An Evolution-Inspired Strategy to Design Disulfide-Rich Peptides Tolerant to Extensive Sequence Manipulation

Jun Zha†‡, Jinjing Li†, Shihui Fan†, Zengping Duan†, Yibing Zhao†, Chuanliu Wu†,*

†Department of Chemistry, College of Chemistry and Chemical Engineering, The MOE Key Laboratory of Spectrochemical Analysis and Instrumentation, State Key Laboratory of Physical Chemistry of Solid Surfaces, Xiamen University, Xiamen, 361005, P.R. China.

‡College of Continuing Education, Guizhou Minzu University, Guiyang, 550025, China

*Corresponding Author; Email: chlwu@xmu.edu.cn.
Table of contents

1. Experimental Section
   1.1 Materials and instruments
   1.2 Synthesis of peptides
   1.3 Oxidation of peptides
   1.4 Construction of phage-displayed peptide libraries
   1.5 Phage panning
   1.6 Fluorescence polarization (FP) assays
   1.7 Surface plasmon resonance (SPR) assays
   1.8 NMR characterization
   1.9 Proteolytic stability of peptides
   1.10 CD characterization

2. Supplementary Data
   2.1 Sequences and mass spectral characterizations of the synthesized peptides (Table S1–S4)
   2.2 Chromatograms of peptides (Figure S1–S8)
   2.3 Characterization of disulfide pairing of 12 and 14 using trypsin digestion (Figure S9–S10)
   2.4 Construction of phage-displayed peptide libraries (Figure S11–S12, Table S5)
   2.5 Sequences obtained from screening of peptide libraries and NMR characterization of the oxidized peptides (Figure S13–S16, Table S6–S7)
   2.6 Binding affinity of the oxidized peptides to target proteins (Figure S17–S23)
   2.7 Proteolytic stability of peptides (Figure S24–S25)
   2.8 CD spectra of the oxidized 15 and oxidized 16 (Figure S26)
   2.8 Mass spectra of peptides (Figure S27)
1. Experimental Section

1.1 Materials and instruments

All the Fmoc-protected amino acids and Rink amide MBHA resin used for peptide synthesis were supplied by GL Biochem (Shanghai, China). Glutathione oxidized (GSSG), glutathione reduced (GSH) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were bought from Energy Chemical (Shanghai, China). Na$_2$HPO$_4$·12H$_2$O, NaH$_2$PO$_4$·2H$_2$O, meta-phosphoric acid (HPO$_3$) and trypsin were purchased from Sigma-Aldrich (Beijing, China). Acetonitrile (ACN), trifluoroacetic acid (TFA), sodium acetate trihydrate (NaAc·3H$_2$O), EDTA disodium salt dihydrate, Sodium dodecyl sulfate (SDS), and all the chemicals used to prepare 1×TAE (Tris-HCl, EDTA disodium salt dihydrate, acetic acid), 1×TE (Tris-HCl, EDTA disodium salt dihydrate), neutralization buffer (Tris-HCl), elution buffer (glycine), 2YT medium (tryptone, yeast extract, NaCl), PEG/NaCl (PEG-6000, NaCl), binding buffer (Tris-HCl, NaCl, MgCl$_2$, CaCl$_2$), washing buffer (Tris-HCl, NaCl, MgCl$_2$, CaCl$_2$, Tween-20) and blocking buffer (Tris-HCl, NaCl, MgCl$_2$, CaCl$_2$, Tween-20 and BSA) were purchased from Sigma-Aldrich (Beijing, China), Sangon Biotech (Shanghai, China) or Sinopharm Chemical Reagent (Beijing, China). Dimethyl sulfoxide (DMSO), acetic anhydride (Ac$_2$O), N,N-dimethylformamide (DMF), piperidine, and phenol were bought from Sinopharm Chemical Reagent (Beijing, China). Klenow fragment, Sfi I, Not I, T4 DNA Ligase, and 20 bp DNA Ladder were bought from Takara (Beijing, China). 20×PBS (Na$_2$HPO$_4$, KH$_2$PO$_4$, NaCl, KCl), DL5000 DNA Marker, ampicillin (Amp), agar, ammonium persulfate and 4S Red Plus were bought from Sangon Biotech (Shanghai, China). EZ-Link™ Sulfo-NHS-LC-Biotin, Dynabeads™ M-280 Tosylactivated, Dynabeads™ M-280 Streptavidin and NeutrAvidin protein were purchased from Thermo Fisher Scientific (Shanghai, China). Streptavidin was purchased from Biosynthesis Biotechnology (Beijing, China). Acetonitrile-D3 was purchased from Cambridge Isotope Laboratories (Beijing, China). Deuterium oxide (D$_2$O) was purchased from Energy Chemical (Shanghai, China). TG1 *E.coli* strain was purchased from Beyotime (Shanghai, China).
All peptides were synthesized using solid-phase peptide synthesis by a CEM Discover Liberty BLUE microwave-assisted peptide synthesizer. High performance liquid chromatography (HPLC, SHIMADZU) equipped with a quaternary solvent manager (QSM), an AQUITY® PDA detector and a column manager was used for analysis of peptides. Reaction progress was monitored by HPLC (a flow rate of 1.0 mL·min⁻¹ flow rate of H₂O (+0.1% TFA) and ACN (+0.1% TFA); isocratic with 10% ACN (+0.1% TFA) for 5.0 min followed by a linear gradient of 10% to 85% ACN (+0.1% TFA) over 30 min). A HITACHI U-3900H UV/Vis spectrometer was used for the quantification of peptides. Surface Plasmon Resonance (SPR) assays were performed by Biacore T200. Fluorescence polarization was recorded by Tecan Infinite® 200 PRO Microplate Reader. Bruker impact II-TOF mass spectrometry, Bruker Esquire 3000 plus ion trap ESI mass spectrometry and Bruker autolex max MALDI-TOF mass spectrometry were applied to identify the formed peptides. NMR experiments were recorded at 298 K on Bruker AVANCE III 600 MHz and 850 MHz equipped with a cryogenic triple-resonance probe. Fast Protein Liquid Chromatography (FPLC) equipped with a Hitrap™ Desalting column (GE Healthcare) was used to purify the biotinylated proteins.

1.2 Synthesis of peptides

All peptides are N-terminally acetylated and C-terminal amidated, which were synthesized at 0.05 mmol scale using the Fmoc solid-phase peptide synthesis (SPPS) on a CEM Liberty blue automated microwave peptide synthesizer (Table S1). Amino acids were coupled onto the MBHA resins using the standard coupling protocol. Peptides were cleaved from the resin and deprotected by treating with a TFA cleavage cocktail for 4 h at room temperature. Then, the cleaved peptides were precipitated in cold diethyl ether, and purified using a HPLC system. All peptides were purified to a purity of >95% before performing the oxidation (Figures S1 and S7).

1.3 Oxidation of peptides

Reduced peptides (>95% purity) isolated by HPLC were identified by mass spectrometry (Table S2). The HPLC-isolated peptides were lyophilized and re-dissolved in a mixture of water and acetonitrile (70%:30% vol/vol) as the stock solution for further use. Concentrations of the reduced
peptide stock solutions were determined by UV/Vis spectroscopy ($\varepsilon_{\text{Trp}} = 5,502 \text{ cm}^{-1} \text{ M}^{-1}$ at 280 nm).

In a typical experiment for peptide oxidation, the reduced peptide was reconstituted in an appropriate amount of phosphate buffer (100 mM, pH 7.4) containing 30% acetonitrile, 0.5 mM GSH and 0.5 mM GSSG to achieve a concentration of 50 μM. After about 4–6 h, the oxidation was complete, and the oxidized peptide was characterized by HPLC and mass spectrometry (Table S3). The Acm-protected peptides were oxidized in the redox buffer in the same way. After the HPLC-purification of the oxidized Acm-protected peptides, the peptide was lyophilized and reconstituted in pure water to achieve a definite concentration (determined by UV/Vis spectroscopy). Then, the peptide solution was diluted by addition of methanol with 0.1% TFA to a concentration of 50 μM, into which 10 eq. I dissolved in methanol was added dropwise. After ~1 h, the Acm was completely removed from the peptide, leading to the formation of the third disulfide bond in the peptide. The final Acm-deprotected product was analyzed using HPLC and mass spectrometry (Table S4). To characterize the disulfide pairing of oxidized 12 and 14, the lyophilized powder (oxidized 12 and 14 isolated by HPLC) was dissolved in a phosphate buffer (100 mM, pH 6.0) containing 50 μg/mL trypsin for 4 h, and then the major digested peptide fragment isolated by HPLC (monitored at 280 nm) was identified using mass spectrometry (Figures S9 and S10).

1.4 Construction of phage-displayed peptide libraries

The peptide libraries were constructed based on a procedure reported previously (Table S5). In brief, DNA libraries encoding random peptide sequences were digested by $Sfi$ I (10 h, 50 °C) and $Not$ I (10 h, 37 °C), which were recovered by gel purification. Then, the purified DNA fragments were
ligated with gel-purified Sfi I/Not I-digested vector pCantab 5E (ratio of insert and vector: 10/1). The ligation mixture was then transformed into *E. coli* TG1 competent cells. The cells were plated on 2 × YT/ampicillin agar plates and incubated at 37 °C for 12 h. The size of the phage libraries was determined by measuring the total number of colonies. The colonies on the plates were then scraped off the plates and propagated for phage production and purification. 20–30 phage clones were randomly picked up for sequencing to evaluate the quality of the phage libraries.

1.5 Phage panning

Proteins (MDM2 and Bcl-2; 5 μM) were biotinylated in phosphate buffer saline (PBS; pH 7.4) with the addition of Sulfo-NHS-LC-biotin (50 μM) for ~0.5 h at room temperature. After the reaction, the unreacted Sulfo-NHS-LC-biotin was removed using a desalt column on an AKTA pure system (running buffer: pH 7.4 PBS). The biotinylated proteins can then be immobilized on streptavidin-coated and neutravidin-coated magnetic beads for screening as described previously.²,³ Briefly, 100 μL of streptavidin-coated magnetic beads was washed three times with binding buffer (150 mM NaCl, 1 mM CaCl₂, 10 mM Tris-HCl, 10 mM MgCl₂, pH 7.4) in a 1.5 mL microcentrifuge tube, which were then re-suspended with 100 μL binding buffer and distributed equally into two 1.5 mL microcentrifuge tubes. Then, the biotinylated protein (first round: 10 μg; second round: 5 μg; third round: 2 μg) was added into one of the two tubes; meanwhile, the same volume of 1 × PBS was added into the other one as a control. The two tubes were incubated on a slowly rotating vortex mixer for 15 min at room temperature. Then, the beads in the two tubes were washed three times with the binding buffer to remove the free proteins, which were then resuspended with 300 μL
binding buffer and 150 μL Blocking buffer (binding buffer containing 0.3% Tween-20 and 3% w/v BSA). The suspension of beads was incubated at room temperature for 2 h on a slowly rotating vortex mixer. Meanwhile, to a tube containing 3.0 mL of phages (>10^{12} t.u.) dissolved in binding buffer, 1.5 mL blocking buffer was added, and the solution was incubated at room temperature for 2 h on a slowly rotating vortex mixer. Then, the phages in solution were split equally into two tubes, into which the BSA-blocked beads with and without the immobilized proteins were added, respectively. After 30 min incubation at room temperature, the unbound phages in the supernatant were removed and the beads were washed for nine times with washing buffer (binding buffer containing 0.1% Tween-20) and twice with binding buffer. During the washing steps, the tubes were replaced at least three time with new ones to avoid the non-specific adsorption of phages. After the last washing step, the beads were resuspended with 200 μL elution buffer (50 mM glycine, pH 2.2) and incubated at room temperature for 5 min. Then, the beads were magnetically precipitated and the supernatant was transferred to a new microcentrifuge tube containing 50 μL neutralization buffer (1 M Tris-Cl, pH 8.9). The eluted phages from both the experimental and control group were diluted to infect exponentially growing TG1 cells to quantify the phage titer, and the eluted phages from the experimental group were propagated for the production of phages used for next-round panning. After three rounds of panning as described above, 20–30 phage clones were randomly picked for sequencing. Note that streptavidin-coated and neutravidin-coated magnetic beads were used to immobilize biotinylated proteins alternatively in each round of selection to prevent the enrichment of streptavidin and neutravidin binders during the panning.²,⁴ For the library screening against streptavidin, streptavidin-coated magnetic beads were directly used for panning.
1.6 Fluorescence polarization (FP) assays

FP assays were performed on 96-well flat-bottom OptiPlate black plates using a Tecan Infinite® 200 PRO Microplate Reader. To examine the binding affinity of oxidized 16 to Bcl-2, a fluorescein-labelled 16 (FITC-βAla-16) was synthesized and purified to a purity of >95% using HPLC. Fluorescein-labelled 16 was first dissolved in DMSO and then diluted with PBS (pH 7.4) for further use. Sumo-Bcl-2 was obtained from an E. coli cell expression system described previously.5 For the FP assay, 20 nM of FITC-βAla-16 was incubated with Sumo-Bcl-2 (0–1.2 μM) in PBS (pH 7.4) on a 96-well plate at room temperature for 10 min, and fluorescence anisotropies were recorded. The K_D value of the binding between FITC-βAla-16 and Bcl-2 can then be obtained by fitting the data with the single-site binding model. For the FP competition assay, 20 nM of FITC-βAla-16 and 400 nM of Sumo-Bcl-2 were incubated with oxidized 16 (0–10 μM) under the same conditions as described above, and fluorescence anisotropies were recorded. The K_i value of the binding between oxidized 16 and Bcl-2 can then be obtained using a data-fitting procedure reported previously.5 The binding of peptides to MDM2 was evaluated using the same procedure described above, which has been described in our previous reports.1, 6

1.7 Surface plasmon resonance (SPR) assays

SPR assays were performed using a Biacore T200. Target proteins (Sumo-Bcl-2 and streptavidin) were immobilized on chip surface using a procedure described previously.5 Briefly, Flow cell Fc4 was activated first with a mixture of NHS and EDC, and then Sumo-Bcl-2 (or streptavidin) dissolved in 10 mM acetate buffer (pH 5.0) flowed through the cell for protein coupling
to reach a target response of 1000 RU. Flow cell Fc3 that was not immobilized with proteins was used as the reference channel. Serially diluted samples were then passed over the flow cells at a flow rate of 30 μL/min (160, 320, 640, 1280, 2560, 640 nM for oxidized 15, 16, and 17; 20, 40, 80, 160, 320, 80 nM for oxidized 11, 15, and 16; 5, 10, 20, 40, 80 nM for oxidized 17). Kinetics data were then analyzed using a 1:1 binding model and local fit to obtain kinetic rate constant and dissociation constant.

1.8 NMR characterization

NMR samples containing 0.5 mM of oxidized 15 and oxidized 16 were prepared in 50% perdeuterated acetonitrile and 50% H2O. NMR experiments were recorded at 298 K on Bruker AVANCE III 600 MHz and 850 MHz equipped with a cryogenic triple-resonance probe for oxidized 15 and 16, respectively. Two dimensional (2D) 1H, 15N/13C HSQC spectra were recorded to obtain chemical shifts of 13C/15N in backbone and side-chains. 2D 1H-1H TOCSY (80 ms) and 2D 1H-1H COSY spectra were acquired to assign NMR signals of the peptide, and 2D 1H-1H NOESY spectra with a mixing time of 300 ms were measured to obtain 1H-1H distance constraints. The NMR data were processed using NMRPipe/NMRDraw and analyzed using NMRFAM-SPARKY.7,8 About 95% of all NOE cross-peaks were assigned manually. Prediction of backbone dihedral angle restraints of the oxidized 16 was performed using TALOS-N based on chemical shifts of backbone resonances. Structural calculations were carried out with ARIA2.3.2 and CNS1.21.9-11 An ensemble of 10 lowest-energy structures was generated from a total of 150 structures. The structures were visualized in PyMol and the quality of structures was analyzed using PROCHECK.12
1.9 Proteolytic stability of peptides

100 μM 15-Ala, 16-Ala, oxidized 15, and oxidized 16 were respectively treated with phosphate buffer (pH = 7.4, 100 mM) containing chymotrypsin at 37 °C. The chymotrypsin concentration used for the digestion of 15-Ala and oxidized 15 is 0.3 μg/mL. The chymotrypsin concentration used for the digestion of 16-Ala and oxidized 16 is 5.0 μg/mL. At predefined times, 50 μL aliquots were taken with a pipette, quenched with 70 μL 10% HPO₃, and analyzed by HPLC.

1.10 CD characterization

Circular dichroism (CD) spectra were measured using a 1.0 mm path length cuvette at room temperature (25 °C). All measured peptides were dissolved in water to reach a concentration of 50 μM. The baseline signal (pure aqueous solution) were subtracted from each spectrum. CD spectra were recorded in a wavelength range of 190–260 nm with a bandwidth of 2.0 nm, a date pitch of 1.0 nm, a response time of 8.0 s, and a scanning speed of 50 nm/min. The thermal denaturation CD spectra were recorded in a temperature range of 25–95 °C with a heating rate of 10 °C/min. For temperature melts, the change of ellipticity at 220 nm was monitored as temperature increased from 25 to 95 °C in an increment of 10 °C. Other experimental conditions were consistent with those of the general method.
2. Supplementary Data

2.1 Sequences and mass spectral characterizations of the synthesized peptides

Table S1. Sequences of the synthesized peptides

| Name   | Sequence                                                                 |
|--------|--------------------------------------------------------------------------|
| 1      | Ac-CGSGSGSGSGCPCCWPELCPWIRSC-NH₂                                         |
| 1-Acm  | Ac-(Acm)GSGSGSGSGCPCC(Acm)WPELCPWIRSC-NH₂                                |
| 2      | Ac-CGSGSGSGSGCPCICPELCPWIRSC-NH₂                                        |
| 2-Acm  | Ac-(Acm)GSGSGSGSGCPCIC(Acm)PELCPWIRSC-NH₂                               |
| 3      | Ac-CGSGSGSGSGCPCIWPCLCPWIRSC-NH₂                                        |
| 3-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPC(Acm)LCPWIRSC-NH₂                               |
| 4      | Ac-CGSGSGSGSGCPCIWPECCPSCRSC-NH₂                                        |
| 4-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPEC(Acm)CPWIRSC-NH₂                               |
| 5      | Ac-CGSGSGSGSGCPCIWPELCPSCRSC-NH₂                                        |
| 5-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPELCP(Acm)RSC-NH₂                                 |
| 6      | Ac-CGSGSGSGSGCPCIWPELCPWCRSC-NH₂                                        |
| 6-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPELCPWC(Acm)RSC-NH₂                               |
| 7      | Ac-CGSGSGSGSGCPCIWPELCPWICSC-NH₂                                        |
| 7-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPELCPWIC(Acm)SC-NH₂                               |
| 8      | Ac-CGSGSGSGSGCPCIWPELCPWIRCC-NH₂                                       |
| 8-Acm  | Ac-(Acm)GSGSGSGSGCPCIWPELCPWIRC(Acm)C-NH₂                                |
| 9      | Ac-CGSGSGCPCIWPELCPWIRCC-NH₂                                            |
| 9-Acm  | Ac-(Acm)GSGGCPCIWPELCPWIRC(Acm)C-NH₂                                     |
| 10     | Ac-CGSGGCPCIWPELCPWIRCC-NH₂                                             |
| 10-Acm | Ac-(Acm)GSGCPCLIWPELCPWIRC(Acm)C-NH₂                                     |
| 11     | Ac-CGSGSGSGCGCPCLIWPELCPWIRSC-NH₂                                       |
|     | Sequence                                      |
|-----|----------------------------------------------|
| 11-Acm | Ac-C(Acm)GSGSGSGC(Acm)GCPCIWPELCPWIRSC-NH₂ |
| 12 | Ac-CGSGSGSCGKGCPICIWPELCPWIRSC-NH₂         |
| 13 | Ac-CGSGSGSGSCPCICWPELCPWIRSCGCGC-NH₂      |
| 13-Acm | Ac-C(Acm)GSGSGSGSCPCICWPELCPWIRSCGCGC(Acm)-NH₂ |
| 14 | Ac-GCPCICWPELCPWIRSCGKGCGSGSGSC-NH₂      |
| 15 | Ac-GCEKATESESPCPICIFPELCPWIVCC-NH₂       |
| 15-Ala | Ac-GAEKATESESPPAIFPELAPWIVAA-NH₂        |
| 16 | Ac-GCMLLDTDIWPCSHPYACPENICC-NH₂         |
| 16-Ala | Ac-GAMILLDTDIWAPASHPYAAPENIAA-NH₂      |
| FITC-βAla-16 | FITC-(β-Ala)-GCMILLDTDIWPCSHPYACPENICC-NH₂ |
| 17 | Ac-GCGSDEACFCPCSHPENCPMAQHC-NH₂         |
| 17-Acm | Ac-GC(Acm)GSDEAC(Acm)FCPCSHPENCPMAQHC-NH₂ |
Table S2. MS characterization of reduced peptides

| Name  | m/z expected          | m/z found     | Molecular formula          |
|-------|-----------------------|---------------|---------------------------|
| 1     | 1287.5029 ([M+2H]²⁺) | 1287.4804     | C₁₀₅H₁₅₇N₃₁O₃₃S₆       |
| 1-Acm | 1358.5400 ([M+2H]²⁺) | 1358.5229     | C₁₁₁H₁₆₇N₃₃O₃₅S₆       |
| 2     | 1251.0052 ([M+2H]²⁺) | 1250.9809     | C₁₀₀H₁₅₈N₃₀O₃₃S₆       |
| 2-Acm | 1322.0423 ([M+2H]²⁺) | 1322.0223     | C₁₀₆H₁₆₈N₃₂O₃₅S₆       |
| 3     | 1279.5236 ([M+2H]²⁺) | 1279.4994     | C₁₀₆H₁₆₁N₃₁O₃₃S₆       |
| 3-Acm | 1350.5607 ([M+2H]²⁺) | 1350.5439     | C₁₁₂H₁₇₁N₃₃O₃₃S₆       |
| 4     | 1287.5029 ([M+2H]²⁺) | 1287.4911     | C₁₀₅H₁₅₇N₃₁O₃₃S₆       |
| 4-Acm | 1358.5400 ([M+2H]²⁺) | 1358.5289     | C₁₁₁H₁₆₇N₃₃O₃₅S₆       |
| 5     | 1251.0052 ([M+2H]²⁺) | 1250.9823     | C₁₀₀H₁₅₈N₃₀O₃₃S₆       |
| 5-Acm | 1322.0423 ([M+2H]²⁺) | 1322.0474     | C₁₀₆H₁₆₈N₃₂O₃₅S₆       |
| 6     | 1287.5029 ([M+2H]²⁺) | 1287.4911     | C₁₀₅H₁₅₇N₃₁O₃₃S₆       |
| 6-Acm | 1358.5400 ([M+2H]²⁺) | 1358.5389     | C₁₁₁H₁₆₇N₃₃O₃₅S₆       |
| 7     | 1265.9944 ([M+2H]²⁺) | 1265.9326     | C₁₀₅H₁₅₆N₂₈O₃₃S₆       |
| 7-Acm | 1337.0315 ([M+2H]²⁺) | 1336.9771     | C₁₁₁H₁₆₆N₃₀O₃₃S₆       |
| 8     | 1300.5289 ([M+2H]²⁺) | 1300.6187     | C₁₀₈H₁₆₃N₃₁O₃₂S₆       |
| 8-Acm | 1371.5660 ([M+2H]²⁺) | 1371.5506     | C₁₁₄H₁₇₃N₃₃O₃₄S₆       |
| 9     | 1156.4754 ([M+2H]²⁺) | 1156.4789     | C₉₈H₁₄₇N₂₇O₂₆S₆       |
| 9-Acm | 1227.5125 ([M+2H]²⁺) | 1227.4617     | C₁₀₄H₁₅₇N₂₉O₂₈S₆       |
| 10    | 1084.4486 ([M+2H]²⁺) | 1084.4396     | C₉₃H₁₃₅N₂₅O₂₃S₆       |
| 10-Acm| 1155.4858 ([M+2H]²⁺) | 1155.4882     | C₉₉H₁₄₉N₂₇O₂₅S₆       |
| 11    | 1300.5289 ([M+2H]²⁺) | 1300.6557     | C₁₀₈H₁₆₃N₃₁O₃₂S₆       |
| 11-Acm| 1371.5660 ([M+2H]²⁺) | 1371.6973     | C₁₁₄H₁₇₃N₃₃O₃₄S₆       |
| 12    | 2728.1454 ([M+H]+)   | 2728.169      | C₁₁₄H₁₇₃N₃₃O₃₃S₆       |
| 13    | 1401.0664 ([M+2H]²⁺) | 1401.2576     | C₁₁₅H₁₇₄N₃₄O₃₆S₆       |
|     | Mass (Da)                  | Charge | Formula       |
|-----|----------------------------|--------|---------------|
| 13-Acm | 1472.6039 ([M+2H]²⁺)     | 1472.8245 | C₁₂₁H₁₸₄N₃₆O₃₈S₆ |
| 14  | 2785.1669 ([M+H]⁺)       | 2785.292  | C₁₁₆H₁₷₈N₃₄O₃₄S₆ |
| 15  | 1444.1128 ([M+2H]²⁺)     | 1444.0485 | C₁₂₄H₁₸₉N₃₉O₃₈S₆ |
| 15-Ala | 2716.3670 ([M+Na]⁺)    | 2716.5180  | C₁₂₄H₁₸₉N₃₉O₃₈ |
| 16  | 1472.1044 ([M+2H]²⁺)     | 1472.0147 | C₁₂₅H₁₸₉N₃₁O₃₇S₇ |
| 16-Ala | 2750.3683 ([M+H]⁺)    | 2750.139   | C₁₂₅H₁₸₉N₃₁O₃₇S |
| FITC-βAla-16 | 3363.2316 ([M+Na]⁺)    | 3362.659   | C₁₄₇H₂₀₁N₃₃O₄₁S₈ |
| 17  | 2567.8820 ([M+H]⁺)       | 2567.474   | C₁₀₀H₁₴₇N₃₁O₃₅S₇ |
| 17-Acm | 2708.9536 ([M+H]⁺)     | 2709.111   | C₁₀₆H₁₵₇N₃₃O₃₇S₇ |
### Table S3. MS characterization of oxidized peptides

| Name   | m/z expected          | m/z found  | Molecular formula                      |
|--------|-----------------------|------------|----------------------------------------|
| 1      | 1284.4794 ([M+2H]^{2+}) | 1284.3791  | C_{108}H_{151}N_{31}O_{33}S_{6}        |
| 1-Acm  | 1356.5243 ([M+2H]^{2+}) | 1356.4338  | C_{111}H_{163}N_{33}O_{35}S_{6}        |
| 2      | 1247.9817 ([M+2H]^{2+}) | 1247.8865  | C_{108}H_{152}N_{30}O_{33}S_{6}        |
| 2-Acm  | 1320.0267 ([M+2H]^{2+}) | 1319.9352  | C_{106}H_{164}N_{32}O_{35}S_{6}        |
| 3      | 1276.5001 ([M+2H]^{2+}) | 1276.4056  | C_{108}H_{155}N_{31}O_{31}S_{6}        |
| 3-Acm  | 1348.5451 ([M+2H]^{2+}) | 1348.4583  | C_{112}H_{167}N_{33}O_{35}S_{6}        |
| 4      | 1284.4794 ([M+2H]^{2+}) | 1284.3811  | C_{105}H_{151}N_{31}O_{33}S_{6}        |
| 4-Acm  | 1356.5243 ([M+2H]^{2+}) | 1356.4200  | C_{111}H_{163}N_{33}O_{35}S_{6}        |
| 5      | 1247.9817 ([M+2H]^{2+}) | 1247.8832  | C_{106}H_{164}N_{32}O_{35}S_{6}        |
| 5-Acm  | 1320.0267 ([M+2H]^{2+}) | 1320.0356  | C_{108}H_{155}N_{31}O_{31}S_{6}        |
| 6      | 1284.4794 ([M+2H]^{2+}) | 1284.3877  | C_{105}H_{151}N_{31}O_{33}S_{6}        |
| 6-Acm  | 1356.5243 ([M+2H]^{2+}) | 1356.5320  | C_{111}H_{163}N_{33}O_{35}S_{6}        |
| 7      | 1262.9709 ([M+2H]^{2+}) | 1262.9076  | C_{105}H_{150}N_{28}O_{33}S_{6}        |
| 7-Acm  | 1335.0158 ([M+2H]^{2+}) | 1334.9560  | C_{111}H_{162}N_{30}O_{35}S_{6}        |
| 8      | 1297.5054 ([M+2H]^{2+}) | 1297.7093  | C_{108}H_{157}N_{31}O_{32}S_{6}        |
| 8-Acm  | 1369.5503 ([M+2H]^{2+}) | 1369.5190  | C_{114}H_{169}N_{33}O_{34}S_{6}        |
| 9      | 1153.4519 ([M+2H]^{2+}) | 1153.4468  | C_{98}H_{141}N_{27}O_{26}S_{6}         |
| 9-Acm  | 1225.4969 ([M+2H]^{2+}) | 1225.4573  | C_{104}H_{153}N_{29}O_{28}S_{6}        |
| 10     | 1081.4252 ([M+2H]^{2+}) | 1081.4202  | C_{93}H_{133}N_{25}O_{23}S_{6}         |
| 10-Acm | 1153.4701 ([M+2H]^{2+}) | 1153.4712  | C_{99}H_{145}N_{27}O_{25}S_{6}         |
| 11     | 1297.5054 ([M+2H]^{2+}) | 1297.7083  | C_{108}H_{157}N_{31}O_{32}S_{6}        |
| 11-Acm | 1370.0507 ([M+2H]^{2+}) | 1370.2456  | C_{114}H_{169}N_{33}O_{34}S_{6}        |
| 12     | 2722.0985 ([M+H]^{+})   | 2722.203   | C_{114}H_{169}N_{33}O_{33}S_{6}        |
| 13     | 1398.0429 ([M+2H]^{2+}) | 1398.2554  | C_{115}H_{168}N_{34}O_{36}S_{6}        |
|     | Molecular Weight | Charge | Experimental Mass | Calculated Mass | Formula          |
|-----|------------------|--------|-------------------|-----------------|-----------------|
| 13-Acm | 1470.5883 ([M+2H]$^2^+$) | 1470.8027 | C$_{121}$H$_{180}$N$_{36}$O$_{38}$S$_6$ |
| 14   | 2779.1200 ([M+H]$^+$)       | 2779.889   | C$_{116}$H$_{172}$N$_{34}$O$_{34}$S$_6$ |
| 15   | 1440.5889 ([M+2H]$^2^+$) | 1441.0     | C$_{124}$H$_{183}$N$_{29}$O$_{36}$S$_6$ |
| 16   | 1468.5805 ([M+2H]$^2^+$) | 1468.5018  | C$_{125}$H$_{183}$N$_{31}$O$_{37}$S$_7$ |
| FITC-βAla-16 | 3357.1846 ([M+Na]$^+$)   | 3356.454   | C$_{147}$H$_{195}$N$_{33}$O$_{41}$S$_8$ |
| 17   | 2561.8351 ([M+H]$^+$)       | 2562.378   | C$_{100}$H$_{141}$N$_{31}$O$_{35}$S$_7$ |
| 17-Acm | 2704.9223 ([M+H]$^+$)       | 2705.077   | C$_{106}$H$_{153}$N$_{33}$O$_{37}$S$_7$ |
**Table S4.** MS characterization of Acm-deprotected peptides

| Name  | m/z expected          | m/z found     | Molecular formula               |
|-------|-----------------------|---------------|---------------------------------|
| 1-Acm | 1284.4794 ([M+2H]^{2+}) | 1284.3796     | C_{105}H_{151}N_{31}O_{33}S_{6} |
| 2-Acm | 1247.9817 ([M+2H]^{2+}) | 1247.8749     | C_{100}H_{152}N_{30}O_{33}S_{6} |
| 3-Acm | 1276.5001 ([M+2H]^{2+}) | 1276.4077     | C_{106}H_{153}N_{31}O_{33}S_{6} |
| 4-Acm | 1284.4794 ([M+2H]^{2+}) | 1284.3839     | C_{105}H_{151}N_{31}O_{33}S_{6} |
| 5-Acm | 1247.9817 ([M+2H]^{2+}) | 1247.9731     | C_{100}H_{152}N_{30}O_{33}S_{6} |
| 6-Acm | 1284.4794 ([M+2H]^{2+}) | 1284.4726     | C_{105}H_{151}N_{31}O_{33}S_{6} |
| 7-Acm | 1262.9709 ([M+2H]^{2+}) | 1262.9082     | C_{105}H_{150}N_{28}O_{32}S_{6} |
| 8-Acm | 1297.5054 ([M+2H]^{2+}) | 1297.5357     | C_{108}H_{157}N_{31}O_{32}S_{6} |
| 9-Acm | 1153.4519 ([M+2H]^{2+}) | 1153.4463     | C_{98}H_{141}N_{27}O_{26}S_{6} |
| 10-Acm | 1081.4252 ([M+2H]^{2+}) | 1081.3791     | C_{93}H_{133}N_{25}O_{23}S_{6} |
| 11-Acm | 1297.5054 ([M+2H]^{2+}) | 1297.7097     | C_{108}H_{157}N_{31}O_{32}S_{6} |
| 13-Acm | 1398.0429 ([M+2H]^{2+}) | 1398.2589     | C_{115}H_{168}N_{34}O_{36}S_{6} |
| 17-Acm | 2561.8351 ([M+H]^{+}) | 2562.348      | C_{100}H_{141}N_{31}O_{33}S_{7} |
2.2 Chromatograms of peptides

Figure S1. Chromatograms of peptides 1–8.
Figure S2. Chromatograms of the products formed after the oxidation of 1–8.
**Figure S3.** Analysis of disulfide pairing in peptide 8 through orthogonal protecting group strategies. 

a) Chromatogram of the products formed after the oxidation of 8. 
b) Chromatograms of the products formed after the oxidation of 8-Acm. 
c) Chromatogram of the products formed from the oxidative folding of 8 (black line) and chromatogram of the products formed from the Acm-deprotection of the oxidized 8-Acm (red line).
**Figure S4.** Chromatograms of products formed after the oxidation of 1-Acm–8-Acm (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized 1-Acm–8-Acm (red line).
**Figure S5.** Chromatograms of products formed after the oxidation of 1–8 (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized 1-Acm–8-Acm (red line).
Figure S6. Chromatograms of the products formed after the oxidation of 9 and 10 (black line) and chromatograms of the products formed after the Acm-deprotection of the oxidized 9-Acm and oxidized 10-Acm (red line).

Figure S7. Chromatograms of peptides 11–14.
**Figure S8.** a) Chromatograms of the products formed after the oxidation of 11 and 13 (black line) and chromatograms of the products formed after the Acm-deprotection of oxidized 11-Acm and oxidized 13-Acm (red line). b) Chromatograms of 12 and 14 (black line) and chromatograms of the products formed after the oxidation of 12 and 14 (red line).
2.3 Characterization of disulfide pairing of 12 and 14 using trypsin digestion

**Figure S9.** Tryptic digestion analysis of the oxidized 12. ↓ denotes the cleavage site of trypsin.

**Figure S10.** Tryptic digestion analysis of the oxidized 14. ↓ denotes the cleavage site of trypsin.
2.4 Construction of phage-displayed peptide libraries

We selected 8 and 11 as model scaffolds to construct phage-displayed peptide libraries.

**Extension primer** 5’CACC GGCGCACCTTGC CGCCGC3’

**Library oligonucleotide**

5’TGGCggeccagegce ATGCAGGTTCGmkkk mkkk mkkk mkkk mkkk mkkk TGCCCCCTGCmkkk CCCa mkkk TGCCCCmkkk mkkk TGCTGCggeccage AAGGTGC CGCCG GTG3’

---

**Figure S11.** Design of phage-displayed disulfide-rich peptide library (taking 8-X₁₆ as an example).
Figure S12. A fluorescence polarization competition assay showing the binding of oxidized 8 to MDM2.

Table S5. Four phage-displayed DRP libraries

| Phage library | The sequence of phage library | Phage library capacity |
|---------------|------------------------------|------------------------|
| 8-X5          | GCX₅CPCIWPELCPWIRCC          | 4.75 × 10⁸              |
| 8-X9          | GCX₉CPCIWPELCPWIRCC          | 1.4 × 10⁹              |
| 8-X₁₆         | GCX₉CPCX₂P X₂CPX₃CC          | 1.10 × 10⁹             |
| 11-X₁₄        | GCX₅CXCPX₂PX₂CPX₄C           | 6.25 × 10⁸             |
2.5 Sequences obtained from screening of peptide libraries and NMR characterization of the oxidized peptides

| Clones | Sequence       | Clone | Sequence       |
|--------|----------------|-------|----------------|
| M-1    | GC ERTDS CPCIWPELCPWIRCC | M-1   | GC NLKLPEDSE CPCIWPELCPWIRCC |
| M-2    | GC SDGES CPCIWPELCPWIRCC | M-2   | GC SYYMICHE CPCIWPELCPWIRCC |
| M-3    | GC EHDDS CPCIWPELCPWIRCC | M-3   | GC RYDREHSE CPCIWPELCPWIRCC |
| M-4    | GC NADES CPCIWPELCPWIRCC | M-4   | GC DNNLDESSLE CPCIWPELCPWIRCC |
| M-5    | GC NDGCS CPCIWPELCPWIRCC | M-5   | GC SESVAFSYH CPCIWPELCPWIRCC |
| M-6    | GC NESES CPCIWPELCPWIRCC | M-6   | GC DDLYFECI CPCIWPELCPWIRCC |
| M-7    | GC NTIEP CPCIWPELCPWIRCC | M-7   | GC ELSSNRFCD CPCIWPELCPWIRCC |
| M-8    | GC NADNL CPCIWPELCPWIRCC | M-8   | GC HMEGALTCE CPCIWPELCPWIRCC |
| M-9    | GC NDDE CPCIWPELCPWIRCC | M-9   | GC GNSEDDSQR CPCIWPELCPWIRCC |
| M-10   | GC QSEEL CPCIWPELCPWIRCC | M-10  | GC SEFNNSSRS CPCIWPELCPWIRCC |
| M-11   | GC QEDDG CPCIWPELCPWIRCC | M-11  | GC RKSPEWCEA CPCIWPELCPWIRCC |
| M-12   | GC QEDEV CPCIWPELCPWIRCC | M-12  | GC PSFDEENE S CPCIWPELCPWIRCC |
| M-13   | GC QERPA CPCIWPELCPWIRCC | M-13  | GC EHVFTLDCE CPCIWPELCPWIRCC |
| M-14   | GC QAPGE CPCIWPELCPWIRCC | M-14  | GC NQETSEING CPCIWPELCPWIRCC |
| M-15   | GC TGSDE CPCIWPELCPWIRCC | M-15  | GC VEEVTRSD CPCIWPELCPWIRCC |
| M-16   | GC TYGSE CPCIWPELCPWIRCC | M-16  | GC ELNTDHNHC CPCIWPELCPWIRCC |
| M-17   | GC PSYVE CPCIWPELCPWIRCC | M-17  | GC EASPDREDI CPCIWPELCPWIRCC |
| M-18   | GC LRREE CPCIWPELCPWIRCC | M-18  | GC KYATIRECE CPCIWPELCPWIRCC |
| M-19   | GC WDERE CPCIWPELCPWIRCC | M-19  | GC NEPHIANNCA CPCIWPELCPWIRCC |
| M-20   | GC DGSKE CPCIWPELCPWIRCC | M-20  | GC APTSLLSE CPCIWPELCPWIRCC |
| M-21   | GC CQQAE CPCIWPELCPWIRCC | M-21  | GC GQSHNSCE CPCIWPELCPWIRCC |
| M-22   | GC GAYTE CPCIWPELCPWIRCC | M-22  | GC LEDVNYRTN CPCIWPELCPWIRCC |
| M-23   | GC GEEFT CPCIWPELCPWIRCC | M-23  | GC DLEIMICQY CPCIWPELCPWIRCC |
| M-24   | GC SEEDP CPCIWPELCPWIRCC | M-24  | GC SVTNHEEED CPCIWPELCPWIRCC |
| M-25   | GC MEPSE CPCIWPELCPWIRCC |       |                |
| M-26   | GC ETPDE CPCIWPELCPWIRCC |       |                |
| M-27   | GC YKDEA CPCIWPELCPWIRCC |       |                |

**Figure S13.** a) Sequences obtained from screening of 8-X$_5$ against MDM2 after three rounds of panning.  
b) Sequences obtained from screening of 8-X$_9$ against MDM2 after three rounds of panning.
**Figure S14.** Sequences obtained from screening of 8-X\textsubscript{16} against MDM2 after three rounds of panning.
| Residue | N       | H N | Cα  | Hα   | Cβ    | Hβ    | Other                        |
|---------|---------|-----|-----|------|-------|-------|------------------------------|
| G1      | 112.7   | 7.927 | 42.85 | 3.838 |       |       |                              |
| C2      | 8.133   | 4.584 | 38.33 | 3.178 | 2.292 |       |                              |
| E3      | 120.6   | 8.082 | 25.96 | 2.029 | 1.906 | 30.1  | (C γ), 2.375 (H γ 2)         |
| K4      | 120.6   | 7.929 | 4.21  | 22.04 | 1.325 | 26.39 | (C δ), 1.589 (H δ 2), 24.62 (C γ), 2.892 (H ε 2), 1.548 (H γ 2), 0.914 (H γ 3), 7.344 (H ζ 1) |
| A5      | 123.7   | 8.074 | 4.255 | 16.69 | 1.352 |       |                              |
| T6      | 109.6   | 4.279 | 57.28 | 3.555 |       |       | 18.96 (C γ 2), 1.11 (H ζ 21), 7.641 (H ν) |
| E7      | 119.2   | 8.07  | 4.183 | 25.59 | 2.085 | 1.95  | 2.389 (H γ 2)                |
| S8      | 112.6   | 7.787 | 55.76 | 4.316 | 61.3  | 3.79  | 3.724                        |
| E9      | 119.8   | 7.708 | 52.94 | 4.299 | 26.43 | 2.027 | 1.88  | 2.357 (H γ 2)                |
| S10     | 115.8   | 7.971 | 52.85 | 4.675 | 61.34 | 3.703 |                              |
| P11     | 4.306   | 29.29 | 2.168 | 1.867 |       |       | 47.85 (C δ), 3.689 (H δ 2), 3.621 (H δ 3) |
| C12     | 7.99    | 49.79 | 4.763 | 39.42 | 3.027 | 2.901 |                              |
| P13     | 4.675   |       |       |       |       |       | 48.15 (C δ), 24.13 (C γ), 3.877 (H δ 2), 3.71 (H δ 3), 2.012 (H γ 2), 1.805 (H γ 3) |
| C14     | 121     | 7.08  | 52.59 | 4.598 | 41.14 | 2.979 |                              |
| I15     | 122.8   | 7.921 | 60.92 | 3.57  | 35.24 | 1.431 | 10.47 (C δ 1), 25.17 (C γ 1), 14.27 (C γ 2), 0.7076 (H δ 11), 1.191 (H γ 11), 0.9895 (H γ 12), 0.3744 (H γ 21) |
| F16     | 7.101   | 51.09 | 5.082 | 36.02 | 2.963 |       | 7.525 (H δ 2), 7.452 (H ε 2), 7.123 (H ζ) |
| P17     | 63.34   | 3.914 | 30.06 | 2.51  | 1.931 |       | 48.2 (C δ), 3.815 (H δ 2), 3.792 (H δ 3) |
| E18     | 113.2   | 8.126 | 54.98 | 3.972 | 24.71 | 1.998 | 1.934 | 30.47 (C γ), 2.426 (H γ 2) |
| L19     | 115.6   | 7.83  | 51.67 | 4.32  | 38.67 | 2.002 | 1.767 | 22.85 (C δ 1), 19.62 (C δ 2), 24.64 (C γ), 0.9517 (H δ 11), 0.8025 (H δ 21), 1.597 (H γ) |
| C20     | 115.4   | 7.445 | 49.32 | 4.447 | 40.97 | 3.01  |                              |
| P21     | 61.76   | 4.119 | 28.85 | 2.015 | 1.519 |       | 47.58 (C γ), 24.3 (C γ), 3.265 (H δ 2), 1.763 (H γ 2), 1.407 (H γ 3) |
| W22     | 115     | 6.761 | 54.31 | 4.591 | 25.59 | 3.325 | 3.173 | 7.054 (H δ 1), 10.04 (H ε 1), 7.255 (H ε 3), 7.191 (H ζ 2), 7.191 (H ζ 3), 7.245 (H ζ 3), 129.5 (N ε 1) |
| I23     | 127     | 8.281 | 62.74 | 3.766 | 18.85 | 1.107 |                              |
| V24     | 117.2   | 7.076 | 4.19  | 35.24 | 1.737 |       | 15.33 (C γ 1), 17.16 (C γ 2), 0.9086 (H δ 11), 1.116 (H γ 21) |
| C25     | 8.047   | 52.8  | 4.537 | 40.23 | 3.364 |       |                              |
| C26     | 7.912   | 53.38 | 4.566 | 36.22 | 3.179 | 2.919 |                              |

Table S6. Chemical shifts of oxidized 15 (BMRB 50900)
| Residue | N  | Hα  | Cα  | Hα  | Cβ  | Hβ  | Other                  |
|---------|----|-----|-----|-----|-----|-----|------------------------|
| G1      | 7.833 | 42.85 | 3.791 |     |     |     |                        |
| C2      | 116.7 | 8.007 | 52.87 | 4.478 | 38.03 | 3.051, 2.884 | 29.59 (C γ ), 2.526 (H γ 2), 2.435 (H γ 3) |
| M3      | 120.3 | 8.142 | 53.77 | 4.324 | 30.39 | 1.993, 1.956 | 10.46 (C δ ), 0.793 (H δ ), 24.9 (C γ 1), 1.393 (H γ 12), 1.094 (H γ 13), 14.84 (C γ 2), 0.807 (H γ 22) |
| I4      | 118.2 | 7.549 | 58.67 | 4.082 | 36.2  | 1.792 | 22.43 (C δ 1), 0.8367 (H δ 12), 20.86 (C δ 2), 0.7793 (H δ 22), 24.42 (C γ ) |
| L5      | 122.6 | 7.724 | 52.23 | 4.335 | 39.74 | 1.568, 1.484 | 22.49 (C δ 1), 20.77 (C δ 2), 0.8307 (H δ 12), 0.793 (H δ 22) |
| L6      | 120.1 | 7.853 | 4.205 | 39.61 | 1.632, 1.516 | 18.91 (C γ 2), 1.113 (H γ 22) |
| D7      | 8.005 | 50.83 | 4.581 | 35.15 | 2.894, 2.798 |     |
| T8      | 111.3 | 7.665 | 4.226 | 66.96 | 4.18  |     | 10.57 (C δ 1), 0.6572 (H δ 12), 24.28 (C γ 1), 1.101 (H γ 12), 0.9157 (H γ 13), 14.63 (C γ 2), 0.5924 (H γ 22) |
| D9      | 118.9 | 8.002 | 50.66 | 4.623 | 35.48 | 2.811, 2.763 |     |
| H10     | 117.6 | 7.516 | 3.989 | 36.14 | 1.624 |     | 7.121 (H δ 1), 9.891 (H ε 1), 7.535 (H ε 3), 7.097 (H η 2), 7.356 (H ζ 2), 7.02 (H ζ 3) |
| W11     | 121.1 | 7.655 | 54.06 | 4.62  | 27.27, 3.253, 3.018 |     |
| C12     | 112.3 | 7.843 | 50.23 | 4.79  | 39.22 | 3.02, 2.952 |     |
| P13     | 61.04 | 4.26  | 29.06 | 1.996, 1.91 | 24.2 (C γ ), 1.793 (H γ 3), 3.581 (H δ 2), 3.356 (H δ 3) |
| C14     | 7.374 | 52.04 | 4.662 | 37.68 | 3.187, 2.877 |     |
| S15     | 117.7 | 8.208 | 57.16 | 4.094 | 60.64 | 3.688, 3.664 |     |
| H16     | 116.8 | 7.335 | 49.68 | 4.913 | 26.37, 2.842 |     |
| P17     | 62.2  | 4.118 | 29.21 | 2.125, 1.758 | 48.1 (C δ), 3.714 (H δ 2), 3.606 (H δ 3), 24.79 (C γ ), 1.956 (H γ 2), 1.911 (H γ 3) |
| Y18     | 118   | 8.07  | 4.122 | 33.85 | 3.102, 3.078 | 7.003 (H δ 2) |
| A19     | 121.3 | 7.696 | 49.95 | 4.28  | 17    | 1.313 |     |
| C20     | 112.3 | 7.857 | 50.25 | 4.934 | 39.84 | 3.213, 3.04 |     |
| P21     | 60.88 | 4.351 | 28.89 | 2.186 |     | 47.7 (C δ), 3.559 (H δ 2), 3.52 (H δ 3), 24.75 (C γ ), 1.926 (H γ 2) |
| E22     | 117   | 7.965 | 4.218 | 25.83 | 2.071, 1.962 | 29.94 (C γ ), 2.358 (H γ 2) |
| N23     | 7.925 | 50.7  | 4.598 | 36.42 | 2.769, 2.718 | 7.333 (H δ 21), 6.543 (H δ 22), 110.9 (N δ 2) |
| I24     | 121.7 | 7.928 | 59.62 | 4.151 | 36    | 1.841 | 10.87 (C δ), 0.8257 (H δ), 24.93 (C γ 1), 1.428 (H γ 12), 1.164 (H γ 13), 15.14 (C γ 2), 0.8938 (H γ 22) |
| C25     | 118.4 | 8.425 | 52.32 | 4.611 | 39.8  | 3.204, 2.857 |     |
| C26     | 118   | 8.039 | 53.06 | 4.465 | 39.72 | 3.216, 3.023 |     |

*S31*
Figure S15. NMR characterization of oxidized 15. a) and b) 2D 1H-1H NOESY (red) and TOCSY (blue) spectra of oxidized 15 in ACN/H_2O (50%/50%, v/v). Only local regions were plotted to show cross peaks of cysteine residues (disulfide connectivity: Cys 2-Cys 25, Cys 12-Cys 16, Cys 14-Cys 20) and cysteine-related assignments were labeled (data deposited in BMRB, accession number 50900).
Figure S16. Sequences obtained from screening of $8\cdot X_{16}$ against Bcl-2 after four rounds of panning.
2.6 Binding affinity of the oxidized peptides to target proteins

![Graph showing binding affinity](image)

**Figure S17.** Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of 16-Ala with Bcl-2.

![Graph showing chromatogram](image)

**Figure S18.** Chromatogram of products formed after the oxidation of 17 (black line) and the Acm-deprotection of the oxidized 17-Acm (red line).
**Figure S19.** Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized 17 with streptavidin.
Figure S20. Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized 11 with streptavidin.

Figure S21. a) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of oxidized 15 with Bcl-2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized 15 with streptavidin.
Figure S22. a) A fluorescence polarization competition assay showing the binding of oxidized 16 to MDM2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized 16 with streptavidin.

Figure S23. a) A fluorescence polarization competition assay showing the binding of oxidized 17 to MDM2. b) Sensorgrams recorded by surface plasmon resonance (SPR) showing the binding of the oxidized 17 with Bel-2.
2.7 Proteolytic stability of peptides

a) Chromatograms of 15–Ala digested by chymotrypsin.

b) Chromatograms of oxidized 15 digested by chymotrypsin.

c) Kinetics of peptide degradation by chymotrypsin in phosphate buffer at pH 7.4. Concentrations of peptides and chymotrypsin were 100 μM and 300 ng·mL⁻¹, respectively.

Figure S24. a) Chromatograms of 15–Ala digested by chymotrypsin. b) Chromatograms of oxidized 15 digested by chymotrypsin. c) Kinetics of peptide degradation by chymotrypsin in phosphate buffer at pH 7.4. Concentrations of peptides and chymotrypsin were 100 μM and 300 ng·mL⁻¹, respectively.
Figure S25. a) Chromatograms of 16–Ala digested by chymotrypsin. b) Chromatograms of oxidized 16 digested by chymotrypsin. c) Kinetics of peptide degradation by chymotrypsin in phosphate buffer at pH 7.4. Concentrations of peptides and chymotrypsin were 100 μM and 5 μg·mL⁻¹, respectively.
2.8 CD spectra of the oxidized 15 and oxidized 16

**Figure S26.** a) CD spectra of the oxidized 15 (50 μM) in water. b) CD spectra of the oxidized 16 (50 μM) in water.
2.9 Mass spectra of peptides

Reduced 1

Oxidized 1

Reduced 1-Acm
Reduced 3-Acm

Oxidized 3-Acm

Acm-deprotected 3-Acm
Oxidized 4-Acm

Acm-deprotected 4-Acm

Reduced 5
Oxidized 7-Acm

Acm-deprotected 7-Acm

Reduced 8

+MS, 0.2min #13

+MS, 0.3min #16

+MS, 0.4min #21

Oxidized - Acm

Acm-deprotected - Acm

Reduced - 8
Acm-deprotected 8-Acm

Reduced 9

Oxidized 9
Oxidized 10-Acm

Acm-deprotected 10-Acm

Reduced 11

+MS, 0.2min #12

+MS, 0.1min #8

+MS, 0.2min #14

Intens. x 10^6

m/z

400 600 800 1000 1200 1400 1600 1800

m/z

400 600 800 1000 1200 1400 1600 1800

m/z

400 600 800 1000 1200 1400 1600 1800
Acm-deprotected 11-Acm

Reduced 12

Oxidized 12
Fragment of oxidized 12 digested by trpsin

Reduced 13

Oxidized 13
Reduced 14

Oxidized 14

Fragment of oxidized 14 digested by trpsin
Reduced 15

Oxidized 15

15-Ala

S63
Reduced FITC-βAla-16

Oxidized FITC-βAla-16

Reduced 17
Figure S27. Mass spectra of peptides.
References

1. Lu, S. M. et al. Directed disulfide pairing and folding of peptides for the de novo development of multicyclic peptide libraries. