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Orthogonal Cysteine–Penicillamine Disulfide Pairing for Directing the Oxidative Folding of Peptides

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Supporting Information

ABSTRACT: Precise disulfide pairing in synthetic peptides usually is achieved using orthogonal protecting group strategies or relies on primary sequence manipulation. Orthogonal disulfide pairing technology should be promising for directing the rational folding of multicyclic peptides from the fully reduced peptides. Here, we report a discovery on the orthogonality between heterodisulfide pairing of cysteine (Cys) and penicillamine (Pen) and formation of Cys-Cys/Pen-Pen homodisulfides. The orthogonal Cys-Pen disulfide pairing can be exploited for highly selective production of certain (multi)cyclic structures (or even a sole structure without isomers) through direct oxidation in air or thiol–disulfide exchanges in redox media. This strategy makes rational folding of multicyclic peptides without protecting groups, sequence manipulation, and complex synthetic reactions a reality, thus providing invaluable assets to peptide communities, and should greatly benefit the development of multicyclic peptide therapeutics and ligands.

The precise pairing of cysteine (Cys) residues is essential for the conformational folding and stabilization of many proteins and natural disulfide-rich peptides.[1] In natural systems, precise disulfide pairing is achieved by multiple oxidation, reduction, and rearrangement steps, which are predetermined by the primary sequence of the polypeptide chains and strategically regulated by molecular chaperones and enzymes such as protein disulfide isomerase.[1a,2] These requisites, however, are notoriously difficult to reconstitute *in vitro* for the synthesis of disulfide-rich/multicyclic peptides, particularly when the primary sequence has to be extensively manipulated for the development of peptide therapeutics and ligands.[3] Consequently, precise disulfide pairing in synthetic peptides is most often achieved using orthogonal protecting group strategies, which are usually sophisticated and laborious, and overall yields are generally low.[3f,4] Orthogonal disulfide pairing technology without protecting groups should be meritorious and hold great promise for the construction of structurally complex peptides, because it enables the oxidative folding of fully reduced peptides into certain multicyclic structures precisely in redox media. However, this kind of technology existed only in fancy until the recent discovery of the orthogonality between CXC motifs (cysteine-*any*-cysteine) and isolated Cys residues, which has been exploited for the directed folding of multicyclic peptides.[3b] Despite achieving the goal of orthogonal disulfide pairing, the requirement of exerting CXC motifs and the bidirectional pairing of CXC motifs (i.e., parallel and antiparallel) significantly limit the applicability of this technology. Novel disulfide pairing strategies that can direct the folding of peptides—without protecting groups, extensive manipulation of primary sequence, and recourse to elaborate postsynthetic reactions—are still greatly desired.

In this work, we report the discovery of orthogonality of disulfide pairing between Cys and penicillamine (Pen) (i.e., heterodisulfides) and that between two Cys or two Pen residues (i.e., homodisulfides). Pen is a Cys-like amino acid with β,β-dimethyl substitution, which has been traditionally used for stabilizing disulfide bridges or to consolidate structural rigidity of peptides.[5] The orthogonality of disulfide pairing can be exploited to rationally direct the folding of peptides containing Cys and Pen residues for highly selective production of certain cyclic structures and peptide dimers from fully reduced peptides through direct oxidation or thiol–disulfide exchanges in redox media.

When a Pen-containing peptide (**1**) was co-incubated under an oxidizing condition with a Cys analogue (**2**), we observed selective formation of a heterodisulfide (**1**-**2**), but negligible formation of homodisulfides (**1**-**1** and **2**-**2**) after the depletion of **1** and **2**, monitored by liquid chromatography and mass spectrometry (LC-MS) (Figures 1 and 2a). This finding clearly

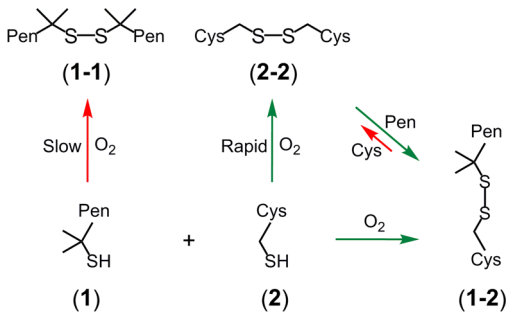

Figure 1. Orthogonal Cys-Pen disulfide pairing: diagram illustrating the orthogonality of disulfide pairing between cysteine and penicillamine (**1**-**2**) and that between two cysteines (**2**-**2**) or two penicillamines (**1**-**1**); note that flanking residues in peptides were omitted for clarity. **1** = GGRPenGW; **2** = GGRCGW.

