kalata B1 is an example for a highly constrained macrocyclic peptide with three disulfide bonds. [B] Examples of biocompatible chemical conditions to induce disulfide bridge formation by oxidation. [C] Formation of cyclic peptides in phage display using disulfide bonds.

An area of significant importance where disulfide bonds have been used routinely to form macrocyclic peptides under biocompatible conditions is phage display (Scheme 2C).[40,79] As the panning and modification of the phage occur outside the cellular environment, the disulfide bond is useful to constrain peptides for screening purposes. Recently, the strategy was expanded to highly constrained disulfide-directed multicyclic peptide libraries.[80]

Thio- and selenoether formation

A modification involving cysteine (and occasionally selenocysteine) that forms chemically more stable conjugates than the previously discussed disulfide bond is thioether formation. Naturally occurring antimicrobial peptides like lanthipeptides and sactipeptides feature such a thioether bridge.[81] In nature, phosphorylated serine or threonine residues undergo enzymatic dehydration and subsequent Michael reaction with a cysteine residue to form the lanthipeptide bridge (Scheme 3A).[82,83] In the past, lanthipeptides were synthesised using Fmoc-SPPS compatible building block strategies;[77,84–87] however, with the advent of biocompatible strategies, libraries of



Scheme 3 Thioether macrocyclisation. [A] Biosynthetic formation of the lanthionine bridge. [B] Examples of biocompatible strategies used to form thioethers in macrocyclic peptides. [C] Macrocyclic Organo-Peptide Hybrid Phage Display (MOrPH-PhD). [D] Example for alternative selenoether formation.

lanthipeptides with multiple thioether crosslinks can be generated using phage and yeast display platforms.[88,89]

Brunel and Dawson[90] synthesised helical peptides with thioether staples by alkylating a cysteine residue with a primary alkyl halide in aqueous buffer at pH 8.4 and room temperature (Scheme 3B). This biocompatible methodology was later adapted in the seminal works of Suga & co-workers that combine an engineered ribozyme (flexizyme) with the *in vitro* mRNA display technique in the Random Nonstandard Peptides Integrated Discovery (RaPID).[39,91–94] The RaPID platform allows reprogramming the genetic code to introduce noncanonical amino acids, for example, displaying α-*N*-chloroacetyl groups that spontaneously form thioethers with a downstream cysteine residue during *in vitro* translation (Scheme 3B).[95–98]

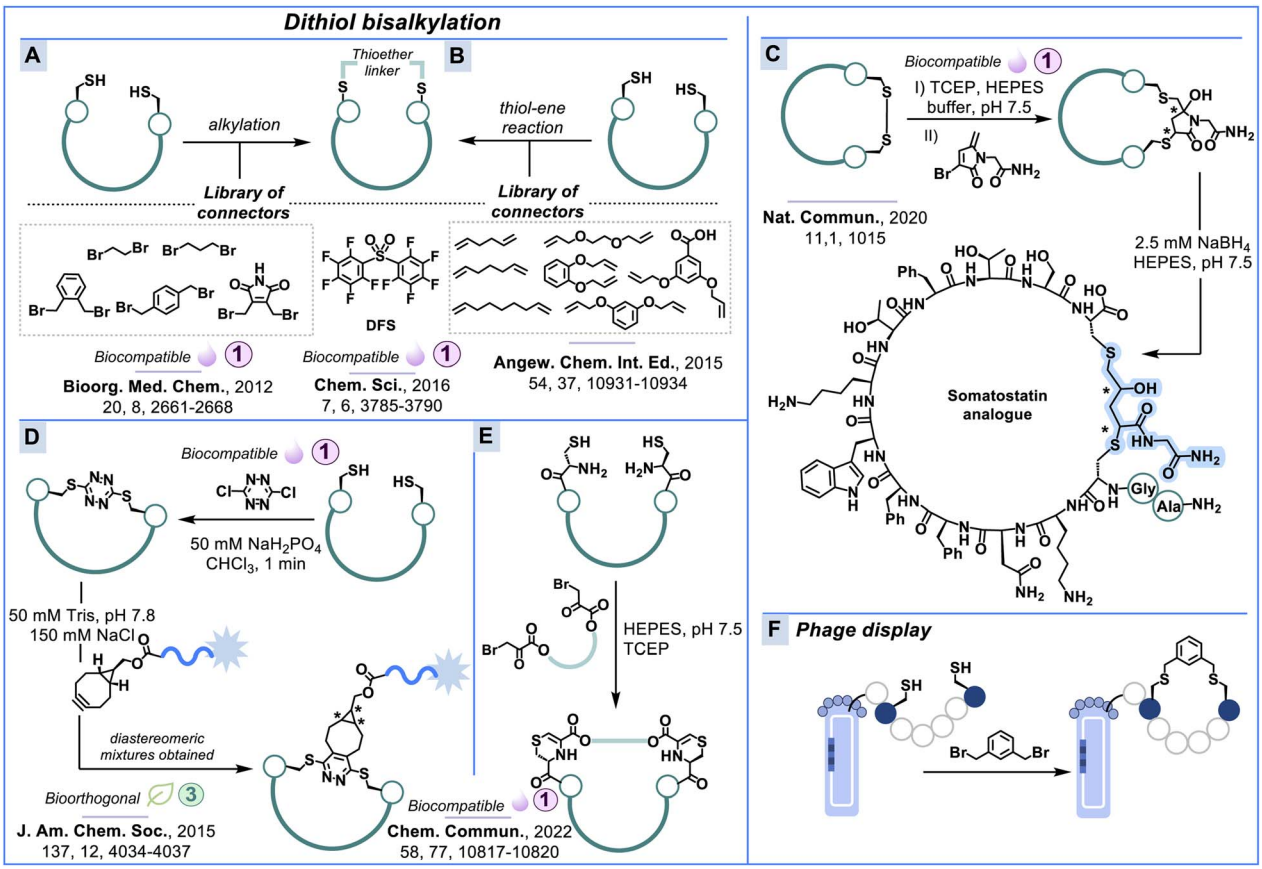
A related chemical approach was recently integrated into the phage display platform by Fasan & co-workers, in which they screened combinatorial libraries of thioether-bridged macrocycles against multiple protein targets and identified high affinity binders.[99] In this strategy titled Macrocyclic Organo-Peptide Hybrid Phage Display (MOrPH-PhD), macrocyclisation of peptide libraries is aided by the regioselective and biocompatible reaction between a cysteine residue and *O*-(2-bromomethyl)-tyrosine (O2beY) (Scheme 3C). The approach requires three plasmids, one of which encodes the tRNA/aminoacyl-tRNA synthetase pair necessary for incorporation of the unnatural amino acid carrying the electrophile.

