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Chemically Diverse Helix-Constrained Peptides Using Selenocysteine Crosslinking

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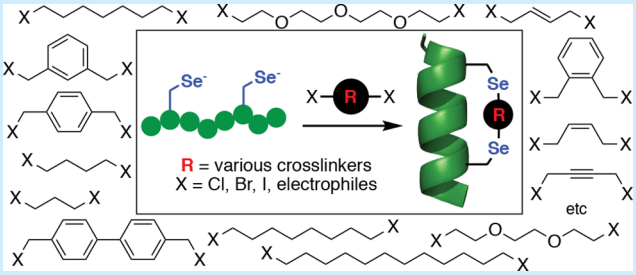
Supporting Information

ABSTRACT: The use of selenocysteines and various cross-linkers to induce helicity in a bioactive peptide is described. The higher reactivity of selenocysteine, relative to cysteine, facilitates rapid cross-linking within unprotected linear peptides under mild aqueous conditions. Alkylating agents of variable topology and electrophilicity were used to link pairs of selenocysteines within a p53 peptide. Facile selenoether formation enables diverse tailoring of the helical peptide structure.



Stapled peptides are a fast-growing class of bioactive peptides that reproduce an α-helix from a protein−protein interaction (PPI). Their ability to bind tightly at shallow PPI interfaces has enabled functional modulation of PPIs considered difficult to target with conventional drugs.[1] Rapid synthetic access to peptide helices with diverse components could accelerate the discovery of PPI inhibitors. Here, we describe a new strategy to induce peptide helicity that expands the scope of peptide stapling by cross-linking pairs of selenocysteine (Sec) residues.[2] The higher side-chain acidity of Sec vs cysteine (pK_a Sec: 5.2−5.6; pK_a vs Cys: 8.2; pK_a Sec within some peptides: 3−4)[2c−e] enables more facile reactions with electrophilic alkanes of varying length and reactivity (see Figure 1).

Many different side-chain to side-chain cross-linking strategies have been reported to induce helical structure in peptides,[1,3−5] including C−C bond formation via Ru-catalyzed olefin metathesis,[1c,6] lactam-bridging between Lys and Asp,[7] Cu-catalyzed 1,3-dipolar cycloaddition,[8,9] and bis-thiolation of peptides containing two cysteines.[10] Olefin metathesis and lactam stapling directly couple side-chains of two amino acids, limiting variations to the cross-link.[5,11,12] Changing staple composition or flexibility often requires elaborate synthesis and/or optimization of on-resin cross-linking reactions.[6,8] In a two-component approach, such as cysteine bis-alkylation (most popular)[10,13−15] or click-bridging with dialkynyl spacers,[9] a bifunctional linker is inserted between the two side-chains and permits chemical diversity in the staple.[3]

However, while connecting cysteine side-chains is simple, it is only practical with highly electrophilic linkers; for example, S-alkylation with bis-bromomethyl[13,14] or bis-haloacetamide[16] aromatic reagents, or S_N-arylation with reactive arylhalides such as perfluoroaryl,[15] palladium-a[17] and tetrazine[18] connectors. Less-electrophilic aliphatic cro[illegible]kers such as dibromoalkanes or diiodoalkanes fail to alkylate cysteines at pH 8 and 20 °C or give low yields[13a,19] and require harsher cyclization conditions that limit utility. Alternatively, cysteines can be modified with bis-diene linkers via photochemical thiol-ene reactions, but such reagents are less accessible than their electrophilic counterparts.[20]

Here, we demonstrate facile alkylation under mild conditions of two Sec inserted at (i, i + 4); (i, i + 7); or (i, i + 11) positions in an unstructured peptide analogue of the tumor suppressor p53 (see Figure 1). The sequence is based on a known peptide inhibitor (ATSP-7041) of the p53-MDM2 interaction in cancer cells.[21,22] We investigate the potential of linkers of differing size, hydrophobicity, and rigidity to cross-link two Sec residues and induce 1−3 helical turns in the peptide.