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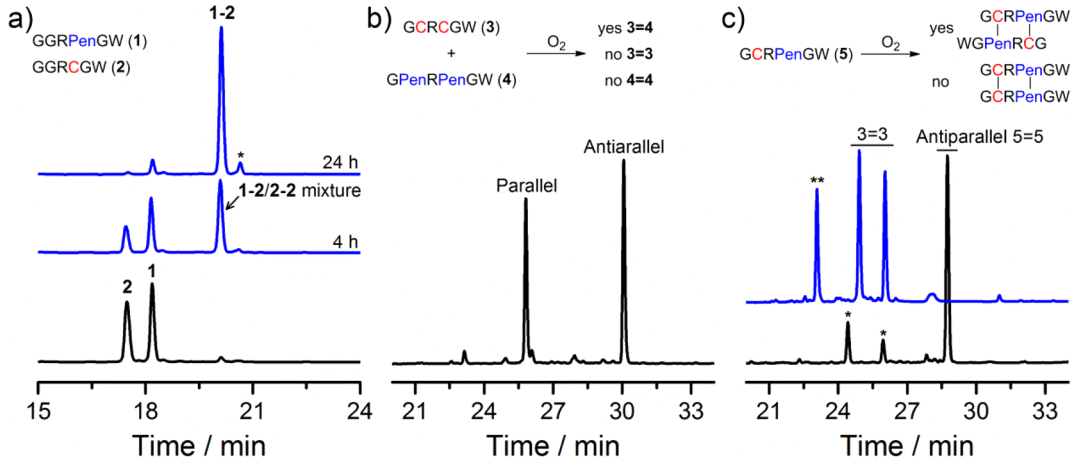

Figure 2. (a) Orthogonal Cys-Pen disulfide pairing: oxidation of **1** (50 μM) and **2** (50 μM) in 30 vol % DMSO/100 mM phosphate buffer for 4 and 24 h (blue line), respectively. Note that **1**-**2** and **2**-**2** have the same retention time in chromatograms, as characterized by mass spectrometry. Asterisk (*) denotes the formation of **1**-**1** from the slow oxidation of **1** (minor product). As calculated from the peak areas, directed disulfide pairing exhibits a high selectivity, the quantity of **1**-**2** exceeds >10 times that of **1**-**1**. (b) Heterodimerization of peptides: products formed after the oxidation of **3** (50 μM) and **4** (50 μM) in 30 vol % DMSO/100 mM phosphate buffer (only asymmetric dimers **3**=**4** formed). (c) Antiparallel dimerization of peptides: products formed after the oxidation of **5** (50 μM; black line) or **3** (50 μM; blue line) in 30 vol % DMSO/100 mM phosphate buffer; **5** oxidized to antiparallel **5**=**5** as the major product, while **3** oxidized to both parallel and antiparallel **3**=**3**. *denotes closed-loop monomer of **5** and parallel **5**=**5** (minor product), respectively. **denotes closed-loop monomer of **3**.