The alkylation of selenocysteine presents an intriguing alternative to using cysteine; however, it is important to consider the increased susceptibility of selenocysteine (pK_a = 5.4) to oxidation and dimerisation compared to cysteine (pK_a = 8.6). Alewood & co-workers successfully constructed a selenoether bridge in oxytocin using biocompatible conditions (Scheme 3D).[100] Importantly, the cysteine analogue failed to form the same linkage, highlighting the valuable applications of selenocysteine alkylation.

Dithiol bisalkylation

Thioether modification of cysteine residues can also be accomplished in a dual manner through bisalkylation of two cysteines. This two-component peptide stapling approach has been employed to access various ring sizes, tackle metabolism, improve bioactivity and stabilise α-helical conformation.[101,102] Cornish & co-workers[103] prepared a series of cyclic amylin-(1–8) analogues with various linkers under biocompatible conditions (Scheme 4A). Dithiol bisalkylation was performed by incubating the linear peptides with two reduced cysteine residues in various aqueous buffers ranging from pH 7.8 to 8.8 with yields between 10% and 67%.

Derda & co-workers introduced decafluoro-biphenylsulfone (DFS) as a new perfluoroarene (fAr) for biocompatible peptide and protein modification (Scheme 4A).[104] While many S_NAr-reagents suffer from nonselective nucleophilic substitution, low reactivity and poor water solubility,[105–107] DFS and perfluoropyridine are capable of site-selective modification in the presence of only low ratios of organic co-solvents, *e.g.*, Tris-buffer with 5% DMF or 10% acetonitrile. DFS reacts quicker with cationic peptides (rate constant of 100–180 M^{-1} s^{-1}) than uncharged peptides (50–80 M^{-1} s^{-1}) and modified 35–45% of thiols in a library of >10^9 disulfide heptapeptides displayed on



Scheme 4 Peptide dithiol bisalkylation. Bridging two cysteine residues using [A] haloalkyl connectors or [B] di-enes to perform thiol–ene chemistry. [C] Cysteine stapling with a 3-bromo-5-methylene pyrrolone derivative. [D] *S,S*-Tetrazine staples allow further modifcation under bioorthogonal conditions. [F] Cysteine stapling during phage display with DBMB.

intact M13 bacteriophage. To select chemically stable peptide binders on phage, Heinis & co-workers have also explored cysteine alkylation using α,α′-dibromo-*m*-xylene (DBMB) (Scheme 4F).[108] Peptide stapling with DBMB resulted in high-affinity ligands of β-catenin with α-helical conformation.

The thiol–ene reaction is an alternative way to alkylate cysteine residues using UV irradiation.[109–111] Examples that implemented thiol–ene chemistry into peptide synthesis include the works of Wang and Chou[109,112] and Beyermann & co-workers.[113,114] Wang and Chou used a radical initiator in NMP as the solvent to afford various dithiol-bisalkylated peptide macrocycles in excellent yields (Scheme 4B). Beyermann & co-workers demonstrated that the thiol–ene reaction can proceed under biocompatible conditions using a photoswitchable amino acid with a vinyl handle. Spring & co-workers used divinyltriazine staples for biocompatible cysteine modification.[115] Buchwald, Pentelute & co-workers further expanded the toolbox of staples by introducing a biocompatible Pd-mediated cysteine arylation.[116]

Another approach for selective cysteine modification in peptides and proteins utilises the thia-Michael addition.[117] Zhou & co-workers introduced 5-methylene pyrrolone and its halo-derivatives as Michael acceptors which form cysteine conjugates at physiological pH (Scheme 4C). This bioconjugation strategy has been used for protein modification and disulfide replacement in the hormone somatostatin (Scheme 4C).[118,119]

Based on the Staudinger phosphonite reaction (SPhR), Hackenberger & co-workers recently introduced vinylphosphonite to cyclise peptides between azide and cysteine residues.[120] They cyclised an azido containing BCL-9-derived peptide and investigated its stability and biophysical properties. At neutral to basic pH, the phosphonamidate was stable, helicity of the stapled peptide was successfully increased, and the stapled peptide inhibited the interaction between native BCL-9 and β-catenin. They further extended this strategy to labelling monoclonal antibodies and GFP.

Smith & co-workers developed a facile incorporation of *s*-tetrazine into peptides containing two unprotected cysteine residues.[121] Peptides can be unstapled with UV light and the Diels–Alder reactivity of *S,S*-tetrazine provides a bioorthogonal handle for the installation of probes (Scheme 4D). Neumann & co-workers have recently reported a related selective tetrazine–thiol exchange (TeTEx) strategy for peptide macrocyclisation which is SPPS compatible and effective without any activation reagents.[122] We reported a peptide staple inspired by the natural product lanthionine ketenamine.[123] This reaction between 1,2-aminothiols and α-bromopyruvates is a combination of cysteine

alkylation and imine formation and allows for the use of various aliphatic or PEG-based linkers (Scheme 4E).

Macrolactone cyclisation

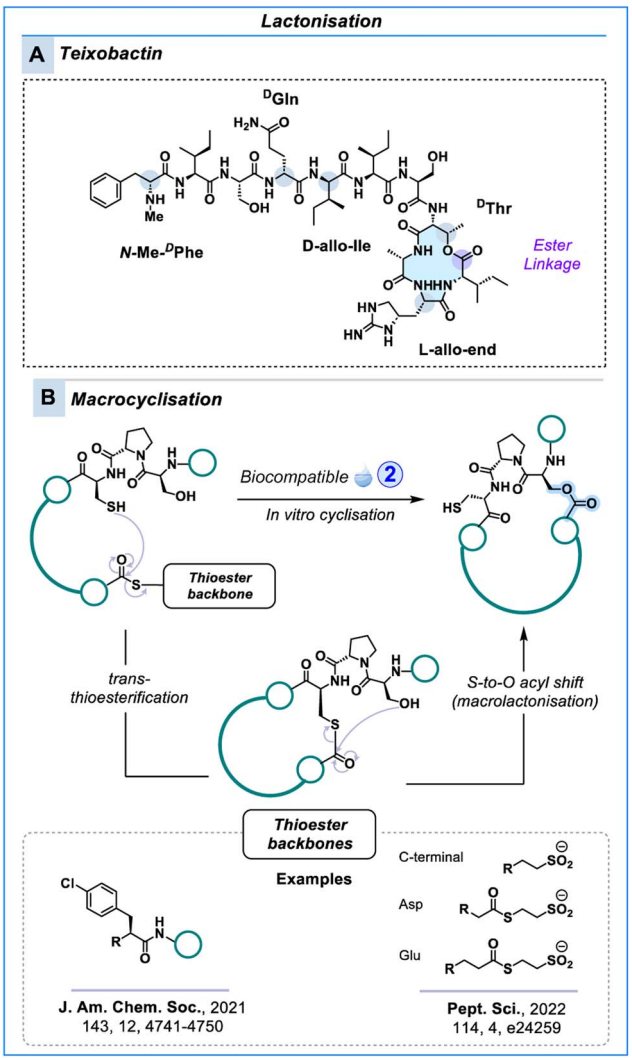
An alternative to the formation of amide bonds is the creation of an ester bond, for example, between serine/threonine and the C-terminal carboxylate. Depsipeptides are natural products with such a characteristic bond in the peptidyl backbone. It has been suggested that replacing the –NH with an O distorts the α-helical and β-sheet structures by decreasing hydrogen bonding.[124–126] Depsipeptides display antimicrobial,[127,128] anti-tumour[129] and antiviral activity.[130] A prominent example is the antibiotic teixobactin (Scheme 5A).

