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Dual In-Tether Chiral Centers Modulate Peptide Helicity

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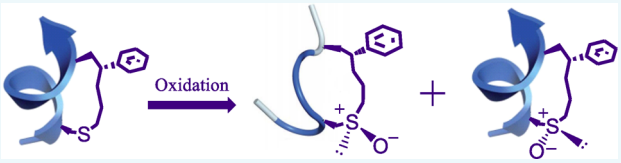
Supporting Information

ABSTRACT: The facile chemical modification on the peptide cross-linking moiety is an important strategy for improving the physicochemical properties of a peptide. Herein, peptides were constrained into helical conformations via the synergistic effects of dual in-tether chiral centers. A pentapeptide minimalistic model was used to determine the correlation between the absolute configurations of the dual in-tether chiral centers and the secondary structures of the peptides. This strategy provides an on-tether modification site that does not interrupt the secondary structure of the peptide.



INTRODUCTION

Constraining a peptide into a helical structure via an additional artificial tether is a common strategy used to develop peptide ligands of protein−protein interactions (PPIs). Well-designed tethers have the potential to enhance the helical content, stability, target binding affinity, and cellular uptake of the peptide. Over the past decade, constraint peptides have been used in a variety of biological studies.[1−8] Constrained peptides have been constructed using disulfide bridges,[9] lactams,[10,11] ring-closing alkene[12−14] and alkyne metathesis,[15] click reactions,[16−18] cys-alkylation,[19] and the incorporation of perfluoroarenes.[20,21] Because the peptide tether has been revealed to interact with the protein binding pocket in some protein−ligand interactions[22,23] (e.g., PDB−3V3B), significant efforts have been invested to develop on-tether modification methods. Dawson et al. developed acetone-linked peptides that could undergo further oxime ligation.[24] Smith et al. developed an inverse electron-demanding Diels−Alder reaction to construct an *S,S*-tetrazine tether for peptide stapling and labeling.[25] Spring et al. reported a double-click strategy to construct peptides that bear different tethers for PPIs ligand screening.[26] However, peptides properties are closely related with peptide secondary structures.[27−30] To date, the effects of on-tether modifications on the peptides' secondary structures were only sporadically studied on case-by-case bases.[26] Based on our knowledge, an elaborate on-tether modification approach that will certainly maintain the peptide secondary structure is still unknown.

In 2016, Moore et al. reported that an in-tether chiral center of stapled peptides influenced the secondary structures and binding affinities of the peptides.[27] Meanwhile, we reported that a carbon chiral center precisely placed in a single-bonded tether dominates the backbone peptide's helicity and biochemical and biophysical properties, including metabolic stability, cellular uptake, and target binding affinity.[28−30] This chirality-induced helicity strategy (CIH) has been expanded from a hydrocarbon chiral center to in-tether sulfur-based chiral centers such as sulfilimines and sulfoxides.[31−34] The CIH peptides provide a unique platform with which to evaluate solely conformational effects on the biophysical properties between peptide epimers. Precedent research indicated that amphipathic helices exhibit far greater cell permeability than similar sequences lacking helicity.[35,36] We also indicated that helical peptide epimers show significantly enhanced cellular uptakes compared with nonhelical epimers.[28,29]Notably, we also reported that the substituent at the in-tether chiral center significantly influences the cellular uptakes and target-binding affinities of the peptides.[30]

The in-tether chiral center was shown to be an intriguing site for further modifications. In positions i and i + 4 in the CIH system shown in Figure 1, the absolute configuration of the chiral center at the C-γ position (γ to the C terminus tethering residue) determines the secondary structure of the peptide. (R indicates helical, and S indicates random coil), while the sole modification on the N-γ position (γ to the N-terminus tethering residue) shows negligible effects on the secondary structure of the peptide.[28] A similar phenomenon was also observed in sulfoxide and sulfilimine CIH systems.[31,34] In this CIH system, we noticed a labile sulfur atom at a nonessential position. Thus, we proposed that this labile in-tether sulfur center would be an ideal modification site to avoid secondary structure perturbation caused by modification.