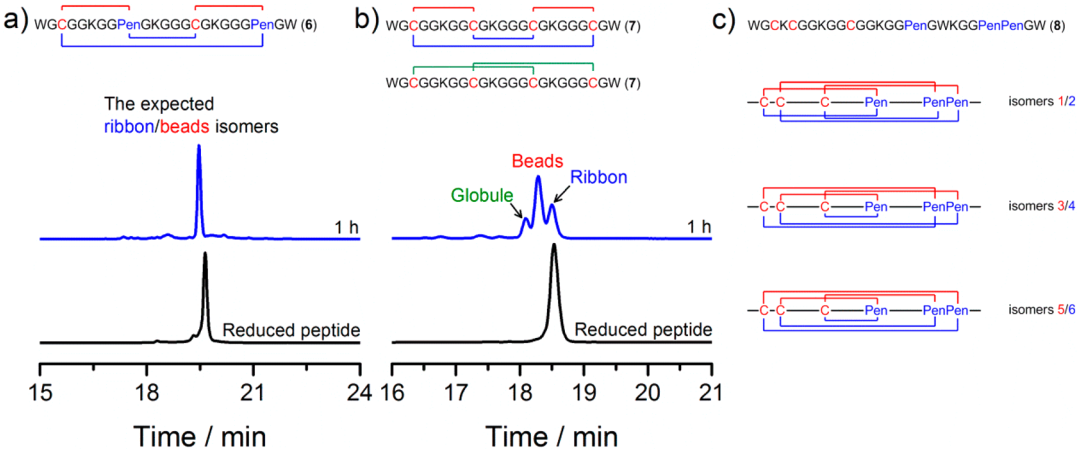

Figure 3. Directed folding of peptides **6**–**8**. (a) Oxidative folding of **6** in 0.5 mM GSH/0.5 mM GSSG redox buffer (~1 h) into the expected ribbon and beads isomers (these two isomers have the same retention time in chromatograms; Figure S7). (b) Oxidative folding of **7** in 0.5 mM GSH/0.5 mM GSSG redox buffer (~1 h). (c) The six possible (also expected) isomers formed after the oxidative folding of **8** in air or in GSH/GSSG buffers.

demonstrates the perfect orthogonality between the disulfide pairing of Cys and Pen and the formation of homodisulfides. Examination of the oxidation process reveals that **1**-**2** was formed not only by direct oxidation of **1** and **2**, but also from thiol–disulfide exchanges between **1** and **2**-**2**. This was indicated by the initially more rapid formation of **2**-**2** during the oxidation compared to **1**-**2**, and its subsequent depletion due to thiol–disulfide exchanges with **1** (Figures 1 and S1). In comparison to cysteine-cysteine (Cys-Cys) disulfides, Cys-Pen disulfides are known to have approximately 10-fold slower rate of thiol–disulfide exchanges due to steric hindrance arising from the two methyl groups adjacent to the disulfide (Figure S2).[5d,e] On the other hand, the thiol group of Pen, as an attacking group to disulfides, possesses relatively comparable activity compared to cysteine thiol (Figure S3). Owing to these regularities, the equilibrium between **2**-**2** and **1**-**2** shown in Figure 1 is strongly directed toward the asymmetric disulfide, which therefore contributes to the orthogonality of Cys-Pen and Cys-Cys disulfides. In addition, the unfavorable trend of formation of **1**-**1**, which is largely resulted from the diploid steric hindrance, on the other hand contributes to the orthogonality for the formation of Cys-Pen and Pen-Pen disulfides. Indeed, the oxidation rate of **1** is ~26-fold slower than that of **2** (Figure S3). The kinetics-driven orthogonal Cys-Pen disulfide pairing represents a novel principle based on which selective formation of disulfide bonds from fully deprotected thiols can be achieved.

The orthogonal Cys-Pen disulfide pairing was first exploited to direct the dimerization of peptides containing two thiols. An instance of particular interest is CXC-containing peptide, which has been known to have the ability of forming an intrinsically stable macrocycle containing two disulfide bonds.[3b,6] This unique feature can be rationally exerted to regulate the folding of peptides and polymers and for stabilizing macromolecular complexes and secondary structure of peptides.[3b,6b,c,7] To examine if the orthogonality of the hetero-/homodisulfide pairing can be extended to the CXC peptides, peptides **3** and **4**

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(GCRCGW and GPenRPenGW, respectively) were co-incubated under oxidizing conditions. Interestingly, homodimeric disulfides (**3**=**3** and **4**=**4**) were negligibly formed after the oxidation, and asymmetric dimers (**3**=**4**, both parallel and antiparallel) dominated the products (Figures 2b, S4, and S5). To examine whether the property of bidirectional CXC/CXC pairing can be regulated through the orthogonal Cys-Pen disulfide pairing, peptide **5** (GCRPenGW) with a CXPen motif was designed and investigated. This peptide oxidized in air to yield an antiparallel dimer as major product (Figures 2c and S6). By contrast, its CXC analogue (**3**) oxidized to form both antiparallel and parallel dimers in a ∼1:1 ratio as expected (Figure 2c). Thus, it is now possible to produce asymmetric CXC dimers and to further restrict the complexity of products to the sole antiparallel dimer without protecting groups and complex synthetic reactions. This study also lends new elements to the CXC toolbox, which would benefit a broad range of applications. In addition, we envisioned the potential of exerting the orthogonal Cys-Pen disulfide pairing technology to other types of peptide dimers with two intermolecular disulfide bonds.[8]