Esterification does not spontaneously occur by mixing a carboxylic acid with an alcohol at neutral pH. Typically, activation of the carboxylate is necessary to allow for ester formation to occur in solution phase.[131] Often this is difficult to perform during Fmoc-SPPS chemistry with many factors impacting acylation, making the synthesis of depsipeptides challenging tasks.[132]

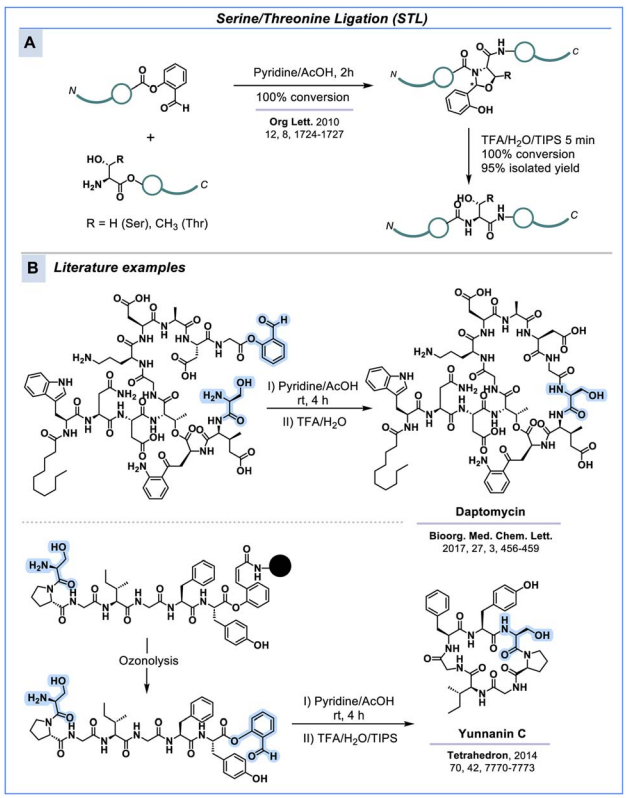
Recently, Suga & co-workers introduced a biocompatible approach to prepare cyclic depsipeptides in line with their work to create macrocyclic libraries for RaPID (Scheme 5B).[133,134] The strategy exploits the spontaneous *S*-to-*O* acyl shift in *N*-acetylated peptides comprising an N-terminal Ser/Thr–Pro–Cys–Gly motif and a C-terminal thioester. The linear peptide is subjected to buffer at pH 7.5 where it undergoes esterification *via* a two-step mechanism. An initial trans-thioesterification between the cysteine and thioester is followed by a rapid *S*-to-*O* acyl shift from the nucleophilic alcohol of Ser/Thr to establish an ester crosslink (Scheme 5B). Although, the methodology is compatible with peptides of diverse ring sizes and sequences, the Ser/Thr–Pro–Cys–Gly motif is pivotal for acyl transfer.

Serine/threonine ligation

A way to use serine or threonine to form backbone amide linkages instead of esters involves the serine/threonine ligation (STL), which involves the reaction between an N-terminal serine or threonine residue and a C-terminal salicylaldehyde ester (Scheme 6A).[135] This technique has been predominantly used to couple two or more peptide fragments to chemically synthesise proteins.[136] Li & co-workers have contributed significantly to developing this technique and could successfully synthesise several well-folded proteins, such as the ACYP enzyme,[135] MUC1



Scheme 5 Macrolactone peptides. [A] Teixobactin is a natural antimicrobial depsipeptide with a macrolactone ring. [B] *In vitro* synthesis of macrolacton peptide libraries using *S*-to-*O* acyl shift.



Scheme 6 Serine/threonine ligation (STL) as an alternative to native chemical ligation (NCL) to [A] conjugate peptide fragments and [B] construct macrocyclic peptides.

glycopeptide antigen,[136] IL-25 [137] and nuclear protein HMGA1a.[138] The ligation proceeds at rather mild conditions in pyridine–acetate buffer (1 : 1) without any metal additives. As a complementary strategy to native chemical ligation (NCL) discussed below, STL has certain advantages such as the relatively high abundance of serine and threonine in proteins (12.7%) compared to cysteine (1.5%), no need for N-terminal modification, and compatibility with most of the amino acids at the C-terminus.[139]

Since STL leads to native amide linkage in the peptide, this strategy has also been explored in the total synthesis of peptide-based antibiotics like daptomycin,[140,141] yunnanin C,[142] (Scheme 5B) cyclomontanin B[143] and their analogues. Liu & co-workers investigated the scope of STL for the synthesis of small and large macrocycles and observed minimal oligomerisation.[144] STL is a two-step process in which a C-terminal aldehyde reacts with the α-amine of N-terminal serine or threonine to from an oxazolidine intermediate. Spontaneous *O*-to-*N* acyl transfer then forms an *N*,*O*-benzylidene acetal which upon acidolysis generates the amide linkage (Scheme 6A). The preferred pH range for the reaction is 4 to 6. Despite some rather harsh conditions, like the final acidolysis, there might be scope for further optimisation to make this strategy biocompatible.