In this work, taking advantage of the well-established carbon chiral-center CIH system and sulfoxide−sulfimine chiral-center

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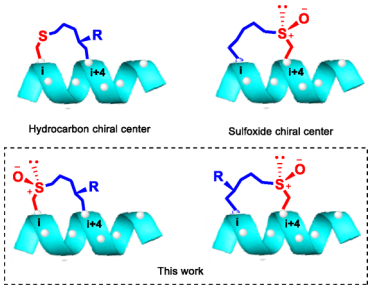

Figure 1. Schematic representation of a helical peptide with dual in-tether chiral centers. Top left: CIH peptides with an in-tether hydrocarbon chiral center. Top right: sulfoxide chiral center. Bottom: Helical peptides with dual in-tether chiral centers.

CIH system, we systematically studied how dual in-tether chiral centers (D-CIH) influence the secondary structures of the backbone peptides on a pentapeptide model system. The model peptides were studied by circular dichroism (CD) spectroscopy and 2D nuclear magnetic resonance (NMR). To our surprise, the nonessential N-γ position in the mono CIH system becomes essential in the D-CIH system, and it was found that N-γ-S and C-γ-R absolute configurations are required for the D-CIH system to induce helicity in the peptide backbone. This induced effect is more pronounced in long peptides. This study provides an approach for on-tether modification method that will certainly maintain the secondary structures of the peptides, which significantly enriches the chemical space for the on-tether modifications of constrained peptides.

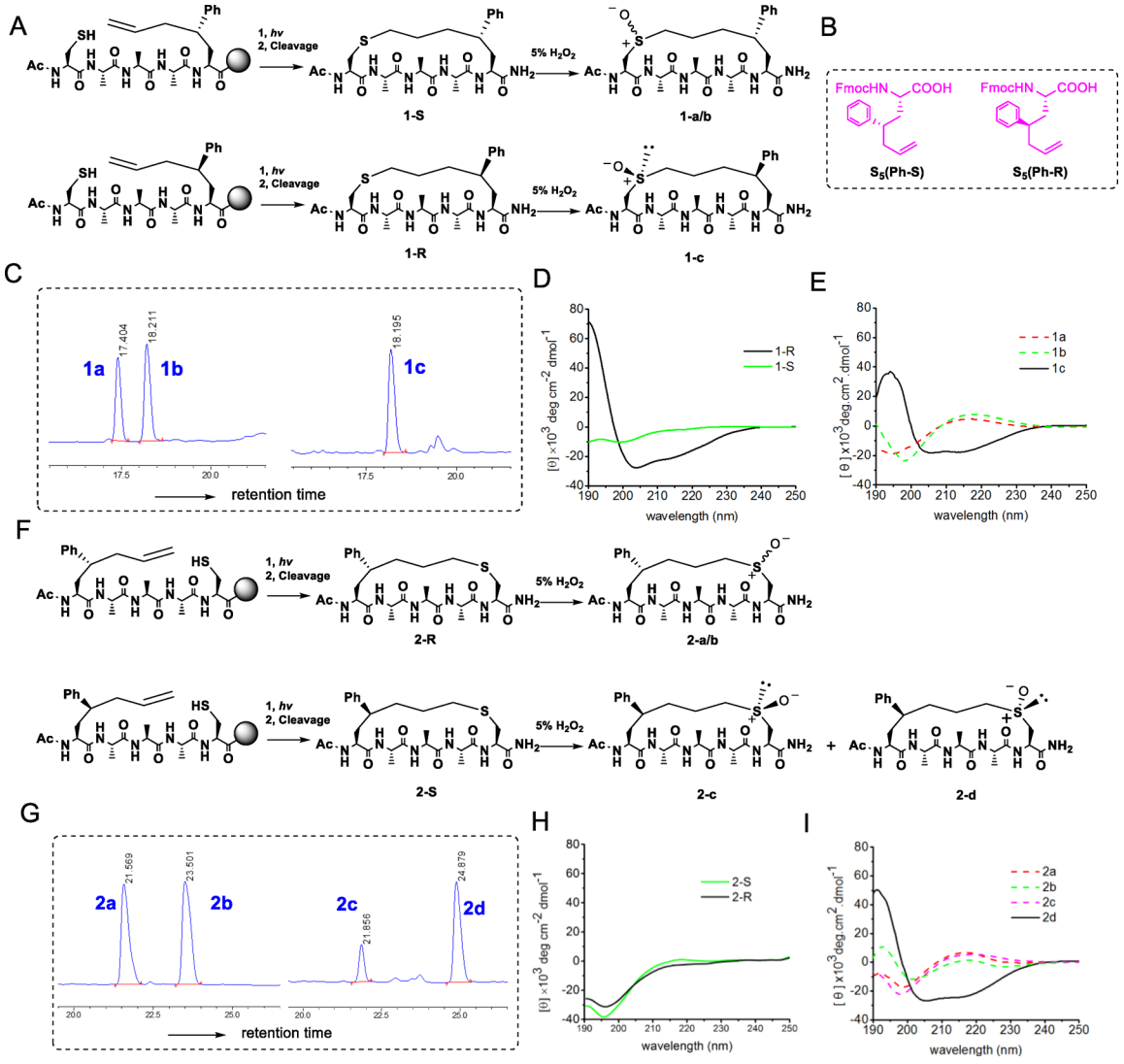

Figure 2. Synergetic effect between the absolute configurations of the dual in-tether chiral centers and the backbone peptide's secondary structures. (A, F) Peptides **1-S**, **1-R**, **2-S**, and **2-R** were synthesized by the thiol−ene cyclization reaction. Peptide sulfoxides **1a−b**, **1c**, **2a−b**, and **2c−d** were obtained by chemical oxidation of thioether peptides **1-S**, **1-R**, **2-R**, and **2-S**, respectively. (B) The γ-phenyl unnatural amino acids S_5(Ph−R) and S_5(Ph−S) were used to determine the absolute configurations of both chiral centers in helical peptides. (C) Representative high-performance liquid chromatography (HPLC) trace figures of peptide epimers **1a−c**. (D) The circular dichroism (CD) spectra of single-chiral-center peptides **1-R** and **1-S** measured in H_2O at 25 °C. (E) The CD spectra of dual-chiral-center peptides **1-a−c** measured in H_2O at 25 °C. (G) Representative HPLC trace figures of peptide epimers **2a** and **2b** and of **2c** and **2d**. (H) The CD spectra of single-chiral-center peptides **2-R** and **2-S** measured in H_2O at 25 °C. (H) The CD spectra of dual-chiral-center peptides **2-a−d** measured in H_2O at 25 °C.

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Table 1. Pentapeptide Library, the Molar Ellipticities and Percentage of Helical Contents of the Helical Epimers (N-γ S, C-γ R), and Product Ratio of Epimers (N-γ R, C-γ R/N-γ S, C-γ R)

	Ac−$Y_1X_aX_bX_cY_2$−NH_2		helical epimer (N-γ S, C-γ R)						
entry	Y_1	$X_aX_bX_c$	Y_2	$[\theta]_{215}$	$[\theta]_{207}$	$[\theta]_{190}$	$[\theta]_{215}/[\theta]_{207}$	helicity[a]	ratio[b]
1[c]	C(O)	AAA	S_5(Ph)	−17 475	−18 000	18 676	0.97	0.69	only product
2[c]	S_5(Ph)	AAA	C(O)	−24 391	−26 337	47 759	0.93	1.00	1:3
3	C(O)	AAA	S_5(Me)	−16 031	−24 127	21 909	0.66	0.64	only product
4	C(O)	AIA	S_5(Me)	−8638	−14 701	11 062	0.59	0.32	1:2
5	C(O)	AQA	S_5(Me)	−9399	−17 578	19 817	0.53	0.35	1:2.5
6	C(O)	ASA	S_5(Me)	−6549	−13 147	5008	0.50	0.26	1:1.5
7	C(O)	AGA	S_5(Me)	−2627	−4577	−3955	0.57	0.10	1:1.5
8	C(O)	AAI	S_5(Me)	−10 556	−18 078	17 483	0.58	0.41	1:1.5
9	C(O)	AFA	S_5(Me)	1671	−8199	7116	−0.20	–	only product
10	C(O)	AAI	S_5(Ph)	−10 956	−16 898	544	0.65	0.41	1:3
11	S_5(Me)	AAA	C(O)	−17 619	−18 033	31 407	0.98	0.72	1:8
12	S_5(Ph)	AIA	C(O)	−14 802	−15 594	37 258	0.95	0.61	1:3

[a]The final relative helical content was calculated based on peptide **2d**, which was defined as 100% helical.[37] [b]The ratios were calculated based on integration of peak areas of peptide epimers c/d in HPLC. [c]Entry 1 and 2 are denoted to peptides 1 and 2 mentioned in Figure 2, respectively. For those entries showing only one peak in HPLC, the ratio is labeled as "only product".

RESULTS AND DISCUSSION

Because the N-γ position is nonessential in the monochiral-center CIH system, a model pentapeptide system was used to study the correlation between the absolute configurations of the two chiral centers in the tethers and the secondary structures of the peptides. The pentapeptide system was used to avoid any possible sequence perturbation (Figure 2A).[37] Peptide Ac−cyclo-[CAAAS$_5$(Ph-R)]−NH_2, **1R**, was synthesized from enantiomerically pure unnatural amino acids (Figure 2B).[38,39] Meanwhile, peptide **1S** was prepared by an analogous route using enantiomerically pure unnatural amino acid S_5(Ph-S). Both peptides were purified and oxidized by stirring in 5% H_2O_2 for 1 h at room temperature to give >95% conversion.[40,41] Interestingly, the peptide epimers **1a** and **1b** (~1:1) were obtained from **1S**, while only one peptide epimer, **1c**, was obtained from **1R** by reverse-phase high-performance liquid chromatography (HPLC; Figure 2C). The ^{1}H NMR spectrum of **1a−c** was recorded in dimethyl sulfoxide (DMSO)-d6; although these peptides possessed same chemical composition, they displayed great variation in amide chemical shifting (Table S1). Circular dichroism spectroscopy revealed that only **1c** displayed α helical structure in water, while peptides **1a** and **1b** showed nonhelical structures (Figure 2E). Then a further question need be confirmed: is the N-γ position still nonessential in the D-CIH system?

To answer this question, we then prepared peptides **2R** and **2S** (Ac−cyclo-[S_5(Ph-R/S)AAAC]−NH_2)with chiral center at the N-γ position of predetermined absolute configuration, as shown in Figure 2F,G. Both peptides **2R** and **2S** were random-coil (Figure 2H) and underwent oxidation smoothly. Interestingly, we obtained all four possible epimers **2a−2d**, and only one peptide epimer **2d** exhibited helical features (Figure 2I). Notably, peptides **2a** and **2b** were generated in a roughly 1:1 ratio, while peptides **2c** and **2d** were generated in a roughly 1:3 ratio (Figure 2G). The ^{1}H NMR experiment of **2a−d** was conducted in DMSO-d6, and detailed chemical shifts assignment of them were summarized in Table S1. These peptides also showed apparent distinctions in their chemical shifts. This control study unambiguously answers our question: the N-γ position is essential in the D-CIH system.

In summary, for peptides **1c** and **2d**, they need a reserved C-γ-position R chiral center and a N-γ-position S chiral center, respectively. Only when both of the absolute configurations were satisfied could the peptides have a helical structure. Notably, altering the nonessential N-γ position in the mono CIH strategy leads to an unexpected synergetic effect in the D-CIH system, emphasizing the importance of the clear elucidation of on-tether modifications' influences on the peptides' secondary structures.

To confirm whether this finding is a generally applicable strategy, a panel of pentapeptides were prepared and tested as shown in Table 1. D-CIH effects were observed for all peptides, and the experimental results are summarized in Table 1 and Figures 3A and S1. Notably, when both of the oxidized peptide epimers were not helical, the epimer ratio was determined to be roughly 1:1 based on HPLC peak integration. However, when one of the two oxidized peptide epimers was helical, the helical epimer was the only product (Entry **1**, **3** and **9**) or the major

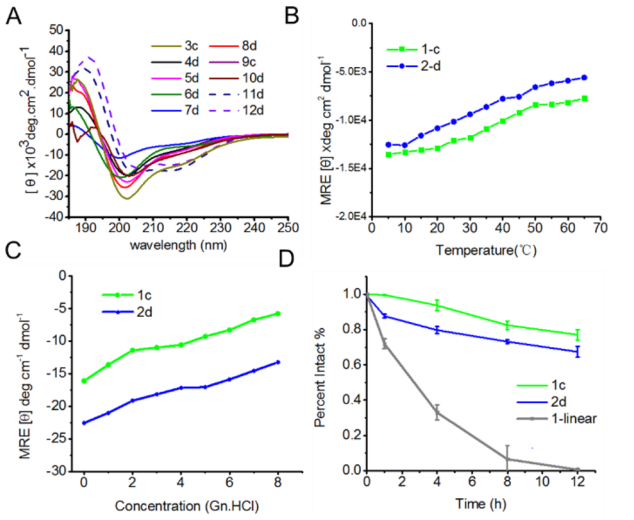

Figure 3. Sequence tolerance and stability study of D-CIH peptides. (A) The CD spectra of the helical products from the oxidation of peptides **3−12** in deionized water at 25 °C. (B) Molar ellipticity at 215 nm of **1c** and **2d** from 5 to 65 °C. (C) Molar ellipticity at 215 nm of **1c** and **2d** over increasing guanidine−HCl content at 25 °C. (D) In vitro serum digestion assay of peptides **1c** and **2d**.

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product in the rest of the entries (see the Table 1 "ratio" column and the Supporting Information for HPLC spectra). The preference of a helical epimer was further proved by a time-dependent HPLC oxidation experiment (Figure S2). Besides, compared to the oxidized precursor peptide **1R**,[26] the dual-chiral-center helical epimer **1c** had similar helical content, and the $\theta_{[215]}/\theta_{[207]}$ values were closer to 1 (Figure S3). A similar phenomenon was observed with other peptides, indicating a more-regular helical turn induced by the D-CIH system. The CD spectra of helical D-CIH peptides were summarized in Figure 3A. Notably, in some cases, such as entry **1** and **3**, the bulky Ph- substituent group exerted a preferable helical inducing effect than the Me-contained peptide, which was consistent with our previous results.[28] It indicated the possibility of tuning a dual-chiral-center peptide's helicity through alternation of the substituent group in the stereocenter.

The stabilities of peptides **1c** and **2d** were investigated by thermal denaturation, denaturation with guanidine−HCl, and in vitro serum degradation experiments (Figure 3B−D). The mean residue ellipticities for peptides **1c** and **2d** were calculated from $[\theta]_{215}$. The ellipticities gradually decreased as temperature or guanidinium concentration increased, but the peptides still retained most of their helicity at high temperature or high guanidine−HCl concentrations (Figures 3B−C and S4). These results suggest that the D-CIH peptides have stable secondary structures. Notably, peptides **1c** and **2d** remained 60% intact after incubating in serum for 12 h (Figure 3D and Figure S5). The linear version (**1-linear**) of peptide **1c** was used as a reference peptide and quickly degraded. Racemization of a sulfoxide chiral center requires 6 M $HClO_4$,[42] which is not likely to happen in physiologically related conditions by chemical means in vitro. A reinjection of aged peptide **2d** onto HPLC also showed no epimerization (Figure S6). Biological oxidation or reduction of tge on-tether sulfoxide of CIH peptides occur with relative difficulty.[34,43−45]

To better illustrate the effect of the D-CIH system on peptide conformation in aqueous solution, a detailed 1D and 2D ^{1}H NMR spectroscopy study of peptides **1c** and **2d** was performed in 90% H_2O: 10% D_2O at 25 °C. The nuclear Overhauser effect (NOE) signals of D-CIH peptides **1c** and **2d** were carefully analyzed, and multiple spectral features were found that are characteristic to a well-defined cyclic pentapeptide with α helicity (Figure 4).[46]

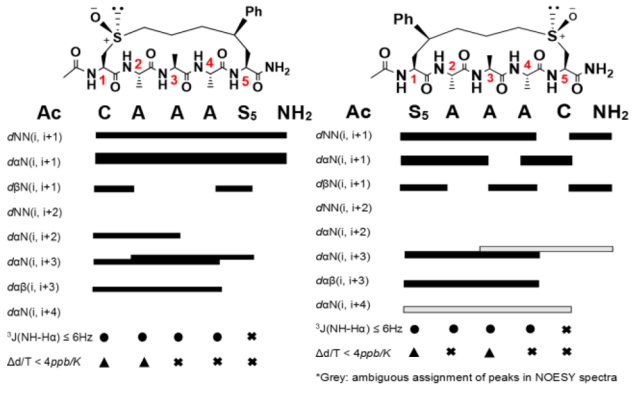

Figure 4. Nuclear Overhauser effect (NOE) summary diagram of **1c** and **2d**. Bar thickness parallels the intensity of the NOE signals. 2D NMR was performed in 90% H_2O/10% D_2O at 25 °C.

Except for the C terminal residue S_5(Ph-R), the other four residues for both peptides **1c** and **2d** had low amide coupling constants ($^3J_{NH-CH\alpha} \leq 6$), suggesting that they were part of helical structures. The observation in NOESY spectra of nonsequential medium-range $d_{\alpha N}(i, i + 3)$ and $d_{\alpha\beta}(i, i + 3)$ NOEs in **1c** and an additional $d_{\alpha N}(i, i + 4)$ signal in **2d** further suggests helical structures. In addition, the low temperature coefficient ($\Delta\delta/T < 4.5$ ppb K^{-1}) of C1 and A2 in **1c** and of $S_5$1 and A3 in **2d** were also indicative of hydrogen bondings typical of a helical structure (Figure S7). Therefore, the NOE and CD spectra of peptides **1c** and **2d** clearly showed that precisely positioned dual chiral centers the can induce helicity of the backbone peptide in aqueous solution.

The effect of the N-γ chiral center of the peptides was discussed. Compared to its nonoxidized precursor **1R**, the chemical shifts of Hα of the residues C1, A4, and $S_5$5 in **1c** were in a higher magnetic field, while residues A2 and A3 were shifted to a lower magnetic field, suggesting that the additional sulfoxide unit could affect the hydrogen bonding pattern of the backbone of the peptide. Peptide **1R** and **1c** showed similar NOE patterns and thermal coefficients (Figure S8), which further proved that the sulfoxide chiral center at the N-γ position in **1c** exhibited a synergetic effect in the D-CIH system and did not interrupts the secondary structure of the peptide. Notably, the peptide **2d** shows a $d_{\alpha N}(i, i + 4)$ NOE signal, which is absent in the **1c** peptides, suggesting that the C-terminal sulfoxide chiral center might have a stronger helix-inducing ability than the hydrocarbon chiral center. This is in agreement with previous CD spectroscopy studies that **2d** has higher helical content than **1c**. We propose that the cause for this phenomenon is that the S═O bond in C-terminus reduced the rotation angle of C−S−C bond and thus diminished the overall cross-linker flexibility of a peptide (Figure S9). This again proves the importance of the C-γ position in helicity enhancement.

To further verify the D-CIH strategy's tolerance of different amino acids and whether it could be translated into longer peptides, peptides **13**, Ac−cyclo(2,5)-[ECAAAS$_5$(Ph-S/R)W]−NH_2, and **14**, Ac−cyclo(2,5)-[ES$_5$(Ph-S/R)-AAACW]−NH_2, were synthesized and processed similarly, as shown in Figure 2. Results were summarized in Figure 5A,B. The only peptide epimers with helical structures were **13c** and **14d**, while the other epimers were random coils. Then the D-CIH strategy was tested with reported estrogen receptor α (ER-α) targeting sequence,[23] cyclo-HCILHS$_5$(Ph-*S*/*R*)LLQ, and PDI sequence-targeting mammal double minute 2 (MDM2) cyclo-TFCHYWS$_5$(Ph-S/R)QLTS.[22,47,48] Only one epimer of these peptides showed helical structures in CD measurements, and the results were summarized in panels c and d of Figure 5, respectively. Thus, we demonstrated that the D-CIH strategy could be translated into longer peptides to construct sophisticated helical peptide ligands.

The modification on the cross-linking moiety could tune the biophysical properties of a peptide, e.g., cell permeability.[35] To test the influence of a sulfoxide on cell permeability, we evaluated the cell penetration ability with peptide **15** Rho−βA−CRRRS$_5$(Ph-R)−NH_2 (βA: β-alanine; Rho: rhodanmine B) and its helical sulfoxide product **16** Rho−βA−C(O)RRRS$_5$(Ph-R)−NH_2. As shown in Figure S10, peptide **16** showed significant decrease of cellular fluorescence compared to peptide **15** in the fluorescence-activated cell sorting (FACS) assays. The incorporation of a sulfoxide is anticipated to add both hydrophilicity and polar surface area of the cross-linker,