We next exploited the orthogonal Cys-Pen disulfide pairing for directing the intramolecular folding of peptides into multicyclic structures. Our design is based on a principle, that is, a Cys residue will be preferentially paired with Pen at equilibrium while half of the Cys residues in a peptide are replaced with Pen residues (i.e., intramolecular Cys-Pen disulfide pairing). A 23-mer peptide **6** containing two Cys residues and two Pen residues was designed with the Cys/Pen residues isolated alternately (Figure 3a). To disfavor sequence-dependent prefolding of the peptide toward a specific isomer, **6** and all peptides thereafter were designed to contain primarily (achiral) glycine residues. The non-glycine amino acids were placed strategically for facilitating the analysis of disulfide pairing by tryptic digestion LC-MS. Oxidation of **6** yielded a single HPLC peak (∼100% conversion) containing the two expected isomers (Figure 3a), that is ribbon and beads topology, respectively (Figure S7). Of note, the globule isomer (Cys-Cys and Pen-Pen pairing) was not observed. In contrast, the oxidative folding of a control peptide containing four Cys residues (**7**) results in formation of all the three isomers as expected (Figures 3b and S8−S11). Particularly, the yield of the globule isomer was >20% for **7**, which is in remarkable contrast to that observed for **6**. These results clearly demonstrate the applicability of the orthogonal Cys-Pen disulfide pairing technology for directing the oxidative folding of peptides.

While the displacement of two Cys residues with Pen in a four-Cys-containing peptide can reduce the total number of isomers from three to two, thus by analogy, three-Pen displacement in peptides containing six cysteines will reduce the complexity of oxidative products by 60% (i.e., from a total of 15 isomers to 6 expected isomers). Indeed, for a peptide (**8**) containing three Cys and three Pen residues, only Cys-Pen pairings (∼100% of total peak areas) were detected in the folded structures, as characterized by tryptic digestion LC-MS (Figures 3c and S12). This efficiency might be more pronounced when the number of Cys residues in peptides further increases to eight (total/expected: 105/24). This result, as well with the selection of proper Cys residues for Pen displacement, would greatly facilitate the synthesis and purification of one or several specific isomers.

Bicyclic peptides with two disulfide bonds are the most concise structures formed from the folding of peptides containing multiple Cys residues, which are widely present in nature and particularly, they are promising models for drug developments.[3a,c,9] However, even when the desired isomer was chromatographically purified (usually the biologically active one), it still suffers from disulfide bond rearrangements to yield the other two possible isomers in biological fluids due to thiol−disulfide exchanges. While the problem of the disulfide bond rearrangement in peptides with two adjacent disulfide bonds (cysteine framework CC−C−C) can be avoided through introducing dithiol amino acids in a recent report,[3c] the problem still remains for their close relatives with a cysteine framework of CXC−C−C, which was found in the family of α-chemokines and in peptide toxins apamin and saratotoxin, and has been exploited as templates for the development of therapeutics and ligands.[9]

Peptide **9** were expected to be oxidized in GSH/GSSG buffers to yield a mixture of bridged and fused bicyclic structures in ∼100% conversion (∼40%/60%; Figure 4a), because the third