Conditions for selenoether formation were investigated by preparing a linear peptide Ac-LTFUEYWAQBUSAA-NH_2

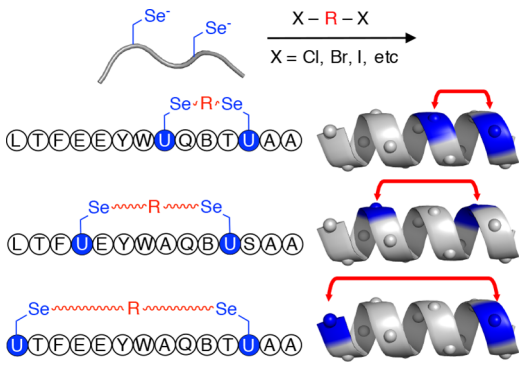


Figure 1. Proposed selenoether stapling of p53 mimicking peptide using different dihaloalkanes to cross-link two Sec, leading to α-helical peptide structures. (B = L-cyclobutylalanine; R = linker.)

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(where B = cyclobutylalanine), containing two Sec residues (U) positioned at (i, i + 7) positions, using standard Fmoc-based solid-phase peptide synthesis protocols and Fmoc-Sec(Trt)–OH[23] as the Sec building block (see Figure 2A). After cleaving

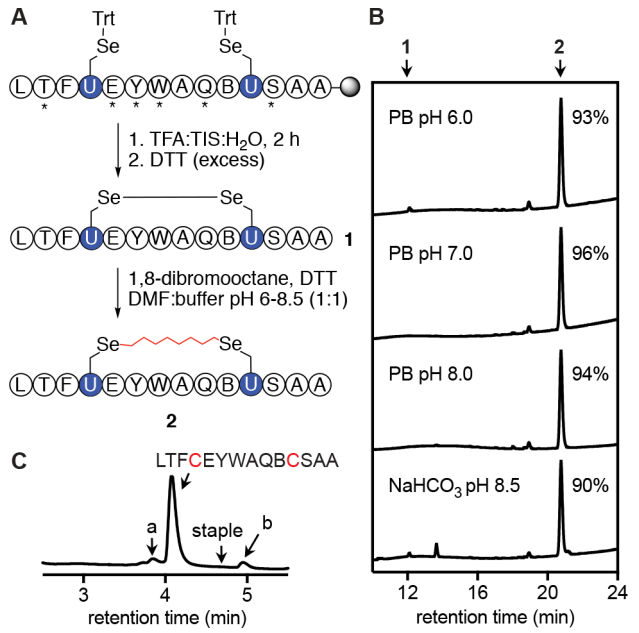

Figure 2. (A) Synthesis of Se-cross-linked peptide **2** via diselenide precursor **1**. Asterisks denote standard side-chain protecting groups. (B) HPLC analysis of Se-alkylation of **1** (100 μM) with 1,8-dibromooctane (800 μM) in the presence of DTT (2 mM) in DMF/0.1 M phosphate buffer (PB) (pH 6, 7, or 8) or DMF/0.1 M $NaHCO_3$ (1:1) for 1 h (5 h in pH 6) at 22 °C. The percent conversion of **1** to **2** is shown. Arrows indicate the retention time of **1** and **2**. (C) UPLC analysis showing lack of cross-linking of LTFCEYWAQBCSAA (1 equiv) with 1,8-dibromooctane (2 equiv) in 0.1 M $NaHCO_3$ (pH 8.5):DMF (1:1) for 18 h at 22 °C. No bis-*S*-alkylation and only traces of monoalkylated material (peak b) were detected. Since the linear peptide did not significantly oxidize (<8% S–S formation after 18 h, peak a), no reducing reagent was necessary (i.e., DTT or TCEP).

from the resin via acidolysis, the selenide moieties were liberated but, instead of two selenols, they formed an intramolecular diselenide bond, in addition to other diselenide oligomers.[2a,24] Adding reducing reagent dithiothreitol (DTT) to crude mixtures increased diselenide monomer **1** that was isolated by reversed-phase high-performance liquid chromatography (RP-HPLC) (see Figures S1 and S2 in the Supporting Information). To investigate the scope of Se-alkylation with less-electrophilic aliphatic linkers, diselenide **1** was combined with 1,8-dibromooctane (8 equiv) in the presence of DTT (20 equiv) in a mixture of DMF and 0.1 M phosphate buffer pH 6–8 or $NaHCO_3$. DMF was added to increase the solubility of the precursor peptide. In all cases, **1** was converted to diselenoether-tethered peptide **2** in excellent yield (Figure 2B). Although cross-linker was present in excess, it was only added once to the peptide, suggesting rapid ring closure with the second Sec residue. Cross-linking of corresponding cysteines in Ac-LTFCEYWAQBCSAA-NH_2 with 1,8-dibromooctane gave no or only traces of bis-thioether cross-linked peptide at pH 8.5 (Figure 2C) or under heating[25] (see Figure S3 in the Supporting Information).