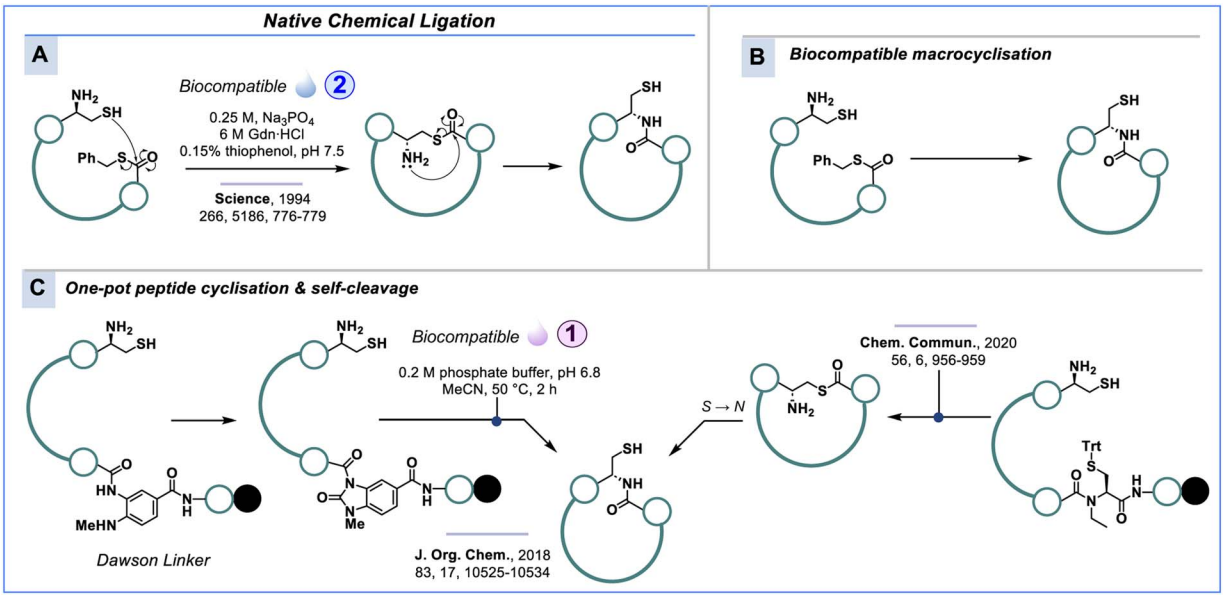
Native chemical ligation

A related technique that uses N-terminal cysteine instead of serine/threonine to form amide bonds is native chemical ligation (NCL), which is broadly employed for protein synthesis but also useful for peptide macrocyclisation. While SPPS is widely used, there remain some technical drawbacks, such as diketopiperazine (DKP) and aspartimide formation, epimerisation of α-carbons and β-elimination of cysteine thiols due to repeated exposure to organic base.[145,146] Another drawback is aggregation from inter-/intramolecular β-sheet formation during SPPS,[147] which can lead to incomplete solvation of the resin-bound peptide and subsequently reduce coupling efficiency. Therefore, it is extremely challenging to efficiently construct peptides comprising more than 50 amino acids through standard SPPS, unless advanced flow chemistry is implemented as shown by Pentelute & co-workers.[148]

Addressing this challenge, Kent & co-workers[149] devised NCL which involves a pair of peptide precursors, one fashioned with a C-terminal thioester and the other with the N-terminal unprotected cysteine. In NCL an initial trans-thioesterification takes place as the nucleophilic cysteine thiol undergoes an S_N2 reaction with the corresponding carbonyl of the thioester fragment, establishing the new thioester crosslink. The proximity of the peptide permits the free amine to undergo an intramolecular rearrangement, which subsequently forms an amide bond and restores a thiol residue (Scheme 7A).[149–153] Selenocysteine has proven an important addition in the synthesis of long polypeptides.[154–157]

The introduction of NCL laid the foundations for constructing larger linear polypeptides linked *via* a native amide backbone, and has inspired researchers like Tulla-Puche and Barany,[158] Olsen & co-workers,[159] Stockdill & co-workers,[160] and Hojo & co-workers[161] to adopt the strategy to generate peptide macrocycles with a native amide crosslink (Scheme 7B).

Gless and Olsen generated a peptide macrocycle in aqueous solution using the Dawson linker (MeNbz).[159,162] After transformation to the urea intermediate, the activated C-terminus reacts with N-terminal cysteine at 50 °C and pH 6.8 to release the cyclic peptide from the resin (Scheme 7C). Surrogate linker systems have also been explored by Hojo & co-workers.[161] The *N*-ethylcysteine linker forms a thioester bond *via* *N*-to-*S* migration to enable intramolecular NCL (Scheme 7C).