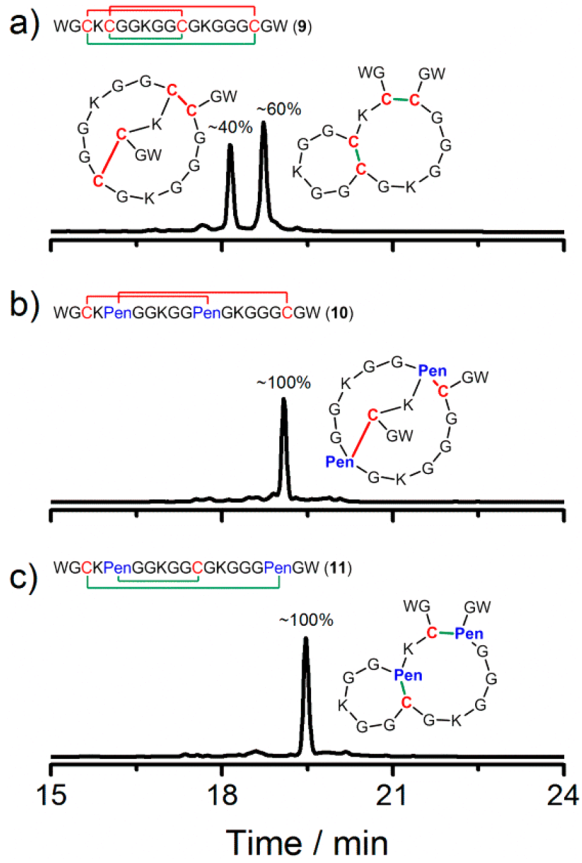

Figure 4. Bicyclic peptides without isomers. (a) Oxidative folding of **9** in 0.5 mM GSH/0.5 mM GSSG redox buffer. (b) Oxidative folding of **10** into a quasi-sole bridged bicyclic structure. (c) Oxidative folding of **11** into a quasi-sole fused bicyclic structure (disulfide pairing analysis, Figures S13−S17).

isomer cannot be formed due to the intrinsic instability of the CXC ring.[3b] Interestingly, while two of the Cys residues within the peptide was strategically replaced with Pen to design **10** and **11** (Figure 4b,c), the oxidative folding of the peptides resulted in rapid and highly selective formation of bridged and fused bicyclic structures, respectively (∼100% conversion), owing to the exclusive disulfide pairing between Cys and Pen residues. As in both case only a single structure formed, the problem of disulfide isomerizations does not exist anymore. Indeed, the obtained bicyclic structures remain stable in GSH buffers (0.5 and 1.0 mM) at room temperature (Figure S18). By contrast, the isolated isomers formed from **9** are subject to rapid disulfide isomer-

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izations in the same redox buffers (Figure S19). To our knowledge, this is the first reported case of the exclusive formation of either bridged or fused bicyclic structures—relying only on the framework of Cys/Pen, but not the primary sequence of peptide—through direct oxidative folding of peptides containing four fully deprotected thiols.

Despite the emergence of the use of stable bridges (or disulfide surrogates) to regulate the oxidative folding of peptides or proteins,[10] using solely oxidative disulfide formation is still most intriguing as this is a naturally evolved strategy and, furthermore it is by far the most efficient and simple reaction to cyclize peptides without side reactions. Effort to more closely mimic the formation of disulfides during the folding has also been praised, which exploited diselenide formation to steer oxidative peptide/protein folding, by replacing cysteine with selenocysteine in the primary sequence.[1b,11] Interestingly, the orthogonal Cys-Pen disulfide pairing strategy explored herein might provide a complementary to existing strategies for more rationally and precisely regulating the folding of peptides (or even proteins). Reducing possible ways of forming disulfide bridges during protein folding should be no doubt beneficial to the selective formation of fewer specific isomers. This would greatly lighten the burdens in oxidative protein folding, where only one of the all possible isomers corresponds to native fold.

In summary, we report a discovery on the orthogonal Cys-Pen disulfide pairing, which has a great potential of being exploited for directing the peptide dimerization (or cyclization) and the oxidative folding of peptides or proteins. The folding was directed by orthogonal Cys-Pen disulfide pairing taking place spontaneously in air or redox buffers; thus, it does not involve complex synthetic reactions and protecting groups. Furthermore, the strategy enables, for the first time, the selective and precise folding of certain bicyclic structures with two distinct disulfide bonds without isomers. The specific structures that are formed after the folding are not determined as conventionally by the primary sequence of peptides, but by the framework of Cys/Pen residues. This feature makes the bicyclic folds particularly suitable as templates for designing peptide libraries. Finally, it would be interesting to further combine the strategy presented herein with other directional disulfide pairing technologies[3b,c] for generating multicyclic structures with not only greater complexity but also less isomeric structures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10779.

Experimental details and Figures S1−S19 (PDF)

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Notes

The authors declare no competing financial interest.

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