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Contemporary strategies for peptide macrocyclization

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Peptide macrocycles have found applications that range from drug discovery to nanomaterials. These ring-shaped molecules have shown remarkable capacity for functional fine-tuning. Such capacity is enabled by the possibility of adjusting the peptide conformation using the techniques of chemical synthesis. Cyclic peptides have been difficult, and often impossible, to prepare using traditional synthetic methods. For macrocyclization to occur, the activated peptide must adopt an entropically disfavoured pre-cyclization conformation before forming the desired product. Here, we review recent solutions to some of the major challenges in this important area of contemporary synthesis.

Ring topology has captivated the imagination of chemists for many years. Of the various bioactive macrocycles known so far, cyclic peptides are of particular significance. In contrast to linear peptides, cyclic variants are more resistant to both exo- and endoproteases[1], which explains the significant therapeutic potential of this class of molecules. The discovery of Gramicidin S in 1944 by Gause and Brazhnikova was the pivotal point in the history of cyclic peptides[2]. Shortly after its discovery in the midst of the Second World War, Gramicidin S was widely used in the treatment of septic gunshot wounds. Since then, the cyclic peptide class of compounds has experienced sustained growth with thousands of cyclic peptides now known. Many of them have been used as therapeutic agents. Examples include octreotide, calcitonin, cyclosporine A, nisin, polymixin and colistin[3].

This Review explores the increasing interest in synthetic macrocyclic peptides. Many members of this vast class of molecules have shown remarkable properties and the capacity for functional fine-tuning. For instance, the receptor selectivity displayed by the RGD (arginine–glycine–aspartic acid) peptide fragment varies dramatically depending on the size of the RGD-containing macrocycle[4]. In another development, the 'stapled peptide' technology applied to the design of inhibitors of the MCL-1 protein delivered potent and cell-permeable peptide molecules with unprecedented selectivity for a target that has been considered 'undruggable' for many years[5].

Synthetic macrocycles are projected to experience exponential growth[6]. Large rings (in the 600–1,500 molecular weight range) equipped with amino acid residues are well suited for capturing extended interactions characteristic of difficult targets, such as protein–protein interactions. Many cyclic peptides are notoriously difficult to prepare. In this Review, we have considered emerging methods directed towards the syntheses of peptide macrocycles. These methods extend well beyond amide bond formation, although amino acid residues are the major components of all molecules discussed herein. In fact, it is the amino acid constituents that are responsible for the major challenges in peptide macrocyclization. In the case of small-to-medium-sized rings, the ground-state *E* geometry of the peptide bond prevents the peptides from attaining the ring-like conformation conducive to cyclization. Larger ring sizes do not pose this problem because they can accommodate *E* peptide bonds. However, synthesis of large macrocycles still requires operationally complex conditions to avoid intermolecular reactivity.

Over the years, several review articles have been published on the synthesis and biological properties of cyclic peptides[7–11]. Herein contemporary synthetic strategies that have emerged in the past 10–15 years will be discussed, with a focus on mechanistic aspects of synthetically useful processes. After a brief mention of general approaches to synthesis, we outline various conformational aspects that have been explored to facilitate peptide macrocyclization. These range from structural elements to external metal-ion promoters. We cover macrocyclizations mediated by sulfur-containing functional groups, ring-contraction strategies, azide–alkyne cycloadditions, ring-closing metathesis, and multicomponent macrocyclization strategies. We conclude with applications that have been designed for the synthesis of cyclic peptide libraries. Metal-catalysed cross-couplings and macrocyclizations of linear precursors that contain linkages other than peptide bonds are beyond the scope of this Review.

General synthetic considerations

Depending on its functional groups, a peptide can be cyclized in four different ways: head-to-tail (C-terminus to N-terminus), head-to-side chain, side chain-to-tail or side-chain-to-side-chain (Fig. 1a).

Of the various methods of synthesizing cyclic peptides, most often the final ring-closing reaction is a lactamization[12], a lactonization[13] or the formation of a disulfide bridge. For example, an effective side-chain-to-side-chain macrocyclization involves a condensation reaction between side chains of aspartic or glutamic acid and lysine residues[14]. Macrocyclizations are best performed under high dilution (typically submillimolar concentrations) to minimize unwanted intermolecular processes such as oligo- and polymerizations. However, these reaction set-ups are often rather sophisticated, involving one or several syringe pumps[15].

Anchoring a reactive molecule to an insoluble polymer can create a pseudodilution phenomenon[16,17]. Functional groups attached to an insoluble polymer are less prone to encounter one another relative to independent molecules in solution that can freely diffuse. The main advantage of solid-supported macrocyclizations is that simple washing and filtration are often enough for purification. To cyclize peptides on a solid support, the linear precursor is most commonly anchored to the support through the side chain of a trifunctional amino acid such as aspartic or glutamic acid. A protecting-group strategy of at least three dimensions of orthogonality is required to construct the linear peptide, deprotect

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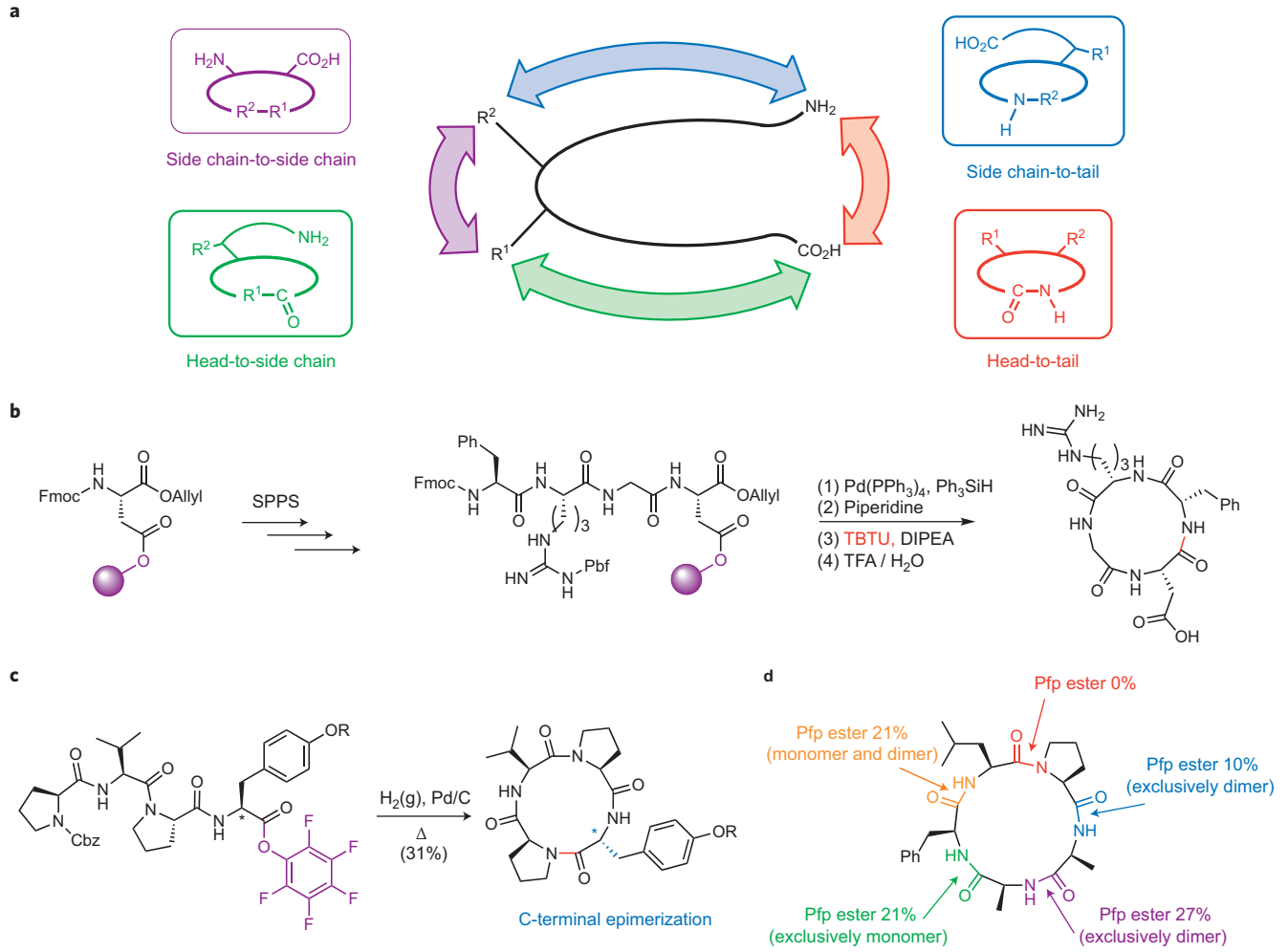

Figure 1 | General synthetic considerations for peptide macrocyclization. a, The four possible ways a peptide can be constrained in a macrocycle. **b**, The solid-phase synthesis of an RGD-containing cyclic tetrapeptide starting from aspartic acid that is bound to the solid support by its side chain, using a protecting-group strategy with three dimensions of orthogonality. **c**, The problem of C-terminal epimerization during the cyclization of the tetrapeptide H-Pro-Val-Pro-Tyr-OPfp. The Cbz-deprotection–cyclization reaction was carried out at a concentration of 0.001 M (Cbz, benzyloxycarbonyl). **d**, All possible ring disconnections of *cyclo*-[Pro-Ala-Ala-Phe-Leu] showing the isolated yields of the combined cyclomono- and/or dimerization through Pfp ester activation. Cylizations were performed in a $CHCl_3$/1 N aq.$NaHCO_3$ (2:1) solution at a concentration of 0.0009 M.

the N- and C-termini, cyclize in a head-to-tail fashion, and finally cleave the product from the solid support[18]. For example, RGD-containing tetrapeptides can be effectively cyclized in a head-to-tail fashion without cyclo-oligomerization by-products[19] (Fig. 1b). Finn's recent study of selective cyclodimerization driven by intramolecular click chemistry on solid-supported peptides makes use of intermolecular reactivity[20]. A myriad of synthetic methodologies have been developed in recent years for the macrocyclization of peptides on solid supports as this area of chemistry has been actively explored[21].

Ring size is the most important factor that governs the success of a head-to-tail peptide macrocyclization. Despite their reputation in the ethos, the cyclization of large peptides containing more than seven amino acids is not problematic and is generally straightforward. However, smaller peptides can often be troublesome, if not impossible, to cyclize. A seminal study by Schmidt and Langner showcased the challenges associated with small peptide cyclizations[22]. They attempted to synthesize various naturally occurring cyclic tetra- and pentapeptides in a head-to-tail fashion. These attempts met predominantly with failure, producing the products of cyclodimerization, trimerization and C-terminal epimerization. For instance, in their attempts to synthesize *cyclo*-[Pro-Val-Pro-Tyr], a known tyrosinase inhibitor, Schmidt and Langner were unable to prepare the all-L-isomer (see end of paragraph for abbreviations). Instead, 31% of the C-terminal epimerization product *cyclo*-[Pro-Val-Pro-D-Tyr] was obtained from the corresponding pentafluorophenyl (Pfp) ester (Fig. 1c). The authors also investigated all possible ring closures of the all-L-cyclic pentapeptide *cyclo*-[Pro-Ala-Ala-Phe-Leu] through Pfp ester activation, and observed a strong sequence dependence for successful cyclization. Only cyclization of H-Phe-Leu-Pro-Ala-Ala-OH resulted in the formation of the monomeric cyclic species, albeit in 21% yield (Fig. 1d). This example demonstrates the difficulties that arise in a seemingly trivial retrosynthetic analysis of a cyclic peptide. The ring disconnection must be chosen carefully and several guidelines have been developed to aid in this[23]. For example, the yield can be optimized if the site of macrocyclization is not sterically encumbered by *N*-alkyl, α,α-substituted or β-branched amino acids (that is, Val or Ile) and if the macrocyclization occurs between two residues of opposite stereochemical configuration. Furthermore, the incorporation of turn-inducing structural elements embedded midway along the linear precursor can also result in a more efficient macrocyclization.

Conformational elements that help bring the ends together

The success or failure of macrocyclization relies on the ability of a linear precursor to conformationally pre-organize its reactive ends in close spatial proximity before ring closure. Such pre-arrangement creates a high effective molarity and results in fewer by-products from intermolecular processes. The importance of spatial proximity of reactive termini has been explored as early as 1963 when the cyclization tendency of peptides was correlated with measured dielectric increments[24]. Molecular conformation of the linear precursor is responsible for the challenging macrocyclizations of all-L and all-D peptides. These prefer to adopt extended conformations as a result of the tendency to minimize allylic strain[25], placing their reactive termini far away from each other. Over the years, various strategies for directing macrocyclizations using conformational pre-organization have been developed and reviewed[26]. Here, these strategies are classified under two categories: (i) 'internal' conformational elements, which encompass covalent modifications of the peptide chain to facilitate the union of its ends, and (ii) 'external' conformational elements, which involve the use of molecular scaffolds that are neither covalently attached to the peptide, nor consumed during the course of the ring closure. The latter falls into the classification of template-mediated macrocyclizations, an active area of chemical synthesis.

Internal conformational elements. In the macrocyclization of peptides, the ring-closure process is favoured when the various structural elements of a linear precursor can accommodate the angular requirements for both termini in the transition state with the least amount of strain[27]. In their study of end-to-end loop-closure kinetics in unfolded polypeptide chains, Daidone and Smith concluded that the loop-closure kinetics in the longer peptides is determined by the formation of intra-peptide hydrogen bonds and transient β-sheet structure, which accelerates the search for contacts among residues distant in sequence[28]. For chains containing more than 10 peptide bonds, loop-closing rate constants on the 20–100 nanosecond time range were found to exhibit a power-law length dependence. Intramolecular hydrogen bonds were found to lower the free energy of loop closure for longer peptides. The observation of a roll-over to slower kinetics and the absence of intra-peptide hydrogen bonds for the shorter peptides provided evidence of intrinsic stiffness of the short polypeptide chains.

To help bring the ends of a linear peptide together, chemists have looked to the secondary protein structures, particularly reverse turns[29]. An elegant way of achieving a minimal end-to-end distance is to introduce a *cis*-amide bond in the middle of the peptide chain, thus forming a motif analogous to a β-turn. By accommodating both the *cis* and the *trans* conformer of the tertiary X_{aa}–Pro amide bond (where X_{aa} represents any L-α-amino-acid), proline has the highest tendency to occur in reverse turns in polypeptides. Although there is little difference between the actual rotational barrier of secondary and tertiary amides, the additional substitution at nitrogen removes a substantial amount of energetic bias of one conformer over the other. *cis*-Amide bonds of proline have been observed in many protein crystal structures. In a classic study, Rothe and co-workers exploited this property in the cyclization of triproline[30].

Linear peptides containing a heterochiral diproline unit are excellent substrates for cyclization due to strong β-hairpin-inducing attributes of the D-Pro-L-Pro template. Using cyclic peptides assembled with the aid of this fragment, Robinson and co-workers were able to accurately reproduce canonical conformations of loops of complementarity-determining regions observed in the crystal structures of antibody fragments[31].

The incorporation of other D-amino acids into all-L peptides is also known to exert turn-inducing effects. Indeed this strategy has been employed to improve the yields of various peptide cyclizations[32,33]. For two diastereomeric short peptide sequences that differ only in α-carbon configuration at the terminal residues, cyclization is favoured for the diastereomer that contains both a D- and an L-residue at its termini[34]. Peptides consisting only of L-residues, devoid of other turn-inducing structures, will often not cyclize until the C-terminal α-carbon has epimerized to the D-configuration[35]. The effect of D-amino acids on peptide macrocyclization have also been modelled theoretically[36].

N-Methyl amino acids have a similar stereochemical impact on the backbone of peptides to that of proline. They have the potential to introduce *cis*-amide bonds into peptide sequences and are well suited to induce β-turns[37]. Their incorporation as turn-inducing elements into cyclic peptides has been well documented by Kessler and co-workers[38,39].

An intriguing example that illustrates the importance of the conformation of linear precursors was documented by Taunton and Deng in their synthesis of the natural product ceratospongamide[40]. This modified cyclic heptapeptide contains two proline residues, a thiazole, and an oxazoline ring. It is biosynthesized as a mixture of two non-interconverting proline amide rotamers. The authors found that the kinetic distribution of the two conformational isomers is governed by the presence or absence of the threonine-derived oxazoline ring in the linear precursor prior to macrocyclization.

Pseudo-prolines are modified heterocyclic amino acids derived from serine and threonine ((4*S*)-oxazolidine-4-carboxylic acid) and cysteine ((4*R*)-thiazolidine-4-carboxylic acid). They were first introduced as structure-disrupting building blocks that prevent the aggregation and self-association of peptides during solid-phase synthesis[41]. They are readily accessible through the acid-catalysed cyclocondensation of serine, threonine or cysteine with an aldehyde or a ketone. When incorporated into a peptide chain, these residues predominantly induce *cisoid* conformations of the amide bond preceding them, establishing type-VI β-turn structures[42]. Several research groups have used these residues as powerful turn-inducing elements in the synthesis of short, constrained cyclic peptides. One of the notable examples is Mutter's synthesis of the cyclic tripeptide *cyclo*-[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] from H-Pro-Pro-Thr($\Psi^{Me,Me}$pro)-OH (Fig. 2a)[43]. This peptide was shown to instantaneously cyclize with PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) in high yields, free of oligomeric structures and at concentrations as high as 0.1 M.

An added benefit of using pseudo-prolines as conformational turn inducers is that following cyclization, they can be cleaved under acidic conditions to free the respective serine, threonine or cysteine residue and thus yield cyclic peptides devoid of turn-inducing elements. Jolliffe and co-workers have applied this strategy to the cyclization of H-(Val-Thr)$_3$-OH, a hexapeptide lacking turn-inducing elements that cannot be conventionally cyclized in a head-to-tail fashion (Fig. 2b)[44]. The authors were also able to show that linear tetrapeptides containing two alternating pseudoproline residues can be cyclized in a head-to-tail fashion to give constrained all-L cyclic tetrapeptides (Fig. 2c)[45]. It should be noted that as useful as pseudoprolines are, one of their major drawbacks is that NH pseudoprolines are extremely difficult to acylate due to their steric congestion. To synthesize these *N*-acylated heterocycles, the most typical approach is to first prepare the dipeptide fragment (containing serine or threonine) followed by formation of the pseudoproline.

External conformational elements. External templates for assisting peptide macrocyclization operate on the basis of a site-isolation mechanism. Polymeric scaffolds can create reaction cavities large enough for only one linear peptide molecule to enter and cyclize at a time. Such distinct nanoenvironments isolate the peptide from the bulk solution. As a result, the isolation effect that these internal cavities create significantly decreases the likelihood of cyclooligomerization. Van Maarseveen and co-workers have applied this strategy

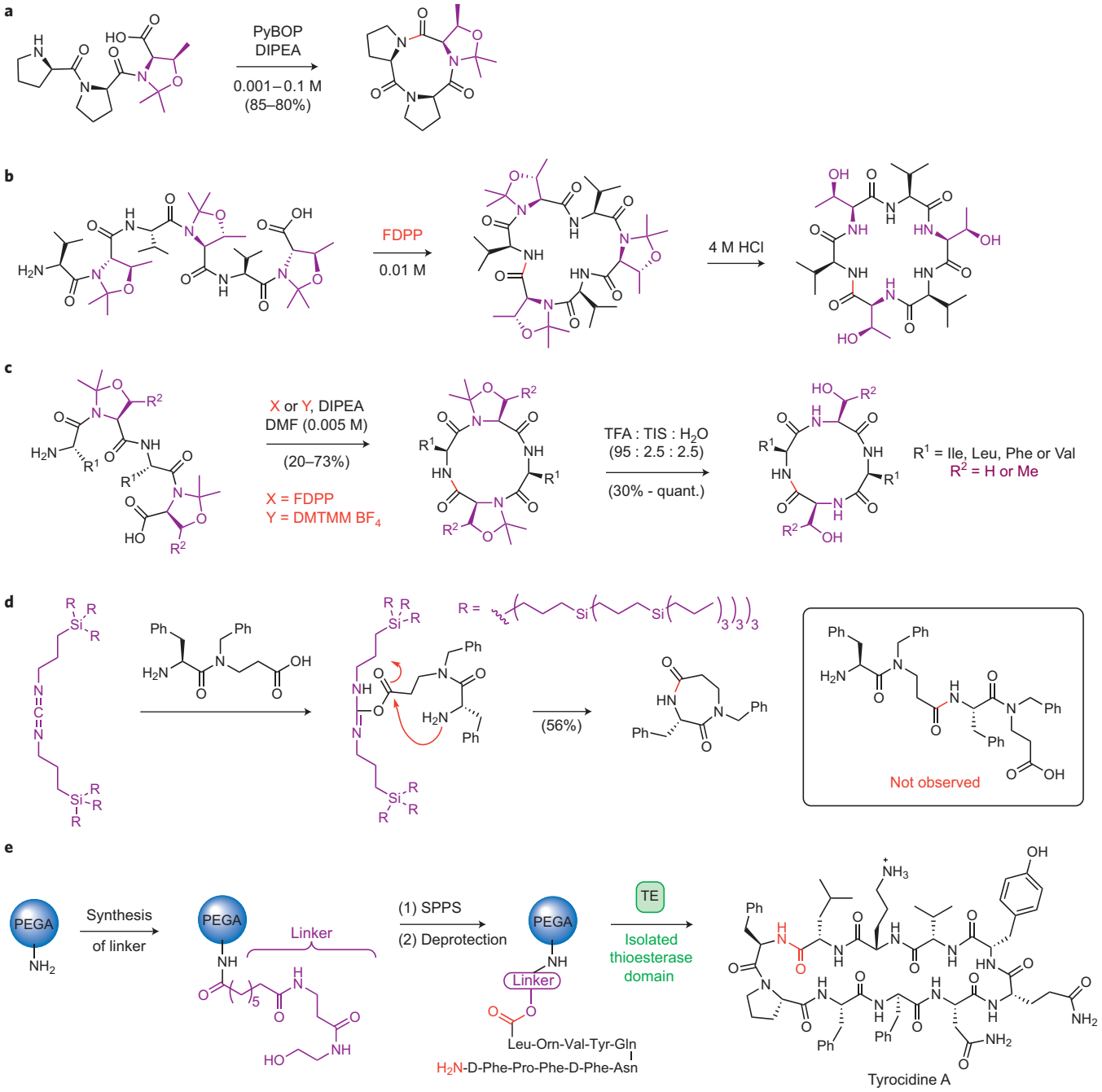

Figure 2 | Conformational control to facilitate peptide macrocyclization. a, Concentration-independent cyclization of a tripeptide equipped with a pseudoproline derived from L-threonine. **b**, The synthesis of a cyclic hexapeptide devoid of turn inducers. A linear precursor with three pseudoproline residues embedded is cyclized with FDPP at a concentration of 0.01M. After cyclization, the turn-inducing pseudoprolines are converted back to the corresponding threonine residues under aqueous acidic conditions. **c**, Synthesis of C2-symmetric, all-L cyclic tetrapeptides containing two alternating pseudoproline residues. After cyclization with either the FDPP or DMTMM BF4 coupling reagents under high dilution (0.005 M), the pseudoprolines are hydrolysed under acidic conditions to liberate the corresponding natural amino acid residues. **d**, Wedge-shaped carbosilane dendrimeric carbodiimides to cyclize homodiketopiperazines through a site-isolation mechanism. The corresponding product of dimerization was not observed. **e**, Isolated thioesterase domains promote a biomimetic synthesis of an antimicrobial cyclic peptide through an enforced precyclization conformation after a transacylation of a solid-supported linear precursor. DIPEA, *N*,*N*-diisopropylethylamine; DMTMM BF4, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate; PEGA, polyethylene glycol polyacrylamide copolymer; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

through the development of carbosilane dendrimeric carbodiimides[46]. These wedge-shaped coupling reagents create a site-isolation effect through their dendritic bulk that traps a lactamization precursor through its C-terminus (Fig. 2d). The authors demonstrated that these dendrimeric carbodiimides could cyclize seven-membered *bis*-lactams (homodiketopiperazines), which are difficult to obtain through traditional lactamization methods.

Tai and co-workers have developed another type of external template for enhancing the difficult macrocyclizations of tetrapeptides. Their method is based on molecularly imprinted cavities[47]. This

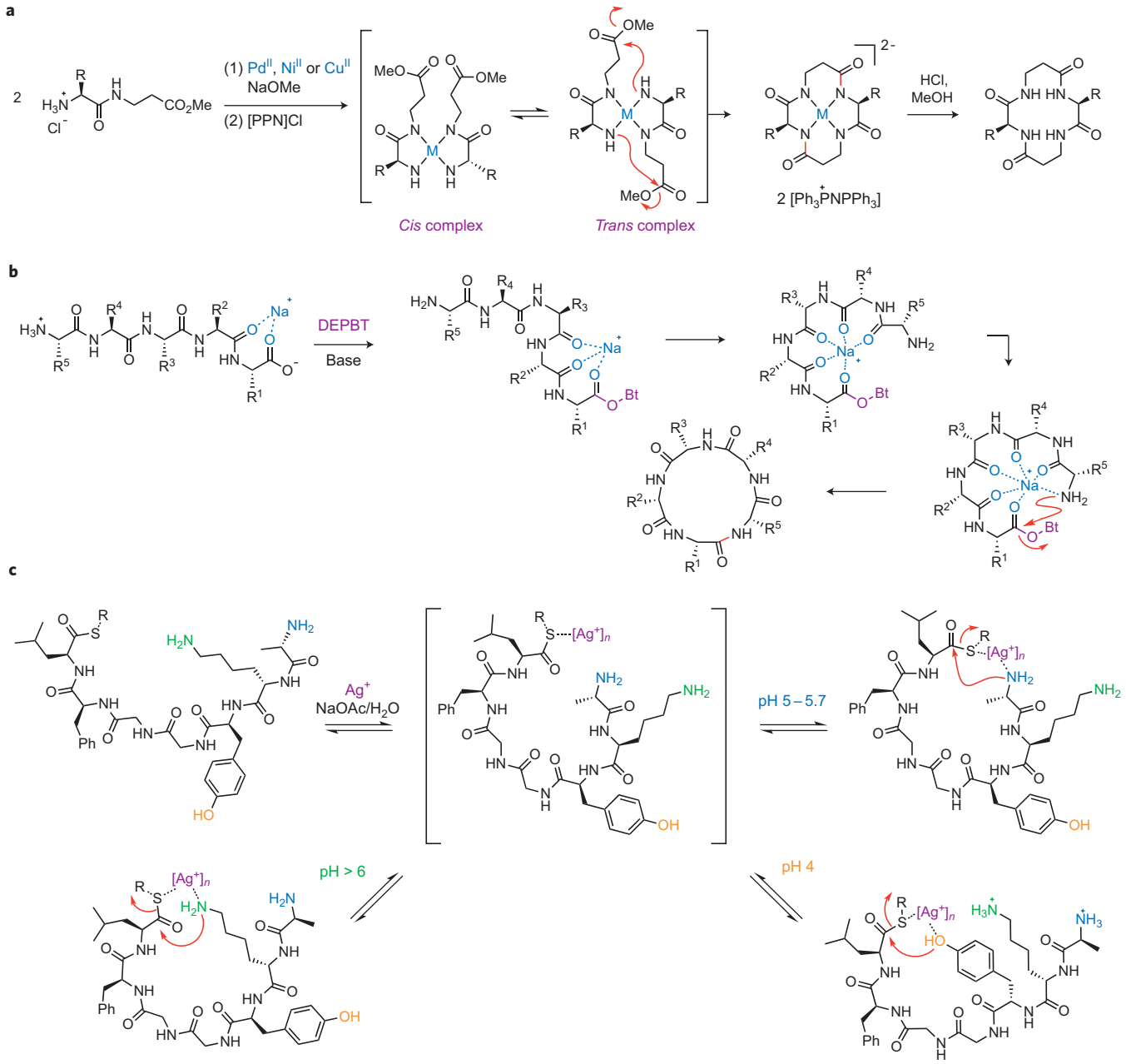

Figure 3 | The use of metal ions to assist in the macrocyclization of peptides. a, Transition metal-mediated cyclodimerization of unactivated dipeptide esters. **b**, Sodium ions used to enhance the head-to-tail cyclization of a peptide through tandem coordinations of amidic oxygen atoms along the peptide chain. **c**, pH-dependent chemoselectivity in the Ag^+ ion-mediated macrocyclization of a peptide thioester. At pH 4, the amino groups of the N-terminus and lysine side chain are protonated and macrocyclization with the phenolic nucleophile of the tyrosine residue is favoured. Head-to-tail cyclization was observed to be favoured between pH 5–5.7. Above pH 6, lactamization with the ε-amine of the lysine residue becomes significant. DEPBT, 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one; PPN; *bis*(triphenylphosphine)imminium.

strategy is built on maintaining a turn structure with *cis*-amide conformations of a linear peptide enforced by a nano-sized cavity.

Many macrocyclic peptides in nature are the products of non-ribosomal biosynthesis. Non-ribosomal synthetases are known to tether the activated linear intermediates through thioester linkages in a way that is analogous to solid-phase synthesis[48]. Walsh and co-workers described a method in which isolated thioesterases promote macrocyclization of linear peptides immobilized on synthetic solid supports[49]. The mechanism involves transacylation to an active-site serine followed by deacylation on intramolecular attack by the amino-terminal nucleophile. This study shows that isolated thioestrerases can enforce solid-phase-bound thioester- and ester-linked linear peptides into pre-cyclization conformation. This versatile chemoenzymatic approach allows production of potent antimicrobials (Fig. 2e). Suga's combined use of a tRNA acylation ribozyme and a reconstituted cell-free translation system has been used towards making peptide macrocycles linked by a thioether bond[50].

Metal-ion-assisted cyclizations

Another external, non-covalent auxiliary-based strategy that has been employed to facilitate macrocyclization of peptides involves the use of metal ions. Inspiration for this strategy is rooted in the knowledge that several naturally occurring cyclic peptides such as

gramicidin, valinomycin and anatamanide are potent ionophores and form stable complexes with metal ions *in vivo*. One of the first examples of the use of metal ions to conformationally pre-organize a peptide for macrocyclization was demonstrated by Beck and co-workers[51] (Fig. 3a). They found that C_2-symmetric cyclic tetrapeptides could be constructed through a metal-ion-mediated dimerization of two unactivated dipeptide methyl esters under basic conditions. A prerequisite for the double head-to-tail lactamization is that both dipeptide esters must first coordinate to the metal centre in a *trans* fashion (in equilibrium with the *cis* complex), thus allowing nucleophilic attack of a coordinated amino group on the neighbouring dipeptide ester. This cyclodimerization method encompasses various ring sizes ranging from 12- to 18-membered cyclic peptides (from α and β amino acids) and does not require high dilution, protecting or activating groups, or coupling reagents. The metal-coordinated dianions can be isolated by precipitation with an appropriate cation and the metal ion can be exchanged for a proton by acidic methanolysis.

Various alkali metals have been shown to enhance the cyclization of peptides. For instance, lithium salts, which were first reported to enhance the solubility of peptides in organic solvents[52], were shown by Robey and co-workers to mediate the selective cyclization of several *N*-chloroacetylated, *C*-cysteine peptides that mimic the C_4 domain of the envelope glycoprotein gp120 found in HIV-1 (ref. 53). Under aqueous conditions, these peptides were shown to only polymerize in a head-to-tail fashion. However in a LiCl/dimethyl formamide solvent mixture, exclusive monomeric cyclization was observed through a nucleophilic displacement of the N-terminal chloroacetyl moiety by the C-terminal cysteine thiol. Ye and co-workers have shown that sodium ions are suitable for promoting the cyclization of linear pentapeptides, whereas larger caesium ions can effectively promote the cyclization of heptapeptides[54,55]. Supported by molecular mechanics calculations, Ye and co-authors propose that these alkali metal ions promote peptide macrocyclization by initially binding the carbonyl and amide groups at the C-terminus of a peptide. Through additional coordination of amidic oxygen atoms along the chain, these ions induce the linear peptide to form a strong turn structure, thus bringing the N- and C-termini in close proximity for cyclization (Fig. 3b).

Tam and Zhang have also exploited the ability of metal ions to facilitate peptide macrocyclizations[56]. Inspired by the S >> N > O order of affinity for silver(I) and seminal work by Blake and Li on enthalpic activation of thioesters for effective segment coupling[57], Tam and Zhang were able to show that linear peptide thioesters can be readily cyclized in the presence of three or more equivalents of Ag^+ ions in aqueous buffered solutions. In this application, Ag^+ ion plays a dual role as an entropic and enthalpic activator for the macrocyclization. By coordinating both the C-terminal thioester and N-terminal amine, Ag^+ captures the reactive functionalities in close proximity for an acyl-transfer ring closure. The thiophilic nature of Ag^+ generates the enthalpic activation on complexation with the thioester, making it a better leaving group and thus facilitating the acyl-transfer reaction. Unprotected peptides can be cyclized individually or as a mixture of peptides in solution[58]. When tyrosine and lysine, which are competitive nucleophilic residues, are incorporated into the backbone of the peptide, the chemoselectivity of the macrocyclization becomes dependent on the pH of the solution (Fig. 3c). However, like any method of macrocyclization that proceeds through a highly activated C-terminal ester, epimerization can be a big problem with Ag^+-ion-promoted activation and this problem is only magnified if the ring closes slowly.

Sulfur-mediated cyclizations

Many synthetic efforts towards the macrocyclization of peptides have taken their inspiration from nature. With the continuing development of state-of-the-art reagents for peptide couplings, these biomimetic approaches employ thioester activation of a C-terminal carbonyl group.

Inspired by the prevalent role imidazole plays in the catalysis of the hydrolysis and transfer of activated acyl groups, Houghten and co-workers have developed a method for the head-to-tail synthesis of cyclic peptides by the direct aminolysis of peptide thioesters in the presence of imidazole[59] (Fig. 4a). The role of imidazole is thought to be that of a nucleophilic catalyst, attacking the carbonyl group of the thioester to form a reactive acyl imidazolyl intermediate, which is subsequently intercepted by another nucleophile. Various peptides ranging from 5 to 11 residues in length were cyclized and the rate was found to be dependent on the ring size. Kahalalide B and its analogues were accessed through the imidazole-catalysed macrolactonization between a C-terminal thioester and the side-chain hydroxyl group of a serine residue in good yields[60].

Crich and Sasaki have developed an amide-bond-forming sequence employing peptide thioacids and Sanger's reagent that is viable for the cyclization of penta- and hexapeptides[61]. Treatment of an N-terminal Fmoc-protected and C-terminal 9-fluorenylmethylthioester peptide with piperidine releases a *seco*-thioacid peptide, which cyclizes in the presence of Sanger's reagent at a concentration of 0.005 M (Fmoc, 9-fluorenyl-methoxycarbonyl). The reaction proceeds through an initial S_NAr reaction with Sanger's reagent to generate a reactive thioester *in situ*. This can then be intercepted by the N-terminal amino group. The methodology is compatible with free carboxylic acids and hydroxyl groups. For example, the authors were able to synthesize *cyclo*-(D-Glu-Ala-D-Val-Leu-D-Trp) in 44% yield over three steps from the corresponding protected linear peptide (Fig. 4b). Tetrapeptides were shown not to be amenable to this cyclization strategy as treatment of Fmoc-Ala-Trp-Gly-Phe-SFm afforded only the cyclodimerization product, in 40% yield (SFm, 9-fluorenylmethylthiolate).

Intramolecular stabilization of peptides through side chain–side chain tethering was pioneered by Felix and co-workers[62]. One of the most convenient and straightforward methods for constraining a peptide into a macrocycle is through the bridging of two internal cysteine thiol groups. The resulting intramolecular disulfide bridges can stabilize secondary structure motifs in peptides. An oxidant (atmospheric oxygen, iodine, DMSO or di(2-pyridyl)disulfide) is required to achieve this cyclization. As the linear peptide sequence is constructed in solution or on solid phase, both thiol groups are capped with identical groups that can be orthogonally cleaved prior to cyclization. Often the thiols are protected with groups that allow for their simultaneous cleavage and disulfide formation. For example, the *S*-acetamidomethyl (Acm) and *S*-triphenylmethyl (Trt) protecting groups can both be removed with molecular iodine generating a sulfenyliodide intermediate that can either react with another sulfenyliodide in a disproportionation reaction or be attacked by a protected thiol to generate the disulfide bond *in situ*. Structural stabilization through side-chain tethering is a highly active field of research but goes beyond the scope of this Review.

The cysteine thiol functionality can also be exploited in an intramolecular thiol-ene reaction to generate peptide macrocycles. This side chain-to-tail cyclization strategy was used by Anseth and co-workers[63]. The macrocyclization involves a radical addition of the thiol group of a cysteine residue at the N-terminus of the peptide to the alkene of an allyloxycarbonyl protecting group on the ε-NH_2 group of a lysine residue at the C-terminus (Fig. 4c). The cyclization performed best on solid support but is amenable to solution-phase chemistry requiring lower concentrations (0.002 M).

The 1,2-aminothiol functionality present on an N-terminal cysteine residue has been applied to a range of macrocyclization strategies. One such method involves intramolecular condensation with an aldehyde to form a stable thiazolidine heterocycle[64]. Tam and co-workers demonstrated that unprotected linear peptides containing an N-terminal cysteine residue and an aldehyde

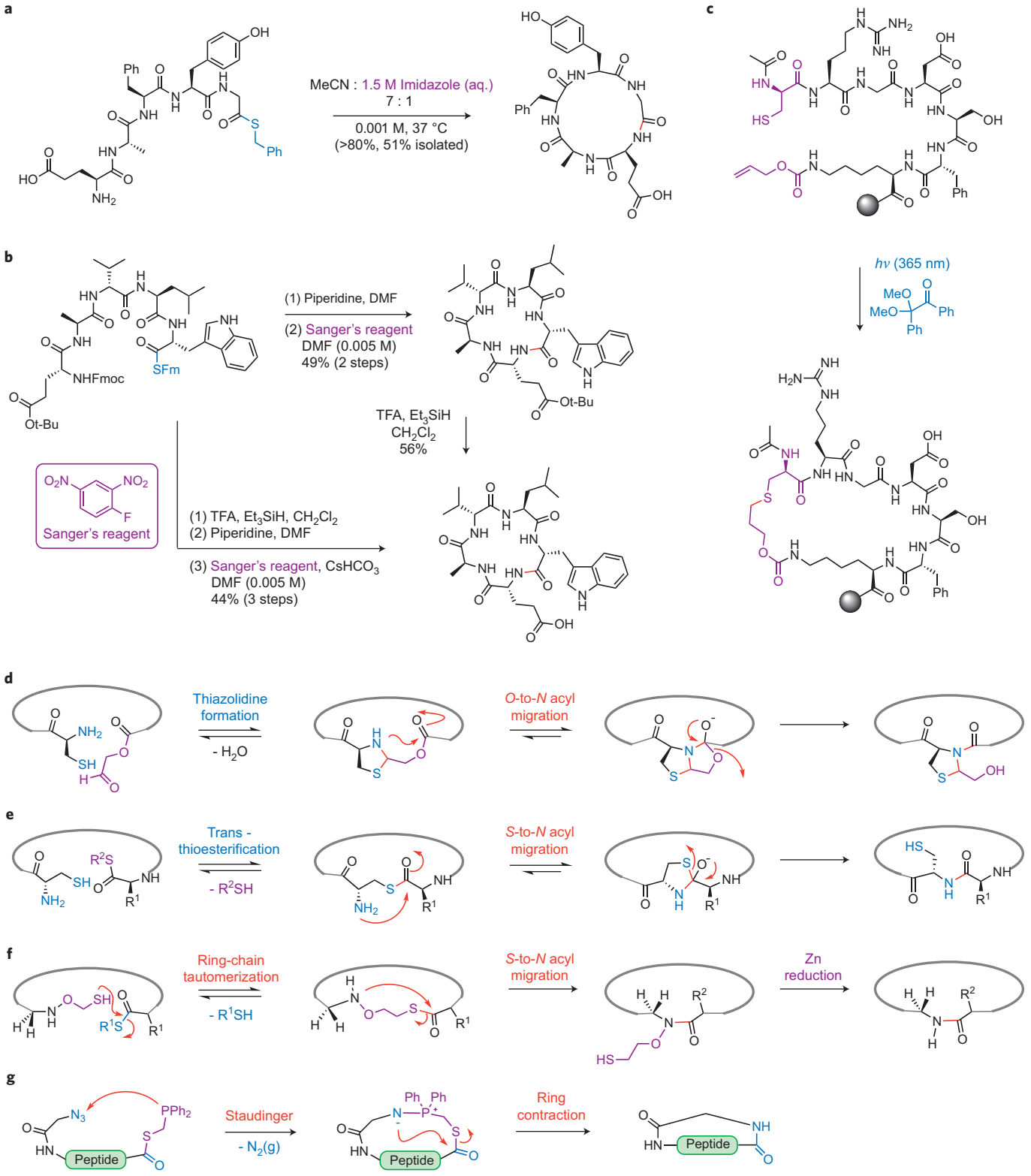

Figure 4 | Peptide macrocyclizations mediated through sulfur-containing auxiliaries. a, The head-to-tail macrocyclization of a pentapeptide thioester catalysed by imidazole. **b**, The head-to-tail macrocyclization of a pentapeptide thioester with Sanger's reagent. The cyclization is compatible with unprotected glutamic acid and tryptophan side-chain residues. **c**, A side chain-to-tail macrocyclization through a thiolene reaction on solid-phase. The cyclization was triggered by the type-I photoinitiator DMPA (2,2-dimethoxy-2-phenylacetophenone) on exposure to 365 nm light. **d**, A head-to-tail macrocyclization of N-terminal cysteine and C-terminal glyceric ester peptides through the formation of a thiazolidine ring followed by an *O*-to-*N* acyl transfer. **e**, Native chemical ligation applied to the head-to-tail cyclization of peptides. An N-terminal cysteine residue undergoes a macrocyclic *trans*-thioesterification with a C-terminal thioester. A subsequent *S*-to-*N* acyl transfer furnishes the desired lactamization product. **f**, A removable N-terminal oxyethanethiol tether applied to the head-to-tail macrocyclization of peptides through a reaction pathway analogous to native chemical ligation without the necessity of an N-terminal cysteine residue. **g**, The head-to-tail macrocyclization of peptides through a traceless Staudinger ligation strategy.

functionality attached to the side chain of a lysine residue readily condense to give side chain-to-tail cyclic peptides embedded with a thiazolidine ring. Furthermore, they were able to show that if the aldehyde was incorporated into the linear precursor as an oxidized C-terminal glyceric ester, then a head-to-tail cyclic peptide can be obtained with an all-amide-bond backbone. This was achieved through a novel ring-contraction mechanism that proceeds via a tricyclic intramolecular rearrangement (Fig. 4d).

1,2-Aminothiols have also found great success in contemporary ligation strategies that rely on a capture/rearrangement mechanism to link two peptide fragments together. In particular, the native chemical ligation vividly displays the power of this mechanism to link peptide fragments under mild conditions[65]. The process involves a reaction between two fragments, one of which is a weakly activated C-terminal thioester, and the other an unprotected N-terminal cysteine residue. The thermodynamic strength of an amide bond over a thioester is the driving force behind this reaction, made possible through a proximity-driven *S*-to-*N* acyl migration. Tam and Pallin were able to show that C-terminal peptide thioesters containing an N-terminal cysteine residue readily cyclized in a head-to-tail fashion (Fig. 4e)[66]. The macrocyclization could be performed in the presence of competitive side-chain functionalities such as the ε-NH_2 group of lysine, the thiol of an internal cysteine or the imidazole of a histidine. Peptides ranging from 5 to 26 amino acid residues in length could be cyclized under the control of ring-chain tautomeric equilibrium resulting in trace amounts of oligomeric products.

Muir and Camarero reported a native chemical backbone cyclization of a fully deprotected, 15-residue peptide containing an N-terminal cysteine and a C-terminal thioester[67]. The head-to-tail cyclization proceeded smoothly under high dilution (0.0005 M) at around neutral pH. PhSH and BnSH were both added during the cyclization step. These reagents serve as nucleophilic catalysts that enable intermolecular transthioesterification pathways to generate a more electrophilic C-terminal active thioester and a better leaving group after attack on the carbonyl by the cysteine thiol. The native chemical ligation protocol has also been extended to the realm of solid-phase macrocyclization chemistry[68].

The native chemical ligation approach to macrocyclization was even used by Dawson in the synthesis of a protein catenane, in which two cyclic peptides have been interlocked with each other[69]. The *S*-to-*N*-acyl migration is also the centerpiece of the 'thia zip reaction' proposed by Tam and co-workers; this strategy has been applied to the end-to-end cyclization of large peptides that are rich in cysteine residues[70]. Key functional groups required for this cascade are an N-terminal cysteine, a thioester and at least one internal free thiol embedded in the peptide chain.

As effective as the above mentioned *S*-to-*N* proximity-driven acyl migration macrocyclization strategies are, their major drawback is the necessity of a cysteine residue at the N-terminus, which limits their synthetic utility. To overcome this structural requirement, Kent and co-workers were able to mimic the side chain of an N-terminal cysteine by attaching an oxyethanethiol moiety onto the α-amino group of the N-terminal amino acid[71]. They were able to effectively run the native chemical cyclization reaction with a C-terminal thioester to synthesize head-to-tail cyclic peptides devoid of a cysteine residue with no detectable cyclo-oligomerization. After the *S*-to-*N* acyl migration, the N-α-oxyethanethiol group can be removed by reduction with zinc dust in dilute acetic acid to yield the native backbone structure of the cyclic peptide (Fig. 4f). More recently, Dawson and Yan have developed a more practical method through the native chemical ligation approach to cyclize peptides that do not contain cysteine residues[72]. After an initial native chemical macrocyclization between an N-terminal cysteine residue and a C-terminal thioester, the cyclic peptide can be devoid of its cysteine sulfur through a desulfurization reaction with Raney nickel.

A thioester-based macrocyclization strategy was developed by Hackenberger and Kleineweischede, whose method is based on a traceless Staudinger ligation[73] (Fig. 4g). A phosphine tethered to a thioester at the C-terminus of a peptide reacts intramolecularly with an azide present at the N-terminus to form a cyclic iminophosphorane. This ring then contracts to form an amide bond as the aza ylide attacks the thioester electrophile. This macrolactamization process was shown to be amenable to the cyclization of several globally deprotected peptides, eleven amino acid residues in length. This provides direct access to a cyclic peptide without further deprotection steps.

Ring-contraction strategies involving lactones

The formation of a larger, more flexible macrocycle followed by an intramolecular ring contraction to yield the desired target is an effective way of alleviating the entropic penalty of macrocyclization. Amblard and co-workers have exploited an *O*-to-*N* migration strategy to synthesize cyclic peptides[74]. Amblard's strategy is rooted in an initial macrocyclization of conformationally flexible depsipeptides followed by an *O*-to-*N*-acyl migration to furnish homodetic cyclic peptides (Fig. 5a). The peptide sequence was first built up from the side chain of an *N*-Boc-protected serine anchored on solid support, creating the *O*-acyl isopeptide bond (Boc, *tert*-butyloxycarbonyl). After cleavage from the solid support, the depsipeptides were cyclized under high dilution to generate their corresponding cyclic analogues. After removal of the *N*-Boc group of the serine residue, the final *O*-to-*N* acyl migration occurred under basic conditions, generating native cyclic peptides in moderate yields. This strategy was shown to be amenable to the synthesis of cyclic penta- and hexapeptides, however Amblard and co-workers observed that the acyl migration step did not proceed for a more conformationally constrained tetrapeptide derivative.

Another ring-contraction strategy involving lactones was developed by Meutermans, Smythe and co-workers and was applied to the head-to-tail cyclization of small peptides[75]. A salicylaldehyde-derived auxiliary is attached to the N-terminus of the peptide chain through reductive amination. Activation of the C-terminus initially results in the cyclization to form the more accessible lactone. This brings the N-terminus into close proximity to the C-terminus and facilitates an *O*-to-*N* acyl transfer (Fig. 5b). This method of acyl capture of activated amino acids followed by an acyl transfer with a 2-hydoxyl-benzyl-based auxiliary was originally developed by Sheppard and co-workers as an efficient way to achieve amide-backbone substitution of hindered peptides[76]. Smythe and co-workers chose 2-hydroxy-6-nitrobenzyl (HnB) as the auxiliary because it is photo-labile and can be removed after macrocyclization. As a proof-of-concept, the difficult-to-cyclize pentapeptide H-Ala-Phe-Leu-Pro-Ala-OH, (Fig. 1d) linked to HnB, was cyclized in 45% yield at a 0.001 M concentration. The authors have also applied this ring-contraction strategy to the synthesis of cyclic tetrapeptide libraries[77]. They were even able to show that this strategy is amenable to the synthesis of highly constrained all-L cyclic tetrapeptides[78]. Such difficult macrocyclizations were shown to be possible if an additional HnB unit was installed onto one of the backbone amide nitrogen atoms to influence *cisoid* amide turn structures to aid in bringing the ends closer together.

A pincer auxiliary strategy for difficult macrolactamizations was developed by van Maarseveen and co-workers[79]. In their approach a salicylaldehyde-derived auxiliary is incorporated into the backbone of a linear peptide, creating a flexible tether, or 'hinge' that allows for a head-to-tail macrolactamization. A subsequent transannular ring contraction involves an *O*-to-*N* acyl transfer reaction (Fig. 5c). Here, the success of the salicylaldehyde-derived auxiliary as a template for macrolactamization is two-fold. First, an enthalpic activation is brought about due to the high reactivity of aryl esters towards aminolysis. Second, an entropic activation results from the

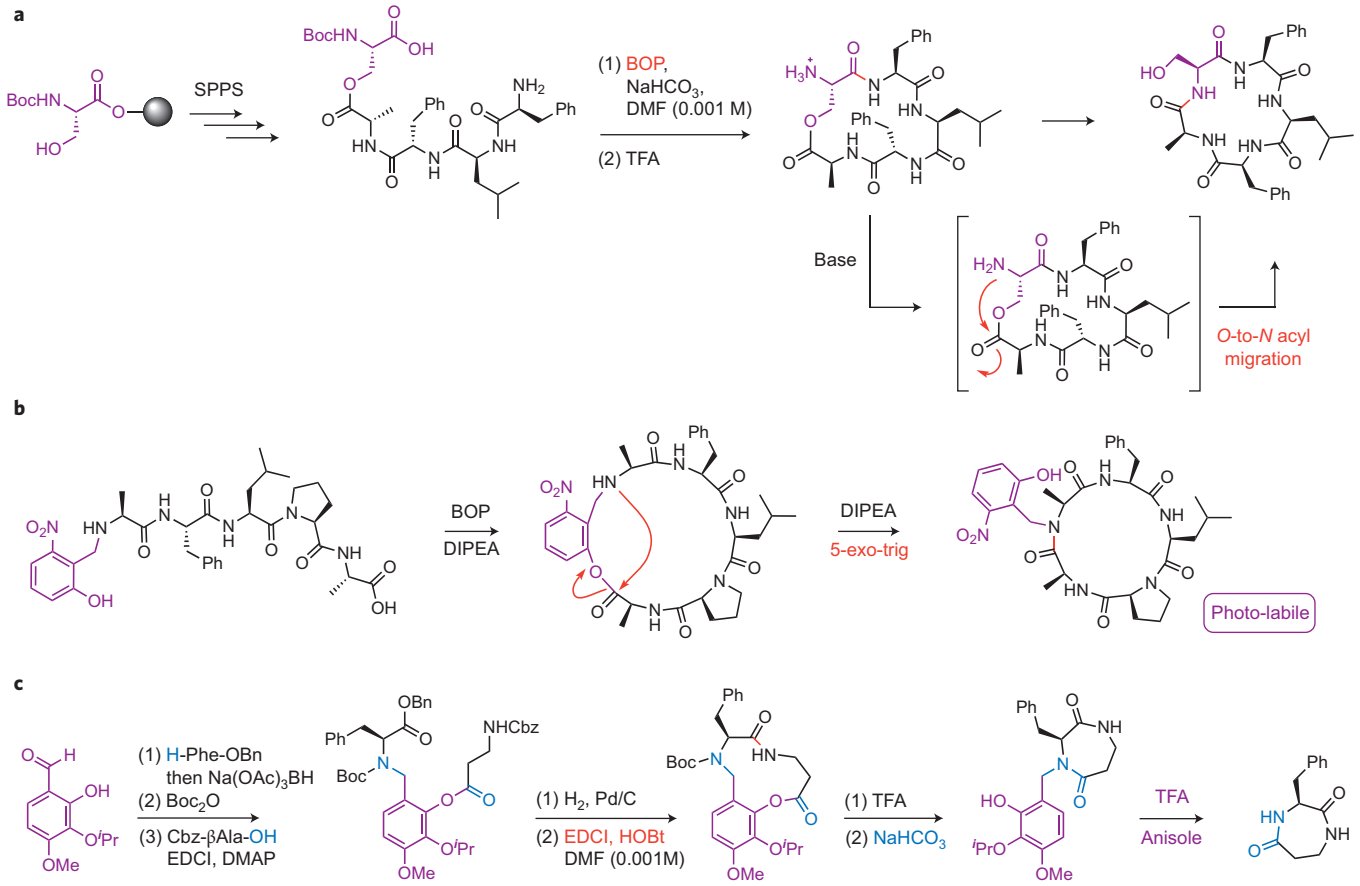

Figure 5 | Peptide lactamizations through ring contractions involving larger lactones. a, A ring-contraction strategy involving an *O*-to-*N* acyl transfer of a depsipeptide to yield a homodetic cyclic pentapeptide. The linear precursor is first constructed from the side chain of a serine residue. After cleavage from the solid support, a macrolactamization furnishes the depsipeptide. A subsequent Boc deprotection liberates the α-amine of the serine residue that undergoes the acyl transfer reaction. **b**, A photolabile auxiliary, HnB used in a ring-contraction strategy to cyclize the challenging pentapeptide, H-Ala-Phe-Leu-Pro-Ala-OH. After the introduction of this auxiliary onto the N-terminus of the peptide, cyclization proceeds through an initial cyclic nitrophenyl ester that preorganizes the peptide for a lactamization that proceeds through an intramolecular *O*-to-*N* acyl transfer to furnish the homodetic cyclic pentapeptide. **c**, A salicaldehyde-derived auxiliary that first serves as a flexible hinge for a peptidic macrolactamization. After a subsequent Boc deprotection, the macrocycle undergoes an *O*-to-*N* acyl transfer under basic conditions through a ring contraction. The displaced auxiliary is then cleaved under acidic conditions to yield the desired homodiketopiperazine. EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

auxiliary correctly positioning the secondary amine and carbonyl functional groups in close proximity for the ring contraction[80]. This strategy has been successfully applied to the synthesis of several homodiketopiperazines that are difficult to synthesize.

Azide-alkyne cycloadditions

There exists a wealth of biologically active structures, isolated from marine organisms, that consist of cyclic peptides embedded with smaller heterocyclic rings. In several examples, the presence of thiazole and oxazole rings imposes conformational restrictions in the corresponding macrocycles[81]. As a result, the incorporation of heterocycles into the backbone of cyclic peptides is growing in interest, especially from a medicinal chemistry perspective. The 1,2,3-triazole is a particularly intriguing heterocycle as it is both thermodynamically and physiologically stable. Depending on its substitution pattern it can serve as an effective isostere for a *trans*- or *cis*-amide bond. This heterocycle, as originally shown by Huisgen, is readily obtained through a 1,3-dipolar cycloaddition between an azide and an alkyne[82]. Recently, the groups of Meldal and Sharpless have developed a highly regioselective variant to generate a 1,4-disubstitution pattern that is catalysed by copper(I) under mild conditions[83,84]. This 'click' chemistry has since been successfully applied to the macrocyclization of peptides.

Triazole-containing analogues of the previously unobtainable *cyclo*-[Pro-Val-Pro-Tyr][10] (Fig. 1c) can be readily synthesized through the cycloaddition of an N-terminal azide and a C-terminal alkyne[85] (Fig. 6a). Importantly, these isosteres were shown to retain their tyrosinase inhibitory activity[86].

Lokey and co-workers have further demonstrated the utility of this reaction as a macrocyclization tool[87]. They were able to perform on-solid support cyclizations of leucine-rich tetra-, penta-, hexa- and heptapeptides. The formation of dimeric and trimeric by-products has been noted in many copper-catalysed azide–alkyne cycloadditions[23]. Ghadiri and co-workers were able to take advantage of this phenomenon; through a tandem dimerization–macrocyclization approach enabled by several tandem click reactions[88], they were able to synthesize C_2-symmetric cyclic peptide scaffolds that were shown to self assemble into peptide nanotubes[89]. This approach was met with greater success than a conventional macrolactamization of a linear precursor already containing both triazole units (Fig. 6b).

1,4-Disubstituted 1,2,3-triazoles are known surrogates for a *trans*-amide bond. Ghadiri and co-workers have shown that the incorporation of one or two of these moieties into cyclic tetrapeptides that are known to display strong binding affinity for the somatostatin (SST) receptor creates conformationally homogeneous analogues[90].

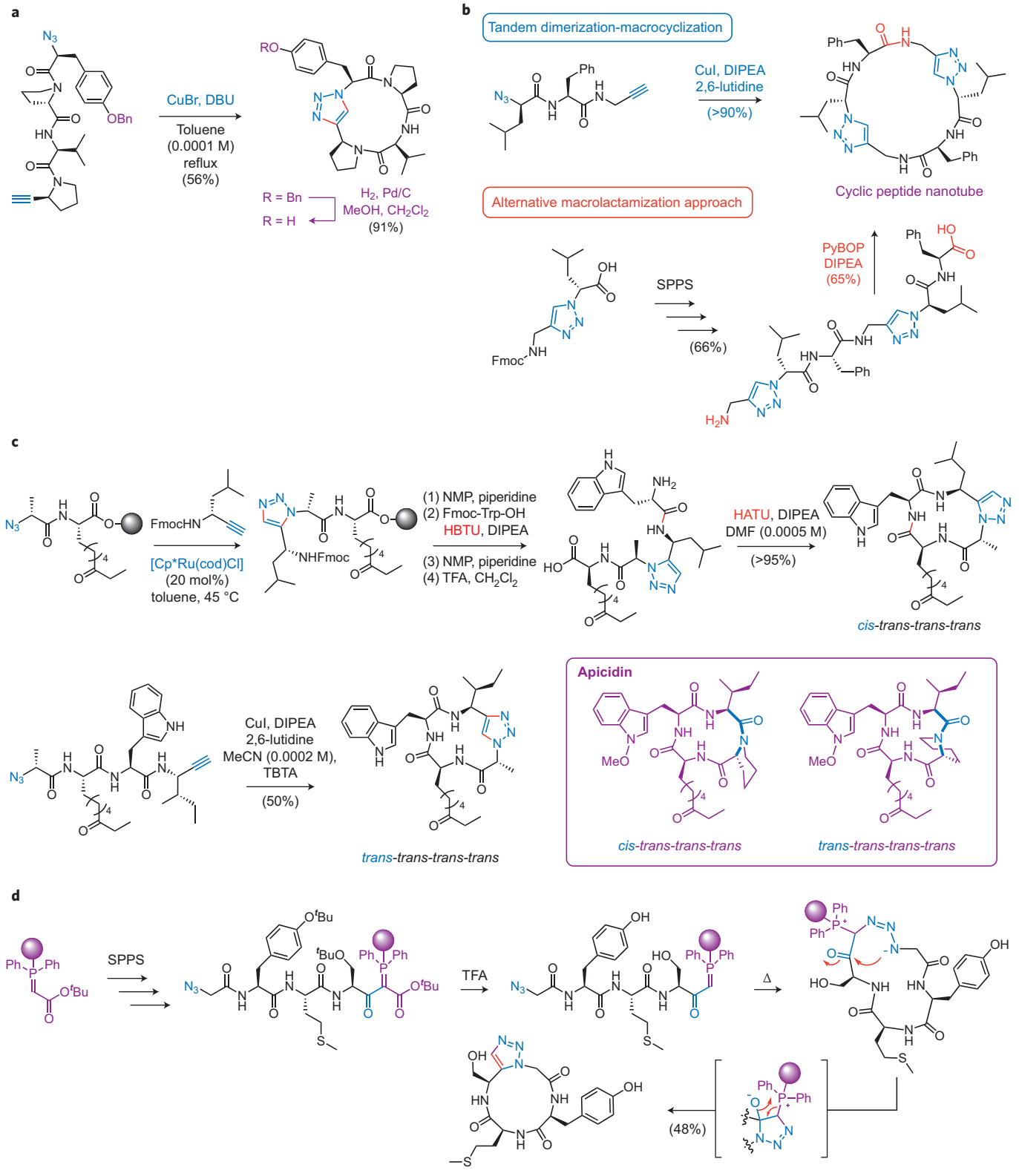

Figure 6 | Azide–alkyne cycloadditions in the synthesis of peptide macrocycles. a, A click-mediated macrocyclization of Tyr-Pro-Val-Pro. **b**, Synthesis of a cyclic peptide nanotube through either a high-yielding tandem dimerization-macrocyclization approach by two tandem click reactions of an azido-dipeptide alkyne (top) or through a less efficient conventional macrolactamization approach (bottom). **c**, The synthesis of triazole-modified analogues of the cyclic tetrapeptide, apicidin. Top: A ruthenium catalysed formation of a 1,5-disubstituted 1,2,3-triazole on solid-phase is followed by a macrolactamization to yield an analogue resembling the biologically active conformation of apicidin. Bottom: A Cu(I)-catalysed intramolecular azide–alkyne cycloaddition to yield an analogue of apicidin resembling its predominant conformation in solution. **d**, Synthesis of a cyclic tetrapeptide analogue containing a 1,5-disubstituted 1,2,3-triazole through an intramolecular cyclative cleavage of a solid-support-bound azidopeptidylphosphorane. HATU, 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMP, *N*-methyl-2-pyrrolidone; TBTA, *tris*[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine.

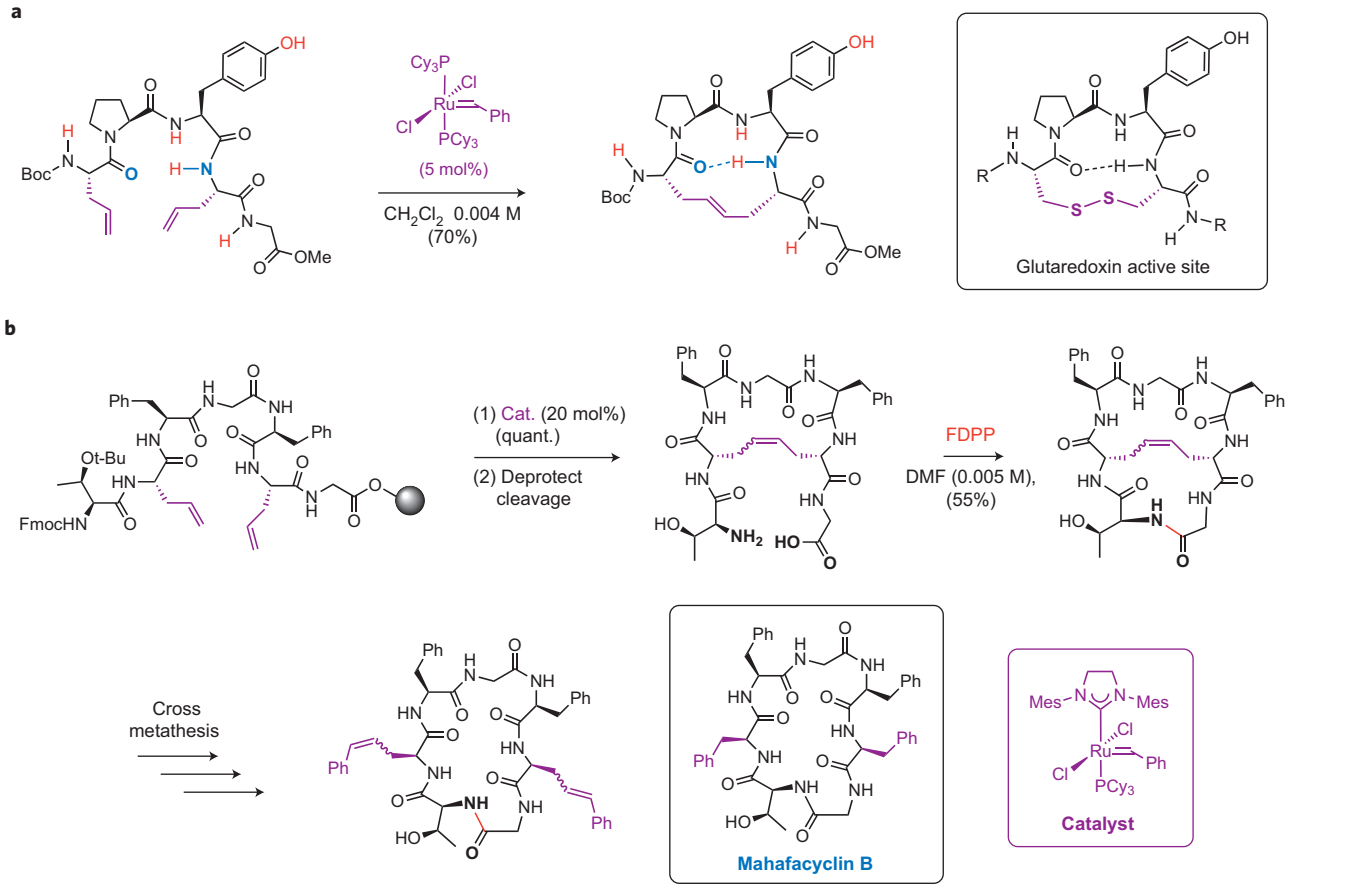

Figure 7 | The use of ring-closing metathesis in the synthesis of peptide macrocycles. a, A side-chain-to-side-chain cyclization and stabilization of a β-turn structure through RCM. Acidic groups labelled in red were shown to be compatible with process. **b**, RCM between two allylglycine residues in a heptapeptide creates a carbon-based tether that brings the N- and C-termini in closer proximity for an effective macrolactamization. The alkene tether is subsequently cleaved and, through several cross-metathesis reactions, a peptide macrocycle resembling the natural product mahafacyclin B can be obtained.

In fact, these pseudotetrapeptides were shown to adopt only one conformation even at 70 °C.

Alternatively, 1,5-disubstituted 1,2,3-triazoles have been shown to be effective *cis*-amide bond surrogates, inducing turn-structure motifs[91]. These can be prepared in a regioselective manner through a ruthenium(II)-catalysed 1,3-dipolar cycloaddition of an azide and an alkyne[92]. Ghadiri and co-workers were able to incorporate these heterocycles as *cis*-amide isosteres in a naturally occurring cyclic tetrapeptide. This was achieved through an initial ruthenium(II)-catalysed formation of a 1,5-disubstituted 1,2,3-triazole containing a linear peptide on solid phase followed by a conventional macrolactamization of the pseudotetrapeptide[93] (Fig. 6c). With this, they were able to produce conformationally homogenous analogues of apicidin. The archetypal cyclic tetrapeptide adopts an all-*trans* amide structure in solution, however, its biologically active conformation incorporates one *cis*-amide rotamer. Indeed, the authors were able to show that their analogue, incorporating a 1,5-disubstituted 1,2,3-triazole, displayed similar biological activity to that of the naturally occurring apicidin.

The first successful cyclization of azidoalkynyl peptides to give 1,5-disubstituted triazole-containing macrocycles was recently reported by Rademann and Ahsanullah, who have developed an on-solid support strategy that is based on a cyclative cleavage in which both cyclization and cleavage from the solid support proceed in the same chemical reaction[94]. This allows for facile purification because the open-chain oligomeric by-products remain attached to the solid support. This chemistry involves a dipolar cycloaddition of polymer-bound azidopeptidylphosphoranes, which avoids the use of amino acid alkynes and is metal-free (Fig. 6d).

Ring-closing metathesis

The advent of the olefin metathesis reaction in forming carbon–carbon bonds has led to a wide range of applications in the area of macrocyclization. The development of the highly functional-group-tolerant ruthenium-based catalysts by Grubbs and co-workers has greatly facilitated the transition of this area of organic chemistry into the realm of peptides and related biological systems. Grubbs and co-workers were the first to apply the strategy of ring-closing metathesis (RCM) to conformationally rigidify amino acids and peptides[95]. Inspired by a class of naturally occurring, disulfide-stabilized, β-turn structural motifs found in a number of redox active proteins, they were able to replace the disulfide bridge within these tetrapeptide sequences with carbon–carbon bonds. This was achieved through an RCM reaction between allylglycine residues, and was found to be tolerant of acidic amide NH protons as well as an unprotected tyrosine phenol group (Fig. 7a).

Following this seminal work, several other research groups have found success in stabilizing secondary structural elements in peptides through alkene-based 'staples', however, these concepts are not the focus of this Review and the reader is directed elsewhere[96–99].

Robinson and co-workers have found a different application of metathesis to facilitate macrocyclization of peptides[100]. Their alkene-containing tethers, formed from the cross metathesis between two strategically placed allylglycine residues, bring the C- and N-termini of a peptide closer together and facilitate macrocyclization by

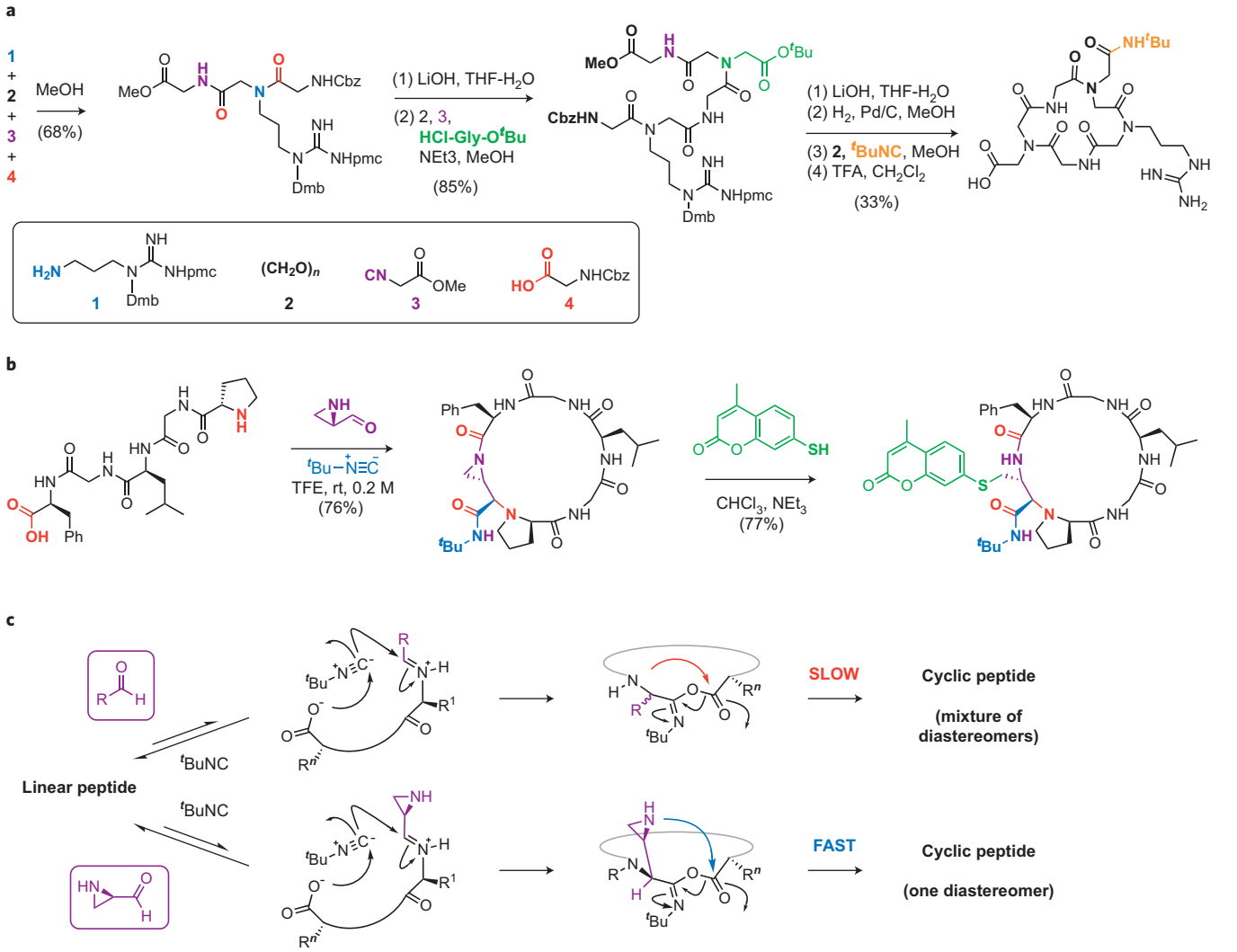

Figure 8 | Peptide ring closures mediated by multicomponent reactions. a, Synthesis of a cyclic RGD pentapeptoid by consecutive U-4CR reactions. Two consecutive U-4CRs were employed for the construction of the acyclic precursor, and after subsequent deprotection of its termini another Ugi three-component four-centre reaction was used for peptoid macrocyclization. **b**, A hexapeptide constrained in a macrocycle through a multicomponent reaction with an amphoteric aziridine aldehyde and an isocyanide. The resulting *N*-acyl aziridine-containing macrocycle can then be site-specifically modified through a ring-opening reaction with 7-mercapto-4-methylcoumarin. **c**, Proposal for the high diastereoselectivites observed in the Ugi-mediated macrocyclizations with aziridine aldehydes.

conventional lactamization chemistry (Fig. 7b). This method was applied to the head-to-tail cyclization of a heptapeptide resembling the natural product mahafacyclin. After the carbocyclic tether was installed, cyclization with FDPP (pentafluorophenyl diphenylphosphinate) afforded the cyclic derivative in 55% yield.

The use of isocyanides and multicomponent reactions

The Ugi four-component reaction (U-4CR) is a versatile tool for the construction of peptoid-based frameworks. Wessjohann and co-workers have applied this multicomponent reaction to the synthesis of RGD-containing cyclic peptoids[101] (Fig. 8a). Their strategy involves the use of three sequential Ugi reactions. The first two consecutive Ugi four-component reactions construct the acyclic RGD-containing precursor. After deprotection, another Ugi three-component four-centre reaction is employed for the peptoid macrocyclization.

Another example using the U-4CR comes from the laboratory of Kazmaier and co-workers. Their approach to cyclic peptidomimetics involved N-alkylated amino acids, which were integrated into a four-component Ugi reaction to construct the linear precursor[102]; this was followed by RCM. Using this method, different polar, hydrophilic and hydrophobic moieties can be placed at any position in a cycle.