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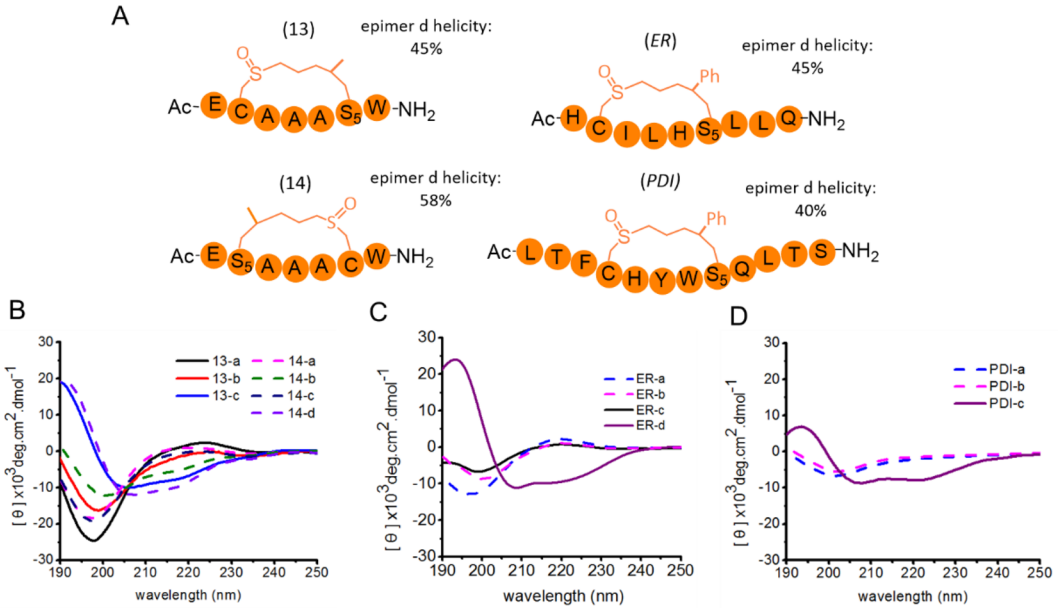

Figure 5. Synergetic effect of the dual in-tether chiral centers in long peptides. (A) The peptide sequence of peptides **13**, **14**, **ER**, and **PDI** and the calculated helicity of their d epimers, respectively. CD spectra of (B) peptides **13a−c** and **14a−d**, (C) peptides **ERa-d**, and (D) peptides **PDIa-c**, measured in 20% TFE buffer solution at 25 °C.

which interrupt the amphipathic structure of thio-ether peptide, leading to the reduction of cellular uptake.[34] These results further emphasized that an on-tether modification method should be carefully evaluated for their effects on the peptides' biophysical properties.

CONCLUSIONS

A systematic investigation of a synergetic D-CIH system to modulate the helicity of the peptide backbone is presented. To maintain helicity, the absolute configurations of the dual in-tether chiral centers necessarily had to be S for the N-γ position and R for the C-γ position. A pentapeptide model system was used to elucidate a correlation between the absolute configurations of the dual in-tether chiral centers. Next, the secondary structures of the peptides were well-profiled by using CD measurements and NMR spectra. This work shows that chemical oxidation of a precisely positioned in-tether sulfur chiral center could be accomplished while maintaining the helical structure of the peptide. The D-CIH peptides showed high stabilities against thermo- and chemo- denaturation and proteolytic digestion. In addition, the D-CIH effect is widely applicable for short peptides with different sequences and can be translated into longer peptides.

The synergetic D-CIH system offers an opportunity for the placement of on-tether modifications based on a labile in-tether sulfur center. This report is a systematic elucidation of the necessary stereochemistry of the two chiral centers on the tether and shows that post-synthesis on-tether modifications can be performed with retention of the secondary structure of the peptide backbone. In this proof-of-concept report, the sulfoxide modification showed detrimental effects on the peptides' cellular uptake. This clearly indicates the importance of careful elucidation of the modification's effects on peptides' biophysical properties. The facile N-γ sulfur site could undergo other modifications, including S-imidization and S-alkylation.[49,50] The D-CIH system is the first systematically elucidated on-tether modification system with a deliberately maintained secondary structure. The D-CIH strategy will significantly broaden the chemical space of on-tether modifications of helical peptide scaffolds. Further development based on the D-CIH system could render the peptides with required biophysical properties or with possibilities for further more-sophisticated modification. Research based on the D-CIH strategy is under current investigation in our laboratory and will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00171.

Additional details on materials and methods. Figures showing auxiliary ligands, chemical and crystal structures, synthetic processes, CD spectra, time-dependent oxidation experimental results, a comparison between peptides and epimers, temperature dependence of the mean residue ellipticity of peptides, HPLC analysis, temperature coefficients, NOE summaries, and cell pentration assays and mean fluorescence intensity. Tables showing chemical shifts and MS values. Selected NMR, HPLC, and LC−MS data. (PDF)

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§K.H. and C.S. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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