Synthetic Cross-linking of Peptides: Molecular Linchpins for Peptide Cyclization

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Abstract: Peptide-derived drugs constitute a significant fraction of therapeutic agents. In 2013, the global market of peptide therapeutics was ca. $19 billion; this value does not include revenue from insulin derivatives of $28 million. The combined sales of insulin and non-insulin peptide drugs is estimated to exceed $70 billion by 2019. A significant fraction of peptide-derived drugs is composed of an amino acid sequence and additional chemical functionalities that improve biological and pharmacological properties of the drug. In this review, we focus on synthetic cross-linkers that we refer to as “linchpins”, which are commonly used to constrain the secondary structure of peptides and equip them with added benefits such as resistance to proteolytic degradation and conformational stability. The latter property leads to an increase in binding potency and increased bioavailability due to increased permeation through biological membranes. Some linchpins can even introduce properties not found in natural peptides such as light-responsive. Peptides cyclized by linchpins can be viewed as a sub-class of a larger family of peptide-derived drugs with desired pharmacological performance in vivo. To understand how chemical modifications by linchpins improve drug discovery, this review also briefly summarizes canonical examples of chemical modification used in modern peptide therapeutics.

Keywords: Peptides, peptide macrocycles, side chain cross-linking, biocompatible chemistry, chemical modification of generically-encoded libraries.

1. INTRODUCTION

Peptides and peptide-derived therapeutic drugs are defined as molecules with less than 50 amino acids. These compounds demarcate a unique chemical space that bridges small molecule therapeutics and biological therapeutics (i.e., antibodies, recombinant proteins and protein domains) [1, 2]. By 2014, there were 60 FDA approved peptide drugs on the market, 140 peptide drugs in clinical trials and more than 500 peptide drug candidates in pre-clinical trial [3, 4]. Within the four years spanning between 2014-2017, 14 new non-insulin peptide entities were approved by the FDA [5]. The sales for the top seven selling peptide drugs (i.e., Copaxone®, Lupron®, Sandostatin®, Zoladex®, Victoza®, Forteo®, Byetta®) were more than $9.0 billion in 2011 [3] and exceeded $11 billion in 2015 [6]. In 2013, the combined sale of peptide drugs and insulin derivatives, was ca. $47 billion and $50 billion in 2015 [6]; in turn, the sales of pharmaceuticals world-wide exceeded $1 trillion in 2014. The market of peptide drugs is smaller when compared to the combined market of small molecule therapeutics and biological therapeutics. For example, antibody-based therapeutics is a rapidly growing class of therapeutics constituting 10% of the newly approved drugs in the past 10 years; however, limited tissue penetration is a fundamental drawback of antibodies. Peptide drugs can penetrate into tissues due to their smaller size [7]. In contrast to many small molecule drugs, peptides accumulate less in the tissues and often show higher specificity [8].

Suboptimal pharmacokinetic properties of the general peptide scaffolds are a major barrier to the use of peptides as drugs. The susceptibility of the peptides to peptidases in the gastrointestinal tract reduces their bioavailability significantly through oral administration. The hydrophilic nature of many peptides dramatically reduces their delivery to the systemic circulation through passive diffusion. Peptides that enter the systemic circulation are subject to renal and hepatic clearance as well as degradation by peptidases. As a result, the half-life of peptides administered directly in the circulation can be as short as a few minutes [9-10]. Such a short half-life may have evolutionary significance: many peptides act as messenger hormones, cytokines, neural transmitters and metabolic indicators. Rapid degradation of the messenger peptides ensures effective turn-off mechanism and high temporal fidelity of the message. However, when peptides are adapted as drugs, in many cases the life-time of the peptide must be extended to avoid frequent or continuous administration.
Both physical and chemical solutions have been devised to increase the bioavailability and resistance of the peptides. Examples of physical modifications include coating the peptide drug with polymers to protect the peptide from gastric degradation and formulation of the peptide with protease inhibitors [11]. Physical protection and peptide formulation are beyond the scope of this review and is reviewed elsewhere [5, 6, 11, 12]. Examples of chemical modification of peptides that improve the pharmacokinetics and pharmacodynamics include conjugation of peptides with fatty acids to increase circulation half-life via interactions with serum albumin and pegylation to increase the hydrodynamic radius. Both modifications delay renal elimination [8, 13]. An example of the first type of modification can be found in Levemir®, chemically modified insulin developed by NovoNordisk, which is an FDA approved form of insulin myristoylated at a lysine residue. A seemingly minor modification – 17 carbons added to a polypeptide with 250 carbons – yields a dramatic increase in half-life from 2.8 h to 8.8 h [14]. This increase is even more dramatic in Victoza®, a 37 amino acid glucagon-like peptide-1 receptor (GLP-1) agonist, also developed by NovoNordisk, which is used for the management of Type 2 Diabetes. Palmitoylation of a lysine residue in this peptide increased the half-life of the subcutaneously injected drug from 1 h to 11-15 h [15]. Examples of the second type of modification are two FDA approved drugs Pegasys®, pegylated interferon developed by Genentech, and Pegintron®, another variant of pegylated interferon distributed by Schering. These technologies highlight the benefits of introducing non-peptide structures, which equip the peptides with beneficial pharmacokinetic properties, while providing only minor interference with their biological function.

Linchpins are an emerging class of chemical modifiers that can be defined as molecules that convert a linear peptide to a cyclic peptide. Many successful natural or synthetic peptide-based therapeutics have cyclic structure and this cyclization provides several advantages: (i) proteolytic stability (ii) restricted conformation mobility and (iii) increased propensity to permeate through bio-membranes. Cyclization via linchpins can be classified as topologically orthogonal to three canonical modes of cyclization of peptides (Figure 1): (i) head to tail cyclization (or macrolactamization) (ii) head-to-side chain or tail-to-side chain cyclization, and (iii) cross-linking of functional groups in side chains, such as the conversion of thiols of cysteines to disulfide bonds(s). Figure 2 depicts the structure of three FDA approved synthetic drugs and three natural peptides highlighting these three types of cyclization. Detailed reviews of these cyclizations can be found elsewhere [16]. To delineate cyclization assisted by linchpins from the canonical approaches of macrocyclization, we briefly review general cyclization approaches and introduce advantages offered by linchpin cyclization.

2. ENRICHMENT OF THE CONFORMATIONAL ENSEMBLES THROUGH MACROCYCLIZATION

Preorganization of the structure of peptides by macrocyclization can increase the binding affinity by decreasing the entropic penalty resulting from binding [17, 18]. A linear flexible molecule exists in multiple conformational states and the contribution of the conformer necessary to achieve the most proper fit for a potent interaction is often small (Figure 3A). Constraining the skeleton of the ligand can preorganize its structure by maximizing the fraction of conformers that exist in the structure optimal for interaction with the receptor (Figure 3B). Therefore, macrocyclization can result in a higher binding association constant ($K_a$) often by increasing the on-rate of binding. Furthermore, dynamic linchpins can change the conformation in response to external stimuli. Such conformational change in a small motif of a macrocycle can dramatically change its structure, favoring a defined set of conformers over the others. When these changes are designed or selected for, they can result in a dynamic increase or decrease in $K_a$ (Figure 3C).

![Figure 1](image171x70.png)  
**Figure 1.** Linchpin based cyclization compared to other types of cyclization.
3. IMPROVING THE PHARMACOKINETICS OF PEPTIDES BY MACROCYCLIZATION

Macrocyclization of the backbone can facilitate formation of internal hydrogen bonds which in turn are known to facilitate masking of hydrophilic areas and exposure of hydrophobic side chains when passing thorough hydrophobic media such as the interior of a lipid bilayer [19]. In this manner, peptide cyclization can overcome difficulties in active or passive intestinal absorption and/or cell penetration. In contrast, a large exposed polar surface area is detrimental to diffusion of a peptide into the cell [20]. Cyclosporine A is a well-known example of an orally bioavailable peptide that permeates the cell membrane by this mechanism. Cyclosporine shows the same conformation when in an aqueous solvent and as a co-crystal structure complexed with its receptor cyclophilin. However, upon entering the hydrophobic environment (cell membrane) cyclosporine changes its conformation to expose its N-methylated hydrophobic backbone to the solvent by making internal hydrogen bonds [18]. Cyclization narrows down the conformational ensemble of a peptide to two classes of conformers: one is abundant in hydrophilic environment and the other conformer is abundant in a hydrophobic environment. Lokey and co-workers generalized this observation and made similar N-methylated peptide scaffolds that exhibit bi-stability upon transfer from polar to non-polar environments [21]. The structures are currently evaluated by Circle Pharma for their potential to serve as orally bioavailable pharmaceuticals [22].

An alternative solution to the cell permeability problem uses macrocyclization of peptides with simultaneous introduction of extended hydrophobic moieties such as a hydrocarbon side chain to yield a so-called “stapled” peptide. Since the original report by Blackwell and Grubbs [23], and later by Verdine and co-workers [24], two decades ago, the production of cell-permeable amphipathic helical peptides via “stapling” has been adopted by several pharmaceutical companies. Aileron, for example, has completed a phase I clinical trial of the stapled peptide ALRN-6924 that binds to the p53 protein for the treatment of advanced solid tumors and Acute Myeloid Leukemia (Clinicaltrials.gov ID NCT02264613 and NCT02909972). The report discloses complete responses, partial responses and evidence of stable disease [25, 26]. Interestingly, cyclization can also improve performance of a charged class of cell-penetrating sequences such as poly-arginine (Rx, where x = 4 – 9). For reasons that are not yet completely understood, cyclic variants of poly-R-peptides exhibit higher cell penetration than their linear counterparts [27, 28]. A recent summary by Pei and co-workers [29] juxtaposes five validated cell permeable bicyclic peptides and other known cell permeable macrocycles.

In the sections below, we will describe how grafting of linchpins introduces similar promising bioavailability fea-

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**Figure 2.** Chemical structure of selected FDA approved cyclic peptide drugs.
tures into peptides while bypassing the challenges associated with macrolactamization or cyclization via cross-linking of unnatural side-chain residues.

3. APPLICATIONS OF MOLECULAR LINCHPINS FOR MACROCYCLIZATION

A linchpin links two or more reactive groups present in amino acid side chains. Connection of $n+1$ anchoring amino acid will result in the production of $n$ cycles. Mono-[30, 31], bi-[32, 33] and tricyclic [34-36] peptides have been produced using linchpins with 2, 3 and 4 anchors, respectively. The advantages of using linchpins for cyclization are: (i) orthogonal and biocompatible functionalities can be programmed in the unnatural structure of the linchpins, thereby bypassing the use of unnatural structures in the peptide chain; (ii) linchpins can introduce extra features into the peptide structure such as hydrophobicity [37, 38], light-responsiveness [39, 40], conformational stability [36, 41], etc.; and (iii) a single linchpin linker can be used for cyclization of diverse peptide sequences.

There are three main routes for cyclization of peptides by linchpins. (i) Chemical modification using native side chain functionalities of the natural amino acids. Nucleophiles in cysteine, lysine and tyrosine [42] have been widely used for such bio-conjugation. (ii) Pre-activation of the amino acid side chains to provide new orthogonal functionalities (e.g., glyoxal [43], dehydroalanine [44, 45]) followed by bio-orthogonal oxime bond ligation [46] or Michael addition

**Figure 3.** The effect of cyclization by linchpins on peptide conformation. A) A linear peptide exists in multiple conformation, most of which are not optimal for interactions with the receptor. B) Macrocyclization can increase the population of the conformers that exhibit the best fit for the receptor and decrease the fraction of conformers prone to undergo degradation. C) Macrocyclization with linchpins that respond to external stimuli, such as light, can enrich the fraction of a set of productive conformer in “stimulus on” (e.g., “light-on”) condition, and deplete it in “stimulus off” (e.g., “light-off”) condition.
[47], (iii) Incorporation of unnatural amino acids with orthogonally reactive functionality [48, 49]. The use of linchpins is particularly attractive in genetically encoded libraries of peptides, such as those displayed on phage or mRNA. Introduction of an unnatural linker that is reactive towards natural amino acids is a convenient strategy to constrain $10^9-10^{10}$ peptides at once. Multiple groups have already shown the linchpin-based cyclization of peptides displayed on the coat protein of bacteriophage M13 [30, 32], T7 [50], and mRNA [51, 52]. In addition to linchpins that convert linear peptides to cyclic through covalent bonds, one can envision and implement strategies that constrain peptide structure via non-covalent interactions, such as hydrogen bonding. [53] coordination by metal such as rhodium [54-56], palladium [57], copper [58], nicker [59] or iron [60, 61] (recent reviews see [62-64]). These strategies exist but will not be discussed in this review.

4. LINCHPINS THAT FORM AMIDE BONDS

Macrolactamization is commonly used for peptide cyclization, since the reactive precursors – amine and carboxylic acid – are readily available in the backbone of the peptide. Amine-reactive linchpins made of bis-succinimidyld esters with different lengths are commercially available or can be readily generated. In sequences that do not contain Lys as part of the recognition sequence, such approach is very convenient. Lys however is a common residue and its use in cyclization is possible only in a narrow class of peptide sequences. In sequences that contain Lys as part of the functional sequence of the peptide, regioselective cyclization mandates the use of orthogonal protecting groups on Lys residues. Despite the need for extra synthetic steps, amide-forming linchpins are popular due to the wide availability of orthogonally protected Lys building blocks and robustness of amide bond forming reactions. We note that non-regioselective cyclization in unprotected peptides can still be a productive avenue in peptide discovery of functional macrocyclic structures. For example, the pioneering work of Roberts and co-workers employed bis-succinimidyld esters cross linkers to derivatize genetically-encoded peptide libraries displayed on mRNA [51] and used these libraries for an effective discovery of macrocyclic ligands for G-protein coupled receptors [65].