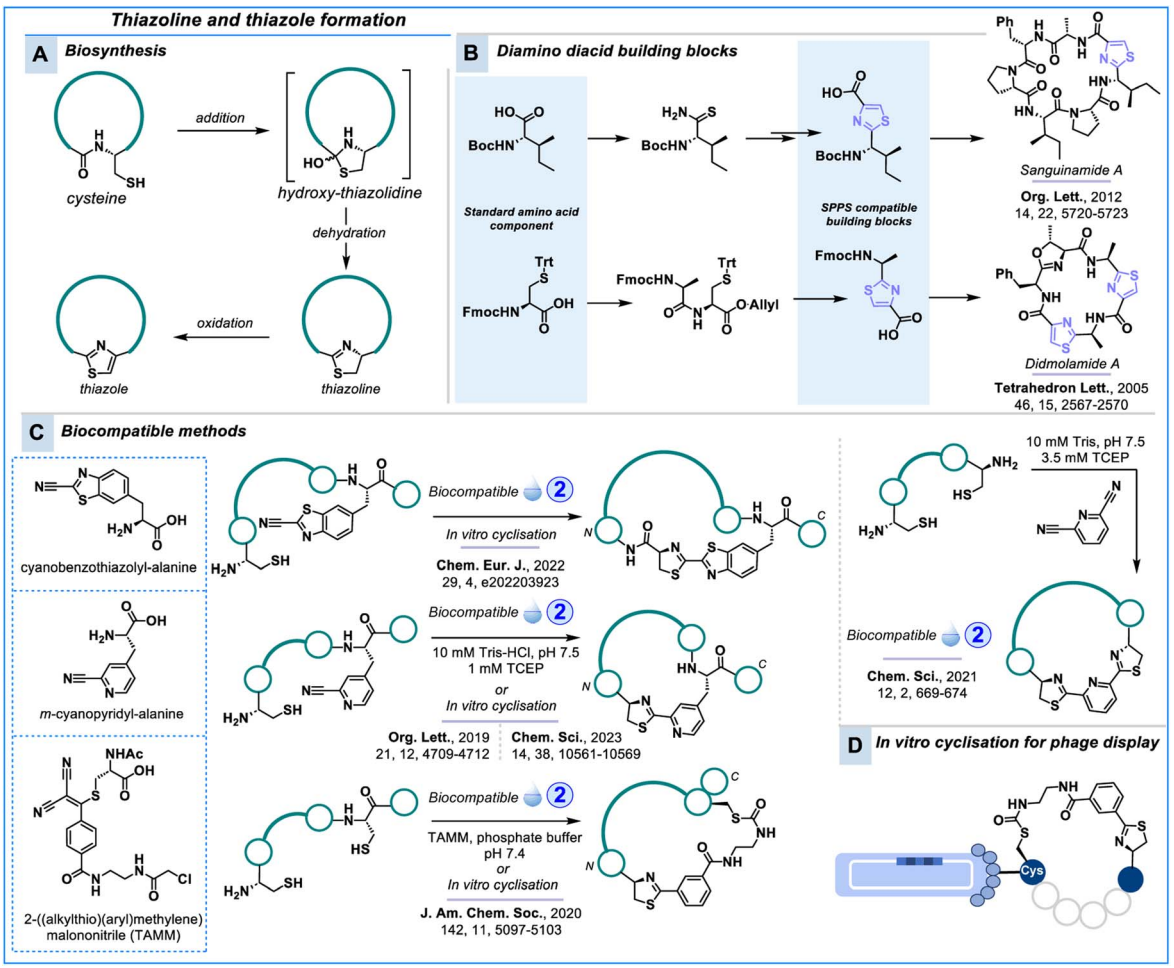
Scheme 7 Native chemical ligation (NCL). [A] Fundamental *N*-to-*S* acyl shift mechanism. [B] Biocompatible NCL with an N-terminal aminothiol and C-terminal thioester. [C] Examples of NCL using safety catch linkers for direct on-resin C-terminal modification.

Thiazoline and thiazole formation

Another method that relies on the unique properties of N-terminal cysteine is the nitrile-aminothiol click reaction used to form thiazolines.[163] Oxidation of the thiazoline leads to thiazole, which is a small heterocycle that appears in a variety of bioactive compounds, including peptides.[164] The unique architecture of the thiazole ring in azole-based peptides makes it an attractive motif in drug discovery.[165] The thiazole unit in the peptide backbone can influence the folding architecture of peptides.[166–169] Flow-on effects from the small thiazole graft can stabilise the 2D structure by improving the intramolecular H-bond networks,[164,170] as well as increasing cell membrane permeability by reducing the number of H-bond donors and minimise water solvation of amide bonds.[171] In nature, thiazole crosslinks are formed through a post-translational transformation of cysteine *via* the mechanism shown in Scheme 8A.[172,173] While no biocompatible synthetic methods for thiazole formation have been reported yet, several peptides containing thiazoles have been synthesised using building blocks with the pre-installed thiazole motif (Scheme 8B), for example, dolastatin 3,[174,175] cyclodidemnamide B,[176] didmolamides A and B,[177] obyanamide,[178] scleritodermin A,[179,180] aeruginazole A,[181] sanguinamide A,[182] marthiapeptide A[183] and venturamides A and B.[184]

The thiazoline heterocycle is yet another amide bioisostere that is part of bioactive natural products and synthetic peptides.[185–188] Inspired by the biosynthesis of firefly luciferin from 2-cyano-6-hydroxybenzothiazole and L-cysteine,[189] the condensation reaction between electrophilic nitriles and 1,2-aminothiols has been exploited for biocompatible macrocyclisation of synthetic and genetically encoded peptide libraries alike (Scheme 8C).[190–193]

Cyanobenzothiazole (CBT) and *m*-cyanopyridine (Scheme 8C) are the two most commonly used moieties. The reaction of 2-cyanobenzothiazole and related nitriles with 1,2-aminothiol has been extensively explored in bioconjugation, enabling applications ranging from nanoparticle design to imaging techniques.[194–203] Most recently, this reaction was employed in the *in vitro* translation of genetically encoded peptide libraries.[204]



Scheme 8 Thiazole and thiazoline peptide macrocyclisation. [A] Biochemical transformation of cysteine *via* a stepwise addition, dehydration and oxidation to the thiazole ring. [B] Thiazole-based peptides prepared from diamino diacid building blocks. [C] Biocompatible peptide macrocyclisation and stapling using the condensation reaction between 1,2-aminothiols and heteroaromatic nitriles or the malononitrile derivative TAMM. [D] Phage display peptide stapling with TAMM.

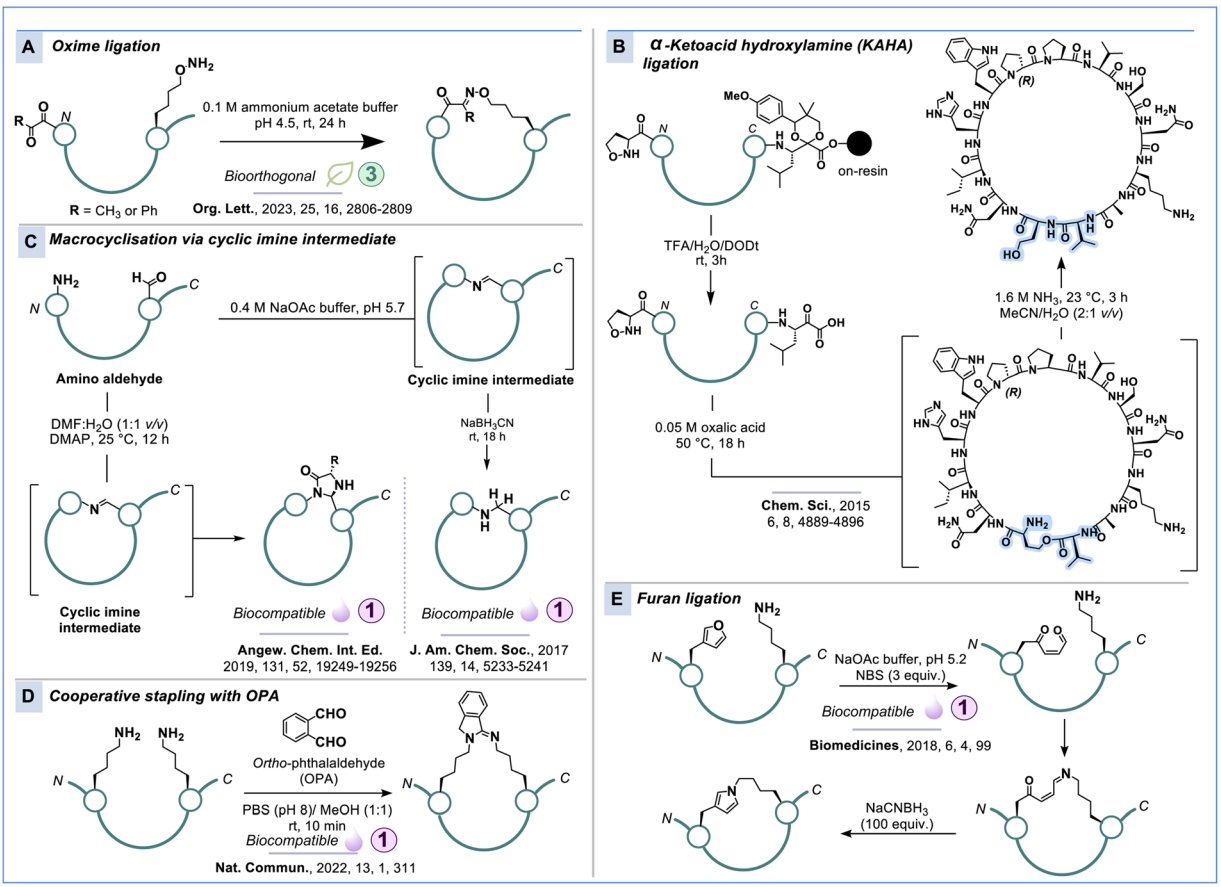
The alternative *m*-cyanopyridyl-alanine (Cpa) can be used directly in SPPS to construct macrocyclic peptides,[205] or the cynaopyridine motif can be constructed on a peptide side chain during SPPS.[206] Cpa has recently also found application in genetically encoded libraries.[207] Additionally, dicyanopyridine reagents can be employed for peptide stapling or to generate bicyclic peptides.[37,208] Cpa can also be selectively incorporated into proteins.[209]

Wu, Tsai & co-workers reported that 2-((alkylthio)(aryl) methylene)malononitrile (TAMM) can also be specific for 1,2-aminothiols.[210] Combination with a chloroacetamide for classic cysteine alkylation delivered a biocompatible handle to staple an N-terminal cysteine and a central cysteine residue which was also applied to phage display (Scheme 8D). Jongkees, Nitsche & co-workers recently reported a similar strategy where they combined the selective reactivity of N-terminal cysteine and non-N-terminal cysteine to construct bicyclic peptide libraries.[211]

Aldehyde and ketone modification

The lack of reactive carbonyl species in canonical amino acids renders aldehydes and ketones alternatives for selective peptide and protein modification. While oxime ligation has been extensively investigated for bioconjugation, it remains relatively underexplored in the context of peptide macrocyclisation, especially concerning genetically encoded peptide libraries. Oxime ligation involves the reaction between an aldehyde or ketone with a hydroxylamine to create a stable oxime bond (C=N–O) in *E* and *Z* isomers (Scheme 9A).[212,213] Compared to general bioconjugation and peptide conjugation,[214,215] examples of peptide stapling and macrocyclisation *via* oxime ligation are scarce in the literature.[216–218] We have demonstrated a peptide cyclisation strategy utilising intramolecular oxime ligation between an alkoxyamine amino acid and an N-terminal α-ketoamide (Scheme 9A).[219]

A related approach involving the use of α-ketoacids at the C-terminus and hydroxy- or alkoxyamines at the N-terminus is the α-ketoacid-hydroxylamine (KAHA) ligation, a method that has been extensively investigated by Bode & co-workers for the chemical synthesis of proteins (Scheme 9B).[220] KAHA ligation offers the advantage of forming a stable amide bond without requiring any additives or coupling reagents. In the context of synthesising peptide macrocycles, Bode & co-workers described the *in situ* cyclisation of linear, unprotected peptides using KAHA ligation, employing (*S*)-5-oxaproline as the hydroxylamine moiety (Scheme 9B).[221] Although KAHA ligation has not yet been integrated into display screening platforms, its ability



Scheme 9 Peptide macrocyclisation techniques based on ketone and aldehyde reactivity. [A] Oxime ligation. [B] KAHA ligation. [C] Reductive amination and "CyClick". [D] OPA stapling. [E] Ligation chemistry based on oxidation of furan to an aldehyde (keto–enal) and subsequent reduction to produce a pyrrole ring with lysine.

to operate under mild aqueous conditions holds promise for biocompatible peptide macrocyclisation.

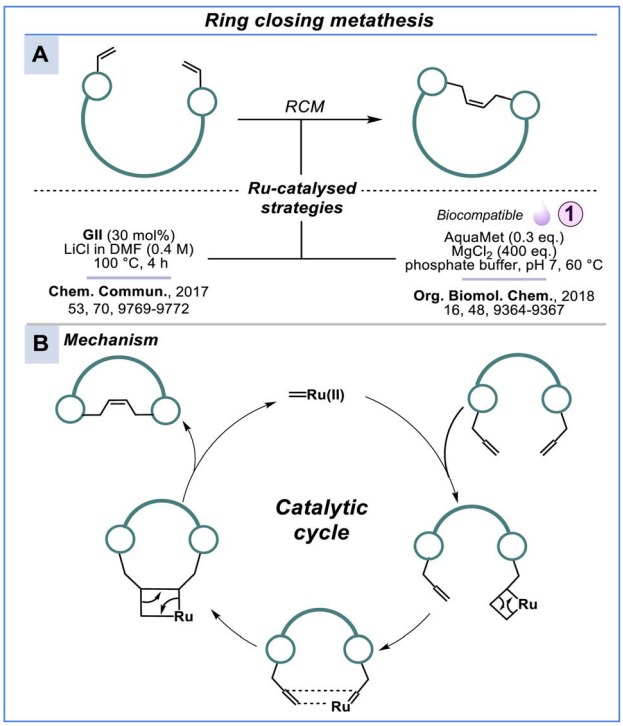
While imines formed through the reaction of aldehydes or ketones with amines are typically transient under biocompatible conditions, they can serve as crucial intermediates for the creation of more stable linkages. Baran, Malins & co-workers have harnessed this phenomenon to synthesise macrocyclic peptides through reductive amination, employing the biocompatible reducing agent sodium cyanoborohydride (Scheme 9C).[222] If the imine intermediate is not trapped by hydride or other externally added nucleophiles, it can engage in a selective intramolecular macrocyclisation with the N-terminus, as reported by Raj & co-workers.[223,224] This "CyClick" strategy forms a 4-imidazolidinone heterocycle from the N-terminal amine of a peptide and an aldehyde (Scheme 9C). Raj & co-workers have further expanded on the use of nitroalkane tethers for the site-specific stapling of aldehydes within peptide chains. This reaction occurs under physiological temperature and pH conditions and has been applied for late-stage peptide bioconjugation and cyclisation.[225] Recently, the same group reported an arene triazene peptide macrocyclisation strategy which is rapid, reversible and biocompatible.[226] Furthermore, this technique introduces an inherent chromophore at the cyclisation site, facilitating peptide detection by UV absorption.

Chen & co-workers have also explored peptide stapling through the utilisation of aldehydes and basic amino acid side chains. Initially, they devised a macrocyclisation method that involves lysine residues in an unprotected peptide, using an excess of formaldehyde with the assistance of nearby tyrosine or arginine residues in close proximity (referred to as KaY/KaR stapling).[227] Although not totally biocompatible, the reaction proceeds under milder conditions and has excellent residue and position selectivity. Further progress towards biocompatible macrocyclisation by Chen & co-workers led to amine crosslinking in a peptide chain using *o*-phthalaldehyde (OPA).[228] This method, an adaptation of the classic OPA–amine–thiol condensation, leverages either two lysine residues arranged side chain-to-side chain or one lysine residue in combination with an N-terminal amine (head-to-side chain) within a peptide chain. Both amines readily condense with OPA to generate an isoindolin-1-imine linker in aqueous buffer at pH 8 (Scheme 9D). Bell & Malins recently reported lysine stapling with 2,6-pyridinedialdehyde linkers and reductive amination.[229]

Furan-based side chains in peptides can undergo oxidation and ring-opening to generate aldehydes or ketones, which can subsequently be further modified, such as for macrocyclisation. Madder, Suga & co-workers developed an *in vitro* translation system for furylalanine, which can react with a lysine side chain to eventually form a pyrrole ring in the absence of competing nucleophiles (Scheme 9E).[230] Recently, this strategy was further expanded by incorporating hydrazine or alkoxyamine-based amino acids in close proximity to the furan moiety.[231]

Ring closing metathesis

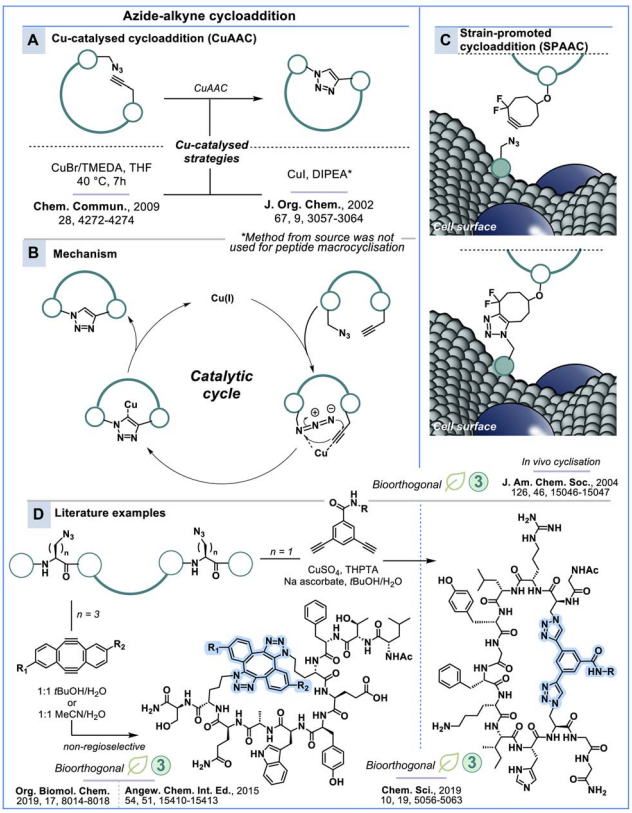
Like the example involving furylalanine, incorporating non-canonical amino acids offers an additional level of chemical orthogonality, enabling, for example, ring closing metathesis (RCM) and azide–alkyne cycloadditions between peptide side chains. Peptide macrocycles with all-hydrocarbon crosslinks accessed by RCM have emerged as useful surrogates of disulfide-bonds and for site-selective peptide stapling (Scheme 10). In pioneering studies, Miller and Grubbs[232,233] installed a rigid substructure into a peptide framework and Clark and Ghadiri[234] prepared β-sheet-like cylinders to explore supramolecular structures that exploit self-assembly of peptide aggregates. Yoshiya & co-workers[235] utilised a water-soluble Ru-catalyst called AquaMet[236] to transform this chemistry towards biocompatible conditions. RCM was performed by incubating unprotected peptides in a neutral buffer followed by the addition of AquaMet at 60 °C for 2 h. While 60 °C might prevent certain applications, this work certainly lays the foundation for biocompatible peptide macrocyclisation using RCM. Work towards olefinic ligation in protein models have been a research of interest of Davis & co-workers[237–239] and have previously been reviewed.[240,241]



Scheme 10 Ring closing metathesis. [A] All-hydrocarbon-crosslink strategies using ruthenium-based catalysis (Grubbs). [B] Simplified catalytic mechanism of a Ru-catalysed RCM reaction.

Azide–alkyne cycloaddition

The pioneering work of Meldal & co-workers[242] and Sharpless & co-workers[243] on the Cu-catalysed [3 + 2]-azide–alkyne cycloaddition (CuAAC) has evolved into an important synthetic strategy to link molecules *via* a [1,2,3]-triazole heterocycle. The triazole heterocycle itself has made significant contributions to a diverse array of bioactivities and has generated substantial interest as a distinctive bioisostere.[244,245] Bertozzi & co-workers



Scheme 11 The azide–alkyne cycloaddition in peptide macrocyclisation and bioconjugation. [A] Cu-catalysed azide–alkyne cycloaddition (CuAAC) [B] The mechanism of CuAAC. [C] Strain-promoted azide–alkyne cycloaddition (SPAAC) on the cell surface. [D] Examples for CuAAC and SPAAC in biocompatible/bioorthogonal peptide stapling.

developed a copper-free, strain-promoted [3 + 2]-azide–alkyne cycloaddition (SPAAC), which has emerged as immensely important reaction in the field of bioconjugation, particularly involving living systems.[246] Meldal, Sharpless and Bertozzi were jointly recognised for the development of 'click chemistry and biorthogonal chemistry' with The Nobel Prize in Chemistry in 2022 (Scheme 11).[41,247–249]

In its classic form, the 1,3-cycloaddition reaction involves a 1,3-dipole and a dipolarophile motif, where the dipolarophile and 1,3-dipole participate in a concerted pericyclic mechanism to form a five-membered ring *via* a [3 + 2]-cycloaddition.[250] In CuAAC, an alkyne and azide serve as the dipolarophile and 1,3-dipole, respectively, catalysed by Cu(I) to selectively form the 1,4-substituted product heterocycle (Scheme 11B).[242,243] While CuAAC is commonly used for conjugation, its reliance on copper makes it incompatible with living systems.[251] Furthermore, copper can non-specifically bind to and precipitate proteins,[252,253] hence, complicating protein conjugation and peptide modifications for screenings in the presence of drug targets. To address these challenges, Bertozzi & co-workers[246] developed copper-free SPAAC inspired by the early works of Wittig and Krebs.[254] They demonstrated that live cells displaying *N*-azidoacetyl sialic acid (SiaNAz) on the cell surface can be conjugated to a cyclooctyne–biotin probe (Scheme 11C). Further advancement of SPAAC employed difluorinated cyclooctyne (termed DIFO) to dramatically accelerate the reaction rate.[255,256]

CuAAc and SPAAC have been used extensively in macrocyclisation and stapling of flexible peptides to render them with defined bioactive conformations (Scheme 11D). Spring & co-workers have stabilised linear diazido peptides in defined α-helical conformations by reacting them with dialkynyl linkers in the presence of Cu-catalyst.[257] This 'double click' stapling approach conferred improved biophysical and pharmacological properties of peptides. Further, they have employed the SPAAC reaction for the *in situ* generation of stapled p53-derived peptides binding to MDM2 in cell culture (Scheme 11D).[36,258] The prototype double-SPAAC derived peptide displayed improved helicity, proteolytic stability and binding affinity. A follow-up study aimed at improving the water solubility of the staples by introducing permanent charges.[259] These peptides displayed low nanomolar affinity and were also found to penetrate cells, thus highlighting the versatile utility of this bioorthogonal macrocyclisation chemistry.

Conclusion & future perspectives

Peptides, whether in their linear or macrocyclic forms, have evolved into promising pharmaceuticals. The discoveries of insulin, ACTH, salmon calcitonin, oxytocin and other constrained peptides ignited significant interest in macrocyclic peptide therapeutics during the early days of drug discovery. The successful translation of numerous peptide drugs into clinical use in recent decades has driven advances in peptide chemistry beyond natural amide and disulfide bond formation.

Researchers have directed their efforts towards alternative strategies for macrocyclisation and late-stage modification to enhance the pharmacokinetic properties of peptides. With the recent broad recognition of click chemistry and bioorthogonal chemistry, we anticipate a surge in research in the years to come, driving further advancements in biocompatible techniques for peptide macrocyclisation and expanding the toolkit of chemical methods for protein and cellular ligation.

With the rise of biological platforms employing mRNA, yeast surface and phage display, there is a growing demand for biocompatible chemistry to synthesise large libraries of cyclic peptides. Attaining mild, selective and orthogonal cyclisation on the nano- and picomolar concentration scale has become an essential requirement. Fortunately, significant progress has been made in the development of biocompatible ligation techniques which we have highlighted here.

In addition to the primarily chemical strategies discussed in this perspective, there has been significant interest in enzyme-mediated macrocyclisation,[260,261] expressed protein ligation[262,263] and SICLOPPS for peptide macrocyclisation within living cells.[40,63] The field of chemoenzymatic ligation has experienced rapid advancements, notably with non-ribosomal peptide synthetases,[264] asparaginyl endopeptidases[265] and subtiligase derivatives,[266] allowing for the cyclisation of various classes of peptides under physiological conditions. While the details of these biochemical methodologies are beyond the

scope of this perspective, it is evident that they carry significant potential for broadening the horizons of biocompatible peptide macrocyclisation.

Data availability

The datasets supporting this article have been uploaded as part of the ESI (Table S1†).

Author contributions

J. H., P. G. and C. N. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

Abbreviations

ACT	Activating group
ACTH	Adrenocorticotropic hormone
ACYP	Acylphosphatase-2
Ade	Adenine
CAS	Chemical Abstracts Service
CBT	2-Cyanobenzothiazole
CDI	Carbonyldiimidazole
Cpa	*m*-Cyanopyridyl-alanine
CuAAC	Copper(I)-catalysed azide–alkyne cycloaddition
DBMB	2,4-Dibromo-5-methoxybenzaldehyde
Dbz	Diaminobenzoic acid
DEBPT	(3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one)
DFS	Decafluoro-diphenylsulfone
DIPEA	*N*,*N*-Diisopropylethylamine
DKP	Diketopiperazine
DMF	Dimethyl formamide
DPPA	Diphenyl phosphoryl azide
fAr	Perfluoroarenes
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GII	Second generation Grubbs' catalyst
HEPES	*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
HBTU	Hexafluorophosphate benzotriazole tetramethyl uronium
HCTU	2-(6-Chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HMGA1a	High-mobility group protein
HOBt	Hydroxybenzotriazole
IL-25	Interleukin 25
α-KAHA	Alpha-ketoacid-hydroxylamine
KaY/KaR	Lysine–formaldehyde–tyrosine/lysine–formaldehyde–arginine
MeNbz	*N*-Methyl benzotriazole
MOrPH-PhD	Macrocyclic organo-peptide hybrid phage display
MUC1	Mucin 1
NCL	Native chemical ligation
NMP	*N*-Methyl-2-pyrrolidone
O2beY	*o*-(2-Bromomethyl)tyrosine
OPA	*o*-Phthalaldehyde
PEG	Polyethylene glycol
RaPID	Random nonstandard peptide integrated discovery
RCM	Ring closing metathesis
RNA	Ribonucleic acid
SiaNAz	*N*-Azidoacetyl sialic acid
SICLOPPS	Split-intein circular ligation of peptides and proteins
SPAAC	Strain-promoted azide–alkyne cycloaddition
SPhR	Staudinger phosphonite reaction
SPPS	Solid phase peptide synthesis
STL	Serine/threonine ligation
TAMM	2-((Alkylthio)(aryl)methylene)malononitrile
TeTEx	Tetrazine–thiol exchange
TCEP	Tris(2-carboxyethyl)phosphine

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