Danishefsky and co-workers have developed a range of novel isocyanide-mediated amide coupling methods towards challenging N,N-dialkylated amides[103]. In a recent tour de force they applied their methodology towards the total synthesis of cyclosporine A. The component peptide building blocks were joined together by a series of isocyanide-mediated coupling reactions.

Electrostatically controlled macrocyclizations

Short peptides are generally found as random coils in aqueous solution, resulting from the capacity of water to disrupt intrapeptide hydrogen bonding[104]. However, in polar organic solvents, short linear peptides prefer a circular conformation, which is driven by ion pairing between the N- and C-termini[105]. Capitalizing on this behaviour could lead to effective macrocyclization strategies, because a reaction geared at maintaining ion pairing between the C- and N-termini throughout the course of the macrocyclization would take advantage of electrostatically induced pre-cyclization

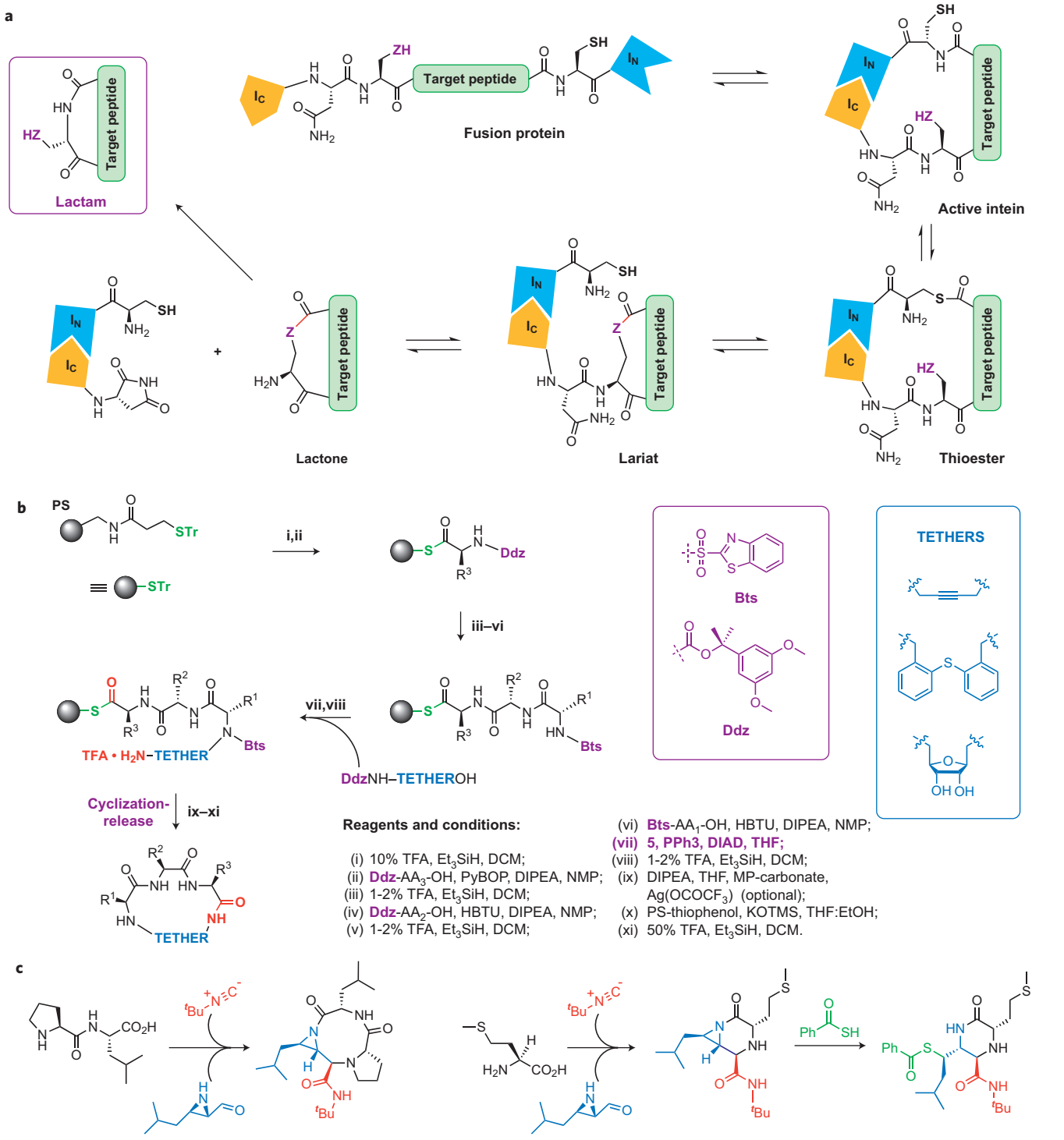

Figure 9 | Strategies for the development of libraries of peptide macrocycles. a, Split-intein-mediated circular ligation for the synthesis of cyclic peptides. **b**, Reaction scheme for the MATCH technology used in the development of libraries of cyclic peptidomimetics. Several examples of the various tethers used are shown in blue. **c**, Digital microfluidics has been used to create libraries of cyclic peptides by combining discrete droplets of reagents. The examples show the dipeptide H-Pro-Leu-OH and amino acid methionine being cyclized in parallel on the same chip reactor by mixing with droplets of an aziridine aldehyde and an isocyanide. A subsequent site-specific modification of the product from methionine is achieved by further mixing with a droplet containing thiobenzoic acid. DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; KOTMS, potassium trimethylsilanolate.

conformers. Towards that goal, Yudin and co-workers have reacted unprotected NH aziridine aldehydes, isocyanides, and linear peptides[106] (Fig. 8b).

This multicomponent method efficiently yields cyclic peptides with high chemo- and stereoselectivities with no signs of epimerization, cyclodimerization or oligomerization. The challenging medium-sized rings are readily prepared; the reaction times are less than 10 hours, proceeding with high yields. The reaction produces peptide macrocycles at high molar concentrations irrespective of the length of the linear peptide precursor. The iminium ion formation between the

aldehyde centre of an aziridine aldehyde and N-terminus of the linear peptide precursor is thought to be delivered by an electrostatically stabilized ion pair. Yudin and co-workers propose that the presence of the nucleophilic centre at the α-position of the amphoteric aziridine aldehyde is responsible for the high yields and diastereoselectivities. When monofunctional aldehydes are used in the reaction with an isocyanide and a peptide, low diastereoselectivities are observed (Fig. 8c). More importantly, the undesired cyclodimerization that typically occurs during the U-4CR cyclization of linear peptides containing less than six residues was not observed. The cyclic peptide product contains an aziridine modification site to which peptidic and non-peptidic side chains can be appended at a late stage of synthesis.

Libraries of peptide macrocycles

Large libraries of macrocycles have been prepared using DNA-programmed chemistry[107]. This versatile platform couples standard organic reactions to DNA hybridization. Each member of the macrocyclic library has a covalently attached single-stranded DNA tag. The first building block, attached to a 59-base template DNA strand, reacts with a second building block attached to a 12-base reagent strand that recognizes a codon sequence within the 59-base template. DNA duplex formation brings the building blocks into close proximity, which serves to increase their effective molarity. Chemical reactions such as reductive aminations, amide bond formation, aldol condensations, and Wittig reactions are compatible with the aqueous conditions of DNA-programmed chemistry. Separation is enabled by the presence of a biotin group covalently bound to the end of the reagent DNA.

Circular ligation was used by Benkovic and co-workers in the synthesis of cyclic peptide libraries in *Escherichia coli*. Randomized peptide sequences can be cyclized under these conditions, enabling biosynthesis of cyclic peptide libraries of up to 10^8 members. The method is based on rearranging the order of the elements of the intein. An active *cis*-intein gives rise to cyclization of the target protein–peptide sequence on splicing. The expressed fusion protein folds to form an active intein. The cyclization reaction is based on an *N*-to-*S* acyl shift at the target N-terminal junction. The ensuing thioester partakes in transthioesterification with a serine or cysteine side chain (Z = O or S, Fig. 9a) at the C-terminus to generate a lariat. The asparagine side chain plays a key role in liberating the cyclic lactone or thiolactone. The latter undergoes a *Z*-to-*N* acyl shift, generating the thermodynamically favoured product *in vivo*.

A versatile parallel synthesis platform aimed at macrocycles (macrocyclic template chemistry, MATCH) has been developed[108]. In this method, macrocycles are made using a non-peptidic tether that forces a peptide sequence into a head-to-tail cyclization. In this method, linear peptide precursors are anchored to a polystyrene solid support via a semi-labile thioester linker. In the course of the cyclization, nucleophilic attack onto the thioester liberates macrocycles from unreacted solid-support-bound intermediates (Fig. 9b). A 40,000-member library was prepared using this technology.

When it comes to making libraries of cyclic peptides, several hurdles must be overcome. Cyclic peptides lack a free N-terminus, which rules out Edman sequencing for sequence elucidation. Also, cyclic peptide molecules can fragment at multiple positions, complicating interpretation of the spectra from tandem mass spectroscopy. Pei and co-workers addressed this problem by encoding beads containing cyclic peptides with a precursor linear sequence[109]. The strategy focused on surface-exposed glutamic acid side chains that were deprotected and cyclized with the terminal amino group of the peptide. The linear peptides in the internal layer served as the encoding strands. Kodadek developed an elegant approach towards peptoid microarrays. In this method, a glutamic acid residue was used to trigger cyclization whereas a cysteine residue allowed specific conjugation of the cyclic peptoid molecule to a maleimide-activated microscope slide. The linear molecule facilitated tandem mass spectrometry-based sequencing[110].

In need of a platform capable of generating spatially resolved droplets containing peptide macrocycles for downstream transfer onto functionalized surfaces, the groups of Wheeler and Yudin employed digital microfluidics. Marocyclization enabled by aziridine aldehydes was used in this method[111]. The movement of discrete nano- to microlitre droplets of the macrocyclization precursors was controlled in parallel by applying a series of electrical potentials to an array of electrodes coated with a hydrophobic insulator. This platform sustained many different multicomponent, multistep reactions in parallel. The device was designed to handle diverse reagents, thirty reaction steps, and was capable of forming five products in parallel (Fig. 9c).

Conclusion and outlook

The post-genomic era will continue to provide new information on biological targets, particularly protein–protein interactions. The difficult-to-tackle problems of this sort cannot be addressed with small molecules. As more protein–protein interactions continue to be validated as targets for therapeutic intervention, there will be a growing demand for molecules that can engage in extended interactions. Flexible synthetic strategies towards molecules of this kind should enable late-stage conformational tuning and solubility optimization. The ultimate goal is to modulate secondary structures in molecules that can pass through cell membranes. As the sophistication of synthetic methods continues to increase, we are certain to witness the identification of novel well-defined surfaces, which we would like to refer to as privileged folds. Peptide macrocycles offer an ideal opportunity to interrogate complex structures of this kind.

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