Figure 4 describes several examples of linchpins that use either one-step incorporation or multiple rounds of protection/de-protection and cyclization for stepwise unmasking of the reactive amines. Pei and co-workers [33] employed a multi-step approach to incorporate trimesic acid (TA) as a linchpin that converted one-bead-two-compound peptide libraries with three amine residues at fixed positions into a library of bicyclic peptides (Figure 4A). First, de-protection of allyloxy carbonyl (alloc)-protected 2,3-diaminopropionic acids (Dap) revealed the free amine used to attach the TA through a single amide bond. Then, FMOC-deprotection of ε-amine of lysine and N-terminal amine exposed two other functionalities that made the other two amide bonds with the anchored TA after exposure to a PyBOP activator. Screening of the bicyclic library against tumor necrosis factor-α (TNF-α) identified two bicyclic peptides C1 and C2 with $K_d = 0.45 \mu M$ and $K_d = 1.6 \mu M$, respectively. C1 exhibited no binding to TNF-α in its monocyclic form and the linear form afforded a $K_d > 10 \mu M$. C1 also increased the LD_{50} of TNF-α from 0.46 ng/mL to 1.8 ng/mL, thereby potentially protecting cells from TNF-α-induced cell death.

Inouye and co-workers scanned 12 variants of dicarboxylic acid linchpins to study the effect of the length of the linker on stabilization of the peptides crosslinked at $i, i+4, i, i+7$ and $i, i+11$ (Figure 4B depicts peptides with lysine residue at $i, i+11$) [66]. They found peptides cross-linked at $i, i+11$ with fluorenyl- and naphthyl-dicarboxylic acid exhibited the highest helicity (70% and 75% helicity, respectively) at 25 °C. In a complementary example, McDowell and co-workers used alkanediamine linchpins (NH$_2$C$_n$NH$_2$, $n = 3-5$) to cross-link between glutamic acids located at $i, i+7$ (Figure 4C) [67]. A 1,5-pentanediyl linker increased the helicity of the linear peptide (Ac-TNEDLARREQQ) from 20% to 100% as measured by circular dichroism (CD). Propanediyli and butandyli linkers increased the helicity to 84% and 63%, respectively. The last strategy mandates simultaneous pre-activation of both side chain carboxylates, making this strategy unreliable. Raines and co-workers developed an esterification approach that bypasses this problem by using a diazonium compound with exclusive reactivity towards native carboxylate nucleophile in the presence of water, amines of Lys, thios of Cys and phenolic hydroxyl groups of Tyr [68]. But, to date, this reaction has only been employed to modify Asp and Glu side in proteins with monovalent diazonium compound [69]; bis-diazonium compounds that cross-link two carboxyl side chain have not been reported.

5. LINCHPINS THAT UNDERGO S$_2$2 NUCLEOPHILIC SUBSTITUTION

Thioether bond formation is a fast and selective reaction between cysteines and halo-acetamide or halo-benzyls. Rate constants of these reactions are 1-20 M$^{-1}$s$^{-1}$, and these values can be influenced by amino acid residues flanking the Cys residue. The reaction is conveniently accelerated at neutral pH (7-8) because the thiolate anion (pK$_a$ of cysteine is 7-9) is $>10,000$ times more reactive as a nucleophile than a thiol [70]. The resulting thioether can mimic disulfide bonds and is not susceptible to undesired reduction and exchange. Replacing disulfides with more stable thioether bonds while preserving the activity of the peptides has been addressed by many research groups over the past 20 years. It was thiol-reactive linchpins that simplified chemical post-translational modifications of peptide libraries and stimulated an explosive development of bis-electrophiles for cyclization of peptides. Due to space limitations, we cannot review all published examples of thiol-reactive linchpins to-date. Rather, we summarize key examples that have historical importance and highlight specific classes of topology or reactivity of linchpins.

First linchpin-mediated cyclization appears in 1985 report of Mosberg and Omnas who introduced S-(CH$_2$)$_2$-S, n=2 or 3, into deprotected [d-Pen$^2$,L-Pen$^5$]Enkephalin, with free C-, N-terminus and side chain of tyrosine [71]. The protocol used by the authors for site-specific alkylation of peptide in the presence of other nucleophilic residues can be traced to 1936 report of de Vignaud and Patterson, who site-selectively modified cystine (Cys-S-S-Cys) with CH$_3$Br$_2$ to yield djenkolic acid Cys-S-S-CH$_2$-S-Cys [72]. The peptide in
1985 report, or cystine in 1936 report were dissolved in liquid ammonia, deprotonated by adding chunks of metal sodium “until a blue color indicating an excess of sodium” and “stapled” by adding Br-(CH₂)₆-Br. As both free NH₂ and COOH residues are present in cystine, the 1936 report of De Vignaurd [72] could serve as a reference to many modern bioorthogonal stapling approaches. De Vignaurd’s Na-NH₃ conditions have some drawbacks, such as damage to aromatic amino acids, epimerization and cleavage of bonds with Proline [73].

First diversity-oriented “linchpin scanning” can be found in the 1992 report of Rich and co-workers, who used cyclization of an analog of pepstatin (pepsin protease inhibitor) with series allylbromide and bromomethyl benzenes (BMB) to produce new pepsin inhibitors (Figure 5A) [74]. Unfortunately, the new cyclized peptides did not show improved inhibition when compared to the linear one. This observation shows that simple constraint of the peptide may not lead to enhanced binding to the target. The peptide has to be constrained in an active conformation. Another challenge is that pepstatin recognizes multiple conformations and such prominent recognition might be the reason why enforced depletion of the conformational pool made no difference in binding affinity.

Timmerman and co-workers reintroduced BMB in 2005 to derivatize unprotected peptides in water. They used this and other linchpins to scan the activity of an overlapping set of a 12-residue fragment of follicle-stimulating hormone (FSH-β, Figure 5B). They showed that cyclization of the sequence CRVPGAAHADSLC with m-DBMB, o-DBMB or p-DBMB resulted in higher affinity (Kₐ = 2-3 µM), while the disulfide-cyclized peptide exhibited Kₐ = 130 µM and the linear peptide had a Kₐ > 1 mM [36]. In this case, the linkers replaced a disulfide bond at i, i+13, which stabilized the shape of the loop of a β-turn. They also found o-DBMB and m-DBMB cyclize faster, most likely because the first thioether formation activates the remaining BMB.

DeGrado and co-workers later expanded this approach to 21 different alkylhalides including BMBs (Figure 5C) to staple a peptide at i, i+4 positions and identified the optimal linchpin for stabilization of the helicity of the peptide Ac-IPPKYGECVELC [41]. m-DBMB cyclized peptide showed the highest helix stabilization based on circular dichroism (CD) spectrometry experiments. Varying the position of cysteines in the chain (while keeping them at i, i+4) also changed the helical content of the peptide, although the authors did not quantify helicity for these peptides [41]. The m-DBMB cyclized peptide exhibited Kᵢ = 11 µM for Kalpain-I protein (a calcium-regulated cysteine protease) while the unmodified linear peptide showed lower inhibition (Kᵢ <100 µM). These results show that the structure of the linchpin is not the only factor important in stabilization of the helix, but careful positioning of the cysteines is required for optimal results.

Lin and co-workers used a di-bromomethylbiphenyl (Bph) and di-bromomethylbipyridine (Bpy) to staple a peptide with cysteines at i, i+7 (Figure 5D). The linear peptide (LTFCHYWQLCS, a dual inhibitor of p53-Mdm2/Mdmx interactions) showed IC₅₀ = 500 nM. After cyclization with Bph and Bpy, the IC₅₀ decreased to 22 ± 4.0 nM and 14 ± 2.0 nM, respectively [75]. In addition, modification of the linear peptide by cyclization with Bph resulted in a 6-fold increase in cell permeability; a 5-fold increase was observed after cyclization with Bpy. To promote an α-helix structure, the linchpin should connect the amino acids located on the
same side of the helix, at $i$, $i+4$, $i+7$ and $i+11$ positions (Figure 7) and have appropriate end-to-end distance. For example, DeGrado found no enhanced helix stabilization by using Bph to connect cysteines at $i$, $i+4$, while Lin showed that the same linker resulted in an active peptide when the connection was at cysteines $i$, $i+7$ (Figure 5D). In another example, DeGrado reported stabilization of an $\alpha$-helix when $m$-DBMB connects cysteines at $i$, $i+4$ (Figure 5C), while the same linker can stabilize a $\beta$-turn, with the connection at cysteines $i$, $i+13$ (Figure 5B). Overall, the interplay of positioning and distance in helix stabilization is not trivial and helicity depend on the attachment point, end-to-end distance of the linker and the composition of the linker [76].

Most of the aforementioned examples utilize resonance-activated bis-haloalkanes as linchpins. Non-activated alkyl
halides are poor electrophiles, but given extended reaction times or increased temperature, they can also be introduced into peptides and proteins. Bednares and co-workers introduced an oxetane pharmacophore via alkylation of cysteines in peptides and proteins by 3,3-bis(bromomethyl)oxetane [77]. Recently Cramer and co-workers [78] and Metanis and co-workers [79] revisited introduction of the "world's smallest linchpin"—methylene thiocetal—by cross-linking two Cys in peptides and proteins with CH2I2 and TCEP in mild biocompatible conditions in water. Earlier reports for introduction -S-CH2-S- bridge exist, including the 1987 reports of Mosberg [80] using Na-NH3 conditions of De Vineguard [72] and 1947 report of De Vineguard that introduced -S-CH2-S- bridge using unprotected cystein and formaldehyde [81]. Departing from these conditions, Ueki and coworkers produced methylene-stapled enkephalin using tetrabutylammonium fluoride (TBAF) in anhydrous dichloromethane for concurrent removal of dimethylphosphinothioyl (Mpt) group and alkylation by Cys by CH2Cl2 solvent [82]. Hallberg and co-workers introduced -S-CH2-S- bridge into unprotected angiotensin, by dissolving the peptide in 1:1 mixture of acetonitrile-dichloromethane in the presence of tetrabutylammonium fluoride and tributyphosphine [83].

While many examples focus on stabilization of α-helices, cysteine reactive linchpins can constrain other conformations such as β-turns or even unstructured loops in larger polypeptides. Recently Kritzer and co-workers devised a LoopFinder software to identify bioactive loops in the Protein Data Bank (Figure 8) [84]. They then used a linchpin scanning approach to identify the linchpin structure that captures the loop in an active conformation and produce inhibitors for several protein targets [85-87].

In the canonical linchpin-scanning approach, it is not always known which conformation should be trapped or even what sequence is a useful binder. Heinis, Winter and co-workers used tri(bromomethyl)benzene (TBMB) to cyclize the peptide libraries at three cysteines to form libraries of bicyclic peptides with the structure of Cx6CxCx6C (Figure 9A) [32]. Screening of the library against human plasma protease kalikrein identified peptides that could inhibit the protease activity with IC50 = 1.7 – 39 nM, when only in the bicyclic form. Non-modified peptides showed at least 250-fold decrease in activity. Heinis and co-workers subsequently combined library screening with a linchpin scanning approach. They observed that in a library, different linchpins synergized with different sets of sequences, and productive ligands for the specific target contained a unique combination of peptide sequence and linchpins [88].

Cysteine-reactive linchpins can accommodate reactive moieties for downstream functionalization. Dawson and co-workers used dichloroacetone (DCA) to constrain the peptide and simultaneously install a ketone functionality. The latter can be used for bio-orthogonal labeling of the peptide or a second cyclization (Figure 9B) [89]. For example, the authors reacted cyclized peptides with aminoxy containing substrates such as biotin, alexafluor 647 or FLAG epitope in the presence of 100 mM aniline at pH 4.5. In most cases, the oxime ligation proceeded in satisfactory yields (>85%) after 16 h. The authors attempted to use the DCA linchpin to form a bicyclic product through a reaction of an N-terminal aminoxy group in the peptide with the ketone. However, formation of a bicyclic product was modest and yielded only 24% of isolated product after 24 h. They later combined DCA and a linchpin scanning approach to show that the peptide sequence Ac-KETAAhCKFehCQHMDS (where hC designates homocysteine) is active after cyclization with the linchpin, whereas neither the analog sequences with natural cysteines, nor its DCA conjugate were active. Ng and Derda subsequently adapted DCA to introduce glycosylated linchpins into the phage-displayed libraries of peptides [90].

All the linchpins mentioned above have static structures. Woolley and co-workers pioneered the use of light-responsive linchpins to alter the structure of helical peptides dynamically in response to light. A classic example used chloroaacetamide functionalities to conjugate light-responsive azobenzene (AzB) linkers to peptides with cysteines at i, i+4, i, i+7 and i, i+11 (Figure 5E, 5F). These light responsive linchpins (LRl) are reviewed in detail below.

S2o2 substitution was recently employed to construct “reversible” linchpins that are installed on unprotected peptides in mild conditions to enhance cell-permeability of the peptide. The linchpins were subsequently removed from the peptide by intracellular enzymes. Building on the fundamental work by Deming and co-workers; who described the scope of reversible alkylation of methionine residues in peptides [91], Li and co-workers employed bis-electrophiles to cross-link two methionine residues [92]. The reaction occurs in a mixed water-acetonitrile solution (7:3) supplied with 1% formic acid. Among a survey of 9 cross-linkers, 16 examples of cyclization (Figure 6A) and one example of bicyclization (Figure 6C) was reported. Of note, was the selective cross-linking of two methionines present in the peptide that contained unprotected Cys residues (Figure 6B). Similar selective alkylation of Met in the presence of Cys was reported four years earlier by Kramer and Deming [91]. The difference illustrates a higher reactivity of the sulfur lone pair in R-CH2-S-CH3 of Met when compared to R-CH2-S-H in Cys and/or a higher stability of a trisubstituted vs. disubstituted sulfonium-like transition state in this reaction. A mechanistic explanation of selective reactivity of Met vs. Cys in acidic pH may be fueled by experimental measurements of a stabilizing effect of methyl on sulfonium cations, combined with poor solvation of S-H bonds [93].

6. LINCHPINS THAT UNDERGO S2AR SUBSTITUTION

High reactivity of perfluorobenzene towards hydroxide, amine and thiolate nucleophiles via S2Ar mechanism was noted by Tatlow as early as 1959 [94]. A few decades later, reaction of Cys in proteins with 1-chloro-2,4-dinitrobenzene via S2Ar reaction was used for modification of proteins [95]. Systematic implementation of these reactions for modification of peptides was empowered by a series of publications by Pentelute and co-workers who employed perfluoroarenes to design effective bidentate electrophiles for cyclization of peptides via S2Ar mechanism [38]. They first used hexafluorobenzene (HFB) and decafluorobiphenyl (DFB) to staple helical peptides at i, i+4 (Figure 10A) [38].
Cross-linking the C-CA (peptide sequence ITFCDLLCYYGKKK, an HIV-1 capsid assembly polyprotein binder) with HFB increased the helicity from 16% to 53%, while the same stapling with the longer linchpin DFB resulted in only a 36% increase. Both stapled peptides showed increased binding to C-CA as determined by surface plasmon resonance. They also qualitatively showed that a mixture of trypsin and chymotrypsin degraded the linear peptide in 3 h, while no significant cleavage was observed for the HFB-stapled peptide and only partial degradation was observed for the DFB-stapled peptide.

Pentelute and co-workers then tested five other variants of perfluoroarenes (fAr) on peptides with cysteines positioned at $i, i+1$ to $i, i+14$ (Figure 10B) [31]. The stapling of cysteines at $i, i+1, i, i+2, i, i+3$ with DFB occurred in modest
yield (7 – 52%), while the more flexible fAr-linchpins exhibited somewhat higher yields (>59%) in reactions with peptides when stapling at \( i \), \( i+1 \) through \( i \), \( i+14 \). The authors hypothesized that the conversions observed in these reactions reflect compatibility between end-to-end distance of the linchpin and the Cys-to-Cys distance in the peptide. Since all reactions were performed in pure DMF, the relevance of these observations to peptide conformation in \( H_2O \) is not clear.

Due to the limited solubility of many S\(_{N}\)Ar reagents in water, these reactions mandate the use of either pure organic solvent or a mixture of water with a high percentage of organic solvent [31, 38]. To overcome this limitation, Derda and co-workers performed SAR analysis of thiol-perfluororene reactions and identified decafluoro-biphenylsulfone (DFS) as an effective reagent for cyclization of Cys-containing peptides (Figure 10D) in water [96]. The reaction yielded quantitative conversion in 5–20 min in aqueous Tris buffer containing as low as 20% acetonitrile or 5% DMF. The rate of up to 180 M\(^{-1}\)s\(^{-1}\) was modulated by the sequence of the peptide: positively charged amino acids such as Arg in the vicinity of cysteine increases the rate by increasing the basicity of the thiol and by stabilizing the carbonan intermediate. DFS effectively cyclized peptide hormones such as oxytocin, urotensin II, salmon calcitonin, melanin concentrating hormone, somatostatin and atrial natriuretic factor in which the number of amino acids between cysteines varied between 4–15. Although S\(_{N}\)Ar between Cys and DFS was significantly faster than the reaction between DFS and the amine of Lys or N-terminal amine [96], Pentelute and co-workers subsequently demonstrated that in peptides devoid of Cys, DFS can perform N-arylation of Lys side chains [97].

One can envision arranging multiple S\(_{N}\)Ar reactive handles to yield linchpins with more than two points of attachment. One challenge with this approach is regiochemical control of multiple reactions. A simple and elegant solution to such a problem using 2,3,5,6-tetrafluoroterephthalonitrile (4F-2CN) as a linchpin with four attachment points recently developed by Chuani Wu and co-workers [98]. One of the critical insights from the authors was the use of peptides that contain two Cys and two penicillamine (Pen) residues to stage a four-step reaction into two distinct steps (i) reaction of two Cys-residues 2,5-fluorines of 4F-2CN and (ii) reaction of thiolate of Pen with the remaining 3,6-fluorines to yield only two out of six possible regioisomers.

As an alternative to perfluorenyl S\(_{N}\)Ar linchpins, Pentelute and Buchwald developed a palladium-aryl reagent to arylate the unprotected thiols of cysteines in peptides and proteins (Figure 10C). The rate of arylation reactions was comparable to the thiol-maleimide Michael addition at pH 7.5 (\( \approx 10^{-4} M \) s\(^{-1}\)), although the authors did not measure the kinetics explicitly. The use of 5% organic solvent (MeCN) was sufficient for an efficient S\(_{N}\)Ar substitution with labeling agents such as biotin and fluorescein; however, the authors had to use 50% of MeCN for cross-linking the cysteine with a bidentate linchpin to cyclize the peptide. The peptide sequence IFKTN\_GLL\_YESLR (10 µM) was stapled with 4,4’-dichlorobenzophenone-palladium complex (20 µM) resulting in the formation of an arylated macrocycle in 10 min [99].
Figure 10. Linchpins that undergo S_N Ar reaction with cysteines to form cyclic peptides. via S_N Ar mechanism. A) hexafluorobenzene [38] and B) diverse perfluoroarenes for cyclization of peptides in DMF [31]. C) Decafluoro-biphenylsulfone-mediated cyclization of peptides in water [96]. D) 2,3,5,6-tetrafluoroterephthalonitrile (4F-CN) combined with peptides that contain two cysteine and two penicillamine residues yields only two out of six possible regioisomers [98]. Arylation of peptides using 4,4'-dichlorobenzopheneone-palladium complex [99] and o-phenylene-bridged digold(III) complex [101]. Dichlorotetrazine introduces a s-tetrazine linchpin into peptides [108]. The resulting s-tetrazine-linchpin can be used for further functionalization of the peptide via an inverse electron demand Diels–Alder reaction.
to perform arylation of unprotected Lys residues. They employed preformed or *in situ* generated [LPd(Ar)X] complexes in the presence of sodium phenoxy in DMSO to cross-link two lysines in peptides using 1,2-bis(4-bromophenoxy)ethane reagent [100]. Spokony and coworkers recently expanded the scope of transition metal mediated Cys-arylation using organometallic Au(III) complexes. The o-phenylene-bridged digold(III) stapling reagent [((Me-DalPhos)AuCl)2(µ2-1,4-C6H4)]+ prepared by the authors, introduced the biphenylene “staple” into the H2N-Cys-Asp-Ala-Ala-Cys-Asp-COX2 peptide, the reaction between peptide (2.8 mM) and organometallic reagent (5.6 mM) was completed in 30 min in biocompatible conditions (1:1 acetonitrile / aqueous Tris-buffered solution, pH 8; room temperature) [101]. Other metal-catalyzed Cys arylation [102-104] and metal free [105] or photocatalytic arylation [106] of Cys residues by diazonium salts exist, but their application towards cross-linking of two Cys residues have not been reported. A notable observation by Molander and co-workers is that Ni/photoredox arylation could use peptide disulfide as starting material and bypass the need for a reducing agent [103]. The use of disulfides in S-arylation is well-known in organic methodology (e.g., see recent review [107]), but examples of simultaneous reduction and modification of peptide disulfides by one reagent are rare.

One of the most versatile yet simple linchpins introduced by S2Ar reaction, described by Brown and Smith, exploits the high reactivity of dichlorotritylazide with thiols to introduce a s-tetrazine linchpin into peptides [108]. The resulting s-tetrazine-linchpin undergoes photochemical ring-opening and it can be used for further functionalization of the peptide via an inverse electron demand Diels–Alder reaction.

### 7. LINCHPINS THAT UNDERGO MICHAEL REACTION

Maleimides can undergo Michael addition with thiols to afford a succinimidyl adduct [109-110]. Pingoud and coworkers used an azobenzene bis-maleimide to attach two cysteines at *i, i+4* in a single chain variant of PvuII (scPvuII); a restriction enzyme containing a total of 157 amino acid residues (Figure 11A) [111]. The scPvuII consists of two identical halves that make it possible to include an azobenzene at each half, after substitution of Tyr49 and Asn62 with cysteines. They found that the doubly stapled *cis*-enzyme shows up to 16-fold higher activity compared to its *trans* variant. The *cis*-azobenzene can promote the formation of a β-turn while the *trans* azobenzene distorts it. Maleimide-based linchpins are attractive because several types of bis-maleimide cross-linkers are commercially available. However the resulting product can undergo retro-Michael reaction and the succinimide moiety is prone to hydrolysis [112]. Another drawback is the simultaneous formation of two stereocenters and up to four diastereomers.

Unlike classical maleimide adducts, the Michael addition to allene derivatives does not form chiral centers. Derda and co-workers employed the allenamide addition strategy developed by Loh and coworkers [113] to construct LR-linchpin 3,3′-bis(sulfonato)-4,4′-bis(buta-2,3-dienylamido) azobenzene (BSBDA) that reacts orthogonally with cysteine residues through a Michael reaction [114]. They showed that the reaction proceeds with a rate constant *k* = 30 M⁻¹s⁻¹ at room temperature to produce the cyclic product (Figure 11B). BSBDA also effectively cyclized peptides displayed on the coat protein of M13 phage yielding 98% conversion in only 20 minutes. Allenamides have been postulated to be more stable towards undergoing retro-Michael addition and hydrolysis (personal communication with T. Loh), but the experimental evidence for the stability of such products is limited.

The Michael addition can be employed for modification of natural peptides by converting the natural cysteine residues to dehydroalanine (Dha) [44]. Again, a drawback of such an approach is the formation of two stereoisomers during the Michael addition of thiols to Dha. Webb and coworkers cleverly employed this drawback to generate all possible (eight) diastereomers of a bicyclic peptide in one reaction. They used 1,3,5-benzotextrimethanol as a C3₉ symmetric tridentate linchpin to cyclize a peptide through Michael addition to a dehydroalanine produced out of cysteines in a linear peptide (Figure 13) [115]. Cyclization of the sequence A*Dha*SDRFRNDha*PADEAL*Dha*G resulted in a mixture of stereoisomers of PK15, a bicyclic peptide reported by Heinis *et al.* that binds tightly to plasma kallikrein protein (Figure 13) [32]. Binding of the mixture of peptide stereoisomers (IC₅₀ = 238 nM) was not higher than the original PK15 (IC₅₀ = 2.7 nM) in which all of the cysteine side chains consisted of L amino acids. Synthesis of each individual peptide isomer, showed no binding improvement (IC₅₀ = 240 nM for the most potent binder) for any other diastereomers of PK15, but the LLL-isomer.

One of the most elegant examples of linchpins that employs a tandem Michael additions is the “PEG-mono sulfone” reagent popularized by Brocchini and co-workers [116]. Its origin can be traced to 1966-1971 reports by Lawton and co-workers that pioneered the use of reagents for sequential alkylation through consecutive Michael additions (see Brocchini, Eberle and Lawton [117] and references within). The core functionality of the linchpin contains a leaving group, such as sulfone, in the alpha position to the conjugated system, such as methyacyclophenone. The first conjugate addition of thiol triggers an elimination of the sulfone leaving group and formation of the second methyacyclophenone moiety, followed by an addition of the second thiol. While “PEG-mono sulfone” is not symmetric, the formed linkage is C2-symmetric. In addition, modern versions of cross-linking thiol residues in proteins, reported first in 1979 [118], introduces value-added moieties such as pegylation.

### 8. LINCHPINS THAT UNDERGO AZIDE-ALKyne [3+2] CYCLOADDITIONS

The copper catalyzed [3+2] cycloaddition of azide and alkyne (CuAAC) is a popular bio-conjugation method because it is orthogonal to all unprotected side chains of natural amino acids [119]. After its introduction by Sharpless and Meldal [120] in 2002, many research groups have used this method to modify peptides, proteins, multi-protein complexes and cells [121]. Azidoalanes, aziidoalanoaline, or aziidoormithine and propargyl glycines can be used in CuAAC mediated cross-linking of synthetic peptides. The
same amino acids can be also incorporated in ribosomally synthesized proteins through metabolic labeling [122].

Bong and co-workers used the CuAAC reaction to conjugate di-alkyne linkers to peptides containing azidoalanine as orthogonal handles (Figure 12A) [123]. Placing the azides at \( i, i+4 \) resulted in helical stabilization by the linker hexa-1,5-diyne. Helical stabilization did not occur with three other rigid structures: ortho-diethynylbenzoic acid, meta-diethynylbenzoic and dipropargyl glycine linkers. Spring and co-workers used poly-arginine substituted dialkynyl benzenes to synthesize cyclic analogs of p53 protein, termed SP1-SP5; peptides contained azidornithine in positions \( i, i+7 \) (Figure 12B) [48]. All of the stapled peptides showed higher binding affinity to the MDM protein (\( K_d = 6.7-44.3\) nM) when compared to the non-stapled precursor (\( K_d = 483 \) nM); however, only SP5 with three Arg substitution could penetrate the cell. Peptides containing less than 3 arginine residues were ineffective in penetrating the cell. In vitro assays showed that binding of SP5 to MDM resulted in a 7-fold increase in p53 activation. Thurber and co-workers used similar approach to cross-link azidohomoanalines incorporo-
9. LINCHPINS THAT USE ALDEHYDE-REACTIVE GROUPS

Horne and co-workers employed a linchpin based on reversible covalent oxime and hydrazide bonds to cyclize peptides (Figure 14). They incorporated aldehyde reactive unnatural amino acids, containing hydroxylamine and hydrazide at i, i+4 and i, i+11 of a 17-mer peptide. They found that m-phthalaldehyde can stabilize the helix when conjugated through an oxime bond at i, i+4 position. In contrast, stapling with three other aldehydes (p-phthalaldehyde, o-phthalaldehyde and 4,4'-biphenyldicarbaldehyde) afforded less stabilization. Conjugation through a hydrazide bond resulted in less helicity compared to the oxime product [49]. These results are similar to the study by DeGrado which showed that only m-DBMB can stabilize the α-helix structure, while o-DBMB and p-DBMB do not show such stabilization.

The same group, in a separate publication, demonstrated that stapling the sequence Ac-WEEWDZEINZYTKEIHKLIRE (where Z is an unnatural aminoxy carrying amino acid) with 1,4-phthalaldehyde can be promoted in the presence of its receptor (HIV gp41-5 protein), suggesting that the receptor can act as a template to enhance macrocyclization (Figure 14B) [131]. Unfortunately, this stapled peptide did not show increased helicity or improved affinity towards the protein. The binding affinity increased (4.5 fold) only after stapling with 2,5-thiophenedicarboxaldehyde, which indicates the effect of the structure of a linchpin on the binding. The cyclization did not increase the helical content of the peptide. This work, however, suggests the potential of using the target receptor as a template for cyclization and imprinting of a productive conformer. To our knowledge these are the only examples of linchpins based on dynamic reversible covalent bonds. Future elaboration of these type of approaches could potentially be used to empower de novo discovery of molecular interactions similar to technologies such as dynamic combinatorial libraries and molecular imprinting [132].

The cross-linkers that react with identical functional groups in peptides are C2V or C3V symmetric. Asymmetric linchpins for cyclization of peptides require two or more sequential reactions in orthogonal conditions. The Fasan group developed a strategy to cyclize peptides fused to N-terminal cysteine of intein GyrA, using two orthogonal reactive groups (Figure 15). Amber suppression was used to genetically encode para-acetylphenylalanine (pAcF) in the peptide sequence which provides the ketone to react with the hydroxylamine end of the linchpin. Upon oxime bond formation between the peptide and the linker, splicing of the intein completes the cyclization (Figure 15). They showed disruption of the interaction between p53 and HDM2 by a 12-mer peptide that was cyclized at i, i+11 position [133].

10. DYNAMIC CONTROL OF PEPTIDE CONFORMATION BY LIGHT-RESPONSIVE LINCHPINS

Light-responsive linchpins (LRL) mentioned in several sections above are a clear example of new functionality that

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**Figure 13.** $C_{3v}$ symmetric linchpins for generation of bicyclic peptides. S$_2$2 substitution of three thiols with bromobenzyls results in a single stereoisomer, while Michael addition of three thiols to Dha produces eight stereoisomers.
a linchpin can add into the peptide backbone. LRL-modified peptides offer a possibility to control many receptor ligand interactions dynamically with spatial and temporal resolution, [134-136] and empower fields of chemical optogenetics and opto-pharmacology. LR-linchpins usually consist of substituted LR-“cores” that change conformation in response to irradiation with light. The conformational change (switching) can be a result of cis/trans isomerization (e.g., azobenzenes) [137], or formation/opening of a covalent bond (e.g., diarylethenes [138-140], spiropyrans [141, 142]). Azobenzenes and diarylethenes are C2v symmetric and are suitable for cross-linking of two reactive groups in peptides. Asymmetric LR-linchpins with two orthogonally reactive groups are also known. While it is not difficult to take any other previously described C2v linchpins and marry them with a light-responsive core, the electronic properties of substituents of the core often influence its photo-switching properties (e.g., switching wavelength, thermal relaxation half-life, photostationary state, etc.). These properties have to be taken into account when designing the reactive handles for the cyclization. Reactive groups can be placed more than two bonds apart from the photo-switching core to minimize the disturbance of photochemical properties. Excessive length of linker that connects peptide and LRL, however, can dampen the transmission of structural changes from linchpin to the peptide. Some elegant solutions yield desired electronic properties after the reaction. For example, Hopmann and co-workers incorporated a fluorinated Azb-amino acid in calmodulin (CaM) protein. This moiety can undergo an intramolecular SNAr reaction with a cysteine inserted in a mutant of the CaM. Such reaction, changed the maximum absorption of the Azb from 326 nm to 340 nm and improved the performance of the photo-switch (Figure 16) [143].

Although photo-switching ligands have been known since the 1950s, the modern design of light-responsive macrocycles started with the pioneering report of Woolley and co-workers demonstrating the first use of an azobenzene linchpin [40]. The sequence Ac-EA\text{CARVA}ibAA\text{CEA}AA\text{RQ} (where Aib is aminobutyric acid) modified by this linchpin at \(i, i+7\) through alkylation of thiols, increased the helical content at 11 °C from 25% to 48% after light-induced isomerization to the cis form. The helical content decreased to 12% at dark photostationary state. Notable subsequent examples by Woolley and Allemann are LRL-stapled HDH3 (a mutant of engrailed homeodomain binding peptide) with trans-BSBCA linker to increase its binding to DNA from \(K_d = 200 \text{ nM}\) to \(K_d = 7.5 \text{ nM}\). After irradiation, the same cyclic peptide shows lower binding affinity (\(K_d = 140 \text{ nM}\)) [144]. Using BSBCA, LR-peptide ligands have been synthesized.
for targets such as streptavidin, [30, 145] PDZ domain of human tyrosine-phosphatase [146], β-adaptin [147], and RNA [148].

A detailed overview of the design of LR-linchpins is beyond the scope of this review and for an in-depth review, we recommend reference [134]. Efforts that went into the design of LRL are significant. For example it was important to vary the span of LR-linchpins to identify optimal length and substituents that yield the most significant change upon irradiation [149-151]. Scanning of various thiol-reactive groups was critical to avoid unwanted reactions with amines [151-154]. SAR studies taught the interplay of substitution position (ortho, meta, para) on switching properties and guided the design of LR-linchpins that have both optimal reactive groups and desired switching properties. The effect of amines, alkyls, amides and other substituents on the thermal relaxation half-life (0.25 ms – 43 h) [155, 156] and switching wavelengths (320 nm – 670 nm) [157, 158], is now relatively well-understood. This knowledge allowed the Woolley group and other groups to design Azb linchpins that respond to red light (635 nm) [159]. Isomerization by red light can provide a platform for photo-switchable peptide drugs, because red light exhibits a higher degree of penetration through the skin to the tissues. Although the stability of Azb in vivo is limited, the fundamental knowledge acquired in the Azb-LRL field will guide future efforts in photopharmacology and chemical optogenetics. Of note, most LR-linchpins have two attachment points to yield a mono-cyclic LR-peptide; C2-symmetric linchpins with three reactive groups that yield light-responsive bicyclic peptides have been reported recently (Figure 9C) [160].

Derda and co-workers [30], and then Heinis and co-workers [145] employed BSBCA linchpins to modify genetically encoded (GE) libraries of peptides and identify light-responsive macrocycles that bind to streptavidin only in the presence (or absence) of light. Both groups demonstrated that the LR-GE libraries of phage can be generated by conjugating BSBCA to billions of peptides. The ligands that are identified from these LR-libraries show different binding to streptavidin in the presence and absence of light. Differences in Kd between cis and trans isomers of the ligand reached only 4-5 fold changes due to, presumably, limited yield of isomerization of the Azb scaffold. Still, these examples suggested that screening of a GE-library of peptides with built-in light-responsive linchpins could serve as one method for lead identification in photopharmacology.

11. OTHER CLASSES OF LINCHPINS

Baker, Caddick and co-workers pioneered the use of dibromomaleimide [161] and dithiomaleimide [162] to produce maleimide-based linchpins (Figure 18A). While dibromomaleimide can react with both thiol and phosphine-based
Today, there exists a wealth of reactions that selectively functionalize the aromatic chains (reviewed in [167]). In theory, any reactive group that has a reported high reaction rate and selectivity towards an aromatic side chain residue should be amenable to production of a C2-symmetric linchpin that cross-links these side chains. Still, only a few reports incorporate the reactive groups into bidentate functionalities and show cross-linking of two aromatic residues explicitly. One example of explicit cross-linking of aromatic residues by an external linchpin is by Johannes and coworkers (Figure 19A): heating 4-bromobenzaldehyde and Camphor-10-sulfonic acid catalyst with a peptide in a sealed tube, introduces a linchpin that bridges two Trp residues in peptides (9 examples) [168]. A report by Albericio, Lavilla and co-workers had all functional attributes of linchpin-like cyclizations [169]: the authors reported that the 1,4-diodobenzene linchpin reacts with two Trp residues, albeit the explicit demonstration was cross-linking of two cyclic peptides, each containing one Trp residue, rather than cyclization of one peptide with two Trp residues (Figure 19B).

12. LINCHPIN-BASED CYCLIZATION OF PEPTIDES: THE BOUNDARIES OF THE DEFINITION

While most examples in this review describe side-chain-to-side-chain cyclizations, a linchpin assisted terminus-to-side-chain and head-to-tail cyclization (Figure 1) are also possible. The example of the former is a reversible macrocyclization of peptides with linchpin derived from Meldrums acid reported by Anslyn and co-workers (Figure 19E) [170]. Yudin and co-workers reported multiple examples of head-to-tail cyclization of peptides using isocyanides and aziridine-aldehydes. These reagents engage N- and C-termini of protected peptides in a disrupted Ugi 4-component reaction (Ugi-4CR) that proceeds via an imidoanhydride intermediate to assemble into a cyclic peptide with aziridine...
linchpin [171]. Yudin and coworkers, also reported an aza-Wittig variant of an Ugi-4CR head-to-tail cyclization of peptide with externally added (N-isocyanimino)phosphorane and aldehydes. The two components and termini of the protected linear peptide assemble into an oxadiazole linchpin which was shown to increase passive membrane permeability (Figure 19C) [172].

Based on the latter two examples, other multicomponent reactions that employ peptide-borne functionalities and externally added component(s) fall under the definition of linchpin-mediated cyclization. The pictorial definition of linchpin-mediated cyclization in Figure 1, thus, can be formalized as: “the transformation of a linear peptide that links side chains, termini, or a combination of thereof, giving rise to a cyclic product that incorporates the elements of the externally supplied reagents”. Recent examples that fall under this definition include a Copper-catalyzed Mannich addition (“A3 macrocyclization”) employed by Lubelle and coworkers (Figure 19D) [173], as well as the Ugi and Passerini reactions employed for peptide cyclization by the Riviera group as described in a recent Account [174]. A report by Malins et al., describing a one-pot Strecker reaction or reductive amination of cyclic imines formed from peptidylaldehydes could also fall under this definition because cyclic products incorporate an externally-supplied CN group (Figure 19F) or hydrogen atoms, respectively [175].

13. LINCHPIN-BASED MODIFICATIONS OF GENETICALLY-ENCODED PEPTIDE LIBRARIES

Introducing linchpins into readily available peptide libraries provided by translational machinery and encoded by DNA or RNA is an emerging general approach for late stage functionalization of millions to billions of compounds at once. Most chemical transformations applied for the modification of genetically-encoded peptide libraries have been mentioned in prior sections. Figure 20A summarizes published examples of stapling of genetically-encoded libraries that use: (i) amide bond formation between two Lys residues in mRNA-encoded library [65]; and (ii) synthesis of phage-displayed cyclic libraries via SN2 alkylations of Cys side chains by bisbromoxylene [176], dichloroacetone oximes [90], chloroacetamide derivatives of azobenzene [30], and crown ether [50], Michael addition by allenamide derivatives [114], and S$_n$Ar arylation by decafluorosulfone [96]. Figure 20B describes examples of bicyclic libraries with linchpins incorporated through Michael addition to three acrylamide groups [88], S$_n$2 reaction between 1,3,5-Tris(bromomethyl) benzene and phage displayed peptides [32], and potentially, mRNA-displayed libraries of peptides [35].

Most aforementioned examples follow the traditional canons of chemistry and strive to design reactions that introduce linchpin and yield a well-defined product or a mixture of a limited number of substitutional isomers or diastereo-
In contrast, Heinis and co-worker inverted the tradition and produced bicyclic peptide by stochastic cross-linking of four cysteines in phage-displayed peptides by C2-symmetric electrophiles [177] (Figure 20B). Although each cross-linking produces a large number of substitutional isomers in an unpredictable ratio, the authors used selection process to find the active mixture of isomers and then deconvoluted the structure of the active isomer after the selection. Similar stochastic synthetic of tricyclic genetically-encoded peptide libraries was proposed a year earlier in the publication by Chuanliu Wu and co-workers (Figure 10D) [98] but the authors have not reported the implementation of this proposal yet.

CONCLUSION

The modification of peptides and peptide libraries with “unnatural” linchpins offers a general and versatile approach for adding new properties to peptide sequences that cannot be added with conventional macrocyclization approaches. The multi-point attachment of linchpins offers topological solutions that are distinct from those achieved by incorporation of unnatural functionalities via UAA-mutagenesis and bioorthogonal reactions that target one amino acid. Examples of the application of linchpins in medium-to-large size proteins are currently limited, but a few published examples of light-responsive linchpins (LRL) built into the proteins highlight the potential advantages of such hybrid post-translational modification of proteins by LRL.
The field is fueled by an ever-expanding palette of unnatural amino acids that can be introduced in peptides via synthetic or biological approaches. Despite the increasing availability of peptides and peptide libraries with UAA, there will always remain a need for introduction of linchpin-type structures into “natural” translationally-made peptides and proteins. We envision that new challenges in this area could be: the identification of uniquely reactive linchpins that cross-link non-traditional residues, i.e., not Cys, Lys and Asp/Glu, in natural peptides and proteins. Linchpins that cross-link Tyr, Trp, His, or Arg residues can already be engineered based on known reactions that target these residues. Even a difficult chemical problem such as design of a linchpin that can cross-link primary or secondary alcohols (side chains of Ser/Thr) in water in the presence of other nucleophiles and electrophiles could be potentially solved via proximity-directed reactions and multi-dentate reactions with boron derivatives. As in classical organic synthesis, the most interesting and practical solutions could emerge from multi-step reactions that capitalize on intra-molecular events at a later stage of the multi-step manifold. For example, a robust inter-molecular reaction between the linchpin and the peptide can be used to introduce the first 1-2 attachment points. Subsequent intra-molecular reactions can drive regiospecific reactions between linchpin and other side chains within the peptide. The DCA linchpin introduced by Dawson can be used as a platform for a “thought experiment” in design of such staged, multi-step cyclizations: for example, robust alkylation of Cys docks the linchpin and places the ketone group in proximity to other side-chain residues. The ketone moiety exhibits low reactivity to all natural side chain residues. But a careful design can build on intra-molecular arrangement of ketone and side chains of Lys, Tyr, Trp or other residues and trigger their reaction in appropriate conditions.

Linchpins could be thought of as co-factors that equip peptides with functions that side chain residues of the peptides alone cannot provide. This analogy helps to illustrate the future impact of linchpins on peptide science. The evolution of life on Earth reached a critical irreversible decision to keep only ~20-amino acid oligomers and five-nucleotide oligomers and add further upgrades to the “oligomer world” via introduction of cofactors and post-translational modifications. These co-factors allowed proteins to perform new tasks without the need to produce and encode new amino acids. Oligomers of a limited set of monomers combined with versatile cofactors and PTMs are the only molecular solution to “life” as we know it. It allows organisms across all domains of life to perform all critical biochemical functions and evolve new functions. Similarly, future molecular solutions in the fields of therapeutics, materials, and bio-

Figure 20. Application of linchpins to modify phage displayed peptides and genetically-encoded peptide libraries displayed on M13 phage, T4 phage and mRNA.
CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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