Next, diselenide **1** was reacted with other bis-alkylators under similar neutral conditions (see Figure 3, as well as Figure S4

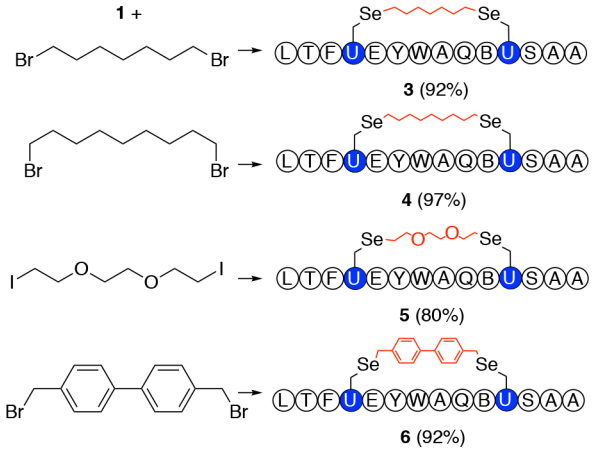

Figure 3. Alkylation of precursor **1** (100 μM) with 1,7-dibromoheptane (800 μM), 1,9-dibromononane (800 μM), 1,2-bis(2-iodoethoxy)ethane (200 μM), or 4,4′-bis(bromomethyl)-biphenyl (200 μM) in the presence of DTT (2 mM) in DMF/0.1 M PB pH 7 (1:1) for 1 h at 22 °C.

and Table S1 in the Supporting Information). Compounds 1,7-dibromoheptane and 1,9-dibromononane efficiently reacted with **1** to give cyclic peptides **3** and **4**. The more-reactive 1,2-bis(2-iodoethoxy)ethane and 4,4′-bis(bromomethyl)-biphenyl similarly produced **5** and **6**, but excess electrophile (beyond 2 equiv) simultaneously alkylated both Sec residues.

Encouraged by the high efficiency of Sec-cross-linking, we also investigated cross-linking of Sec residues at (i, i + 4) and (i, i + 11) positions. For these series, commercially available Fmoc-Sec(Mob)–OH was employed as Sec building blocks and subsequent Sec-deprotection occurred with 2,2′-dithiobis-(5-nitropyridine) (DTNP) in TFA during resin detachment, as previously described.[26] To simplify the synthetic protocol and improve yields, alkylating reagents were directly reacted with crude peptides after cleavage from the resin (see Figure S5 in the Supporting Information).[2a] A range of dichloroalkyl, dibromoalkyl, or diiodoalkyl or aryl linkers of different length and flexibility were used to cross-link Sec residues to form macrocyclic peptides **7**–**17** (see Figure 4, as well as Tables S2 and S3 in the Supporting Information).

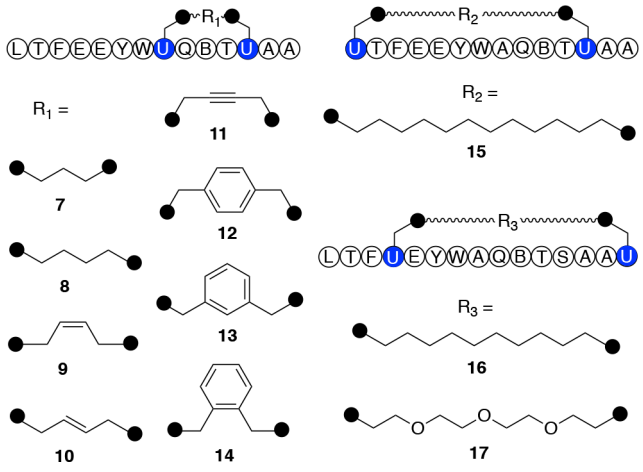

Figure 4. Examples of (i, i + 4) and (i, i + 11) selenoether cross-linked peptides **7**–**17**. Solid black circles represent Se atoms.

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The α-helicity of diselenoether peptides in phosphate buffer pH 7.2 was assessed by circular dichroism spectroscopy (see Figure 5). The peptides displayed varying degrees of α-helicity,

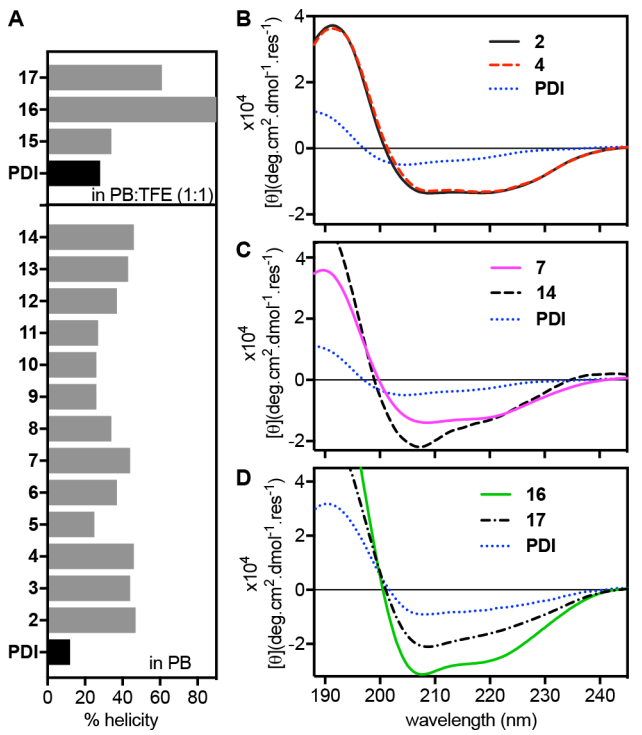

Figure 5. (A) α-Helicity (%) of selenoether-cross-linked peptides **2**–**17** (50 μM) in 10 mM phosphate buffer pH 7.2 (PB) or 10 mM PB:trifluoroethanol (TFE) (1:1) determined by CD spectroscopy. (B–D) CD spectra of PDI and the two most helical peptides in each series: (i, i + 7) (panel (B)), (i, i + 4) (panel (C)), and (i, i + 11) (panel (D)) in 10 mM PB (panels (B) amd (C)) or in 10 mM PB:TFE (1:1) (panel (D)) at 22 °C.

but are significantly higher (2–4 fold), compared to the parent linear sequence Ac-LTFEHYWAQLTS-NH_2 (PDI)[22] (Figure 5A). The longer alkane linkers showed the highest helix induction for (i, i + 7) constructs (**2** and **4**; see Figure 5B). For the (i, i + 4) series, helical stabilization was less prominent using alkene- or alkyne-constrained tethers than their aliphatic counterparts; whereas an *o*-xylene linkage (**14**) was the most α-helix-inducing aromatic connector of positions Sec8-Sec12, although with accompanying 3_{10}-helix formation (Figure 5C). Among (i, i + 11) stapled peptides, the 10-atom-long aliphatic cross-linker in **16** displayed the strongest α-helical stabilization (Figure 5D).

MDM2 binding of selenoether peptides with various linkers was evaluated by competition fluorescence polarization assay, where **2**–**17** competed against MDM2 complexed with a fluorescein-labeled PDI (FITC-PDI). Most cross-linked peptides showed enhanced affinity, compared to PDI (1.2–2.5-fold increase for 11 of the 16 stapled peptides; see Figure 6A, as well as Table S4 in the Supporting Information). Next, we examined the ability of the selenoether-stapled peptides to affect viability of MCF-7 breast cancer cells, determined by XTT assay after incubating peptides at 3, 10, and 30 μM concentrations (Figure 6B). The control linear peptide PDI showed no activity in this assay. From the (i, i + 7) series, peptides **2** and **4** were the most active and reduced cell viability in a dose-dependent manner. Interestingly, polyethylene glycol (PEG)-stapled peptide **5**

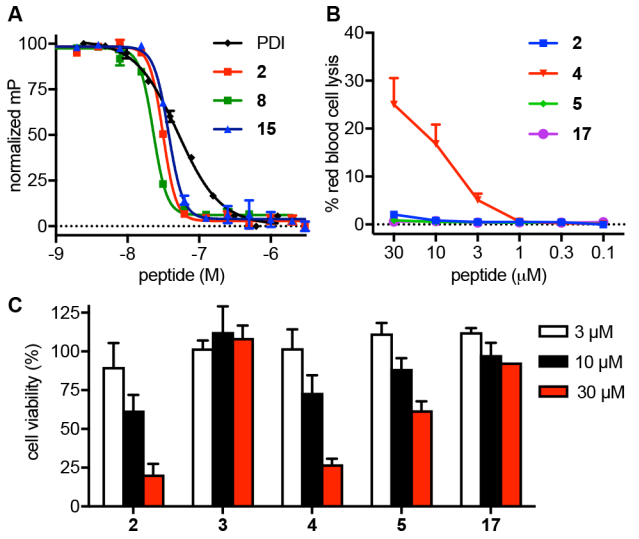

Figure 6. (A) Inhibitory activity of peptides PDI (IC_{50} = 57.7 ± 4.9 nM), **2** (IC_{50} = 31.2 ± 0.5 nM), **8** (IC_{50} = 22.8 ± 0.8 nM), and **15** (IC_{50} = 37.3 ± 0.9 nM) against a FITC-PDI/MDM2 complex. (B) Cell viability of MCF-7 cells after incubation at 37 °C with peptides **2**–**5** and **17** for five days. Remaining live cells after peptide incubation were colorimetrically determined by reducing the dye XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) by cellular enzymes of damaged cells. (C) Hemolysis of human red blood cells after incubation with peptides **2**, **4**, **5**, and **17** in PBS for 1 h at 37 °C.

showed moderate activity at 30 μM, indicating that PEGylated cross-linked peptides can also penetrate cells and act as PPI inhibitors. Compounds of the (i, i + 4) series did not reduce cell viability. Peptides **15** and **16** of the (i, i + 11) series showed poor solubility in an assay buffer and were not tested, while **17** was soluble but had no significant activity (Figure 6B).

To establish that the reduced cancer cell viability was not a consequence of nonspecific membrane disruption by the peptides, we tested the peptides for lysis of healthy human red blood cells (RBC). As shown in Figure 6C, octane-cross-linked peptide **2** and PEGylated peptides **5** and **17** exhibited no significant lytic activity in these cells, while the more-hydrophobic peptide nonane-cross-linked **4** induced moderate RBC lysis at higher μM concentrations.

We have demonstrated a simple synthetic strategy to rapidly construct structurally diverse helix-constrained peptides by purposely varying both the positions of two selenocysteine residues along the sequence and the composition of the aliphatic linker between them. Two-component selenoether stapling occurred under mild conditions, in neutral aqueous media at room temperature, with unprotected peptide precursors that are readily prepared by standard solid-phase peptide synthesis. Se-alkylation can be performed under a wider pH range than Cys-alkylation and is remarkably effective using less-reactive electrophiles such as dichlorobutane and dibromooctane. At neutral pH, DTT was an effective reductant for Sec without significantly reacting with electrophilic linkers (except for stronger alkylating reagent 1,2-bis(2-iodoethoxy)-ethane). Alternatively, Se-alkylation may be performed in the presence of ascorbate instead of DTT.[27] In this study, we found that a modified peptide epitope constrained at positions 4 and 11 via a 12-atom aliphatic cross-linker (**2**) was the most promising derivative from a diverse series of bis(selenoether) peptides. Considering the abundance of commercially available

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bifunctional electrophilic cross-linkers, the selenoether stapling approach demonstrated here permits facile access to peptide macrocycles of diverse chemical and topological composition that can be explored to fine-tune biophysical properties of peptide inhibitors of different PPIs. Such peptides hold the promise to target yet undruggable intracellular oncogenic proteins, triggering apoptosis and modulate cancer progression.[1] Finally, the Se-stapling approach adds to the growing repertoire of strategic applications of Sec in peptides and proteins, including advances in peptide folding, metabolic stability, intramolecular cyclization, and protein synthesis.[2,24,28]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b00233.

Experimental procedures for preparing selenoether stapled peptides, characterization of compounds by HPLC and MS analysis, and biochemical assays (PDF)

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Author Contributions

A.D.A. designed the chemistry and synthesized and characterized peptides. S.R.P. performed cell-based assays. D.P.F. directed the research. A.D.A. and D.P.F. wrote the paper.

Notes

The authors declare no competing financial interest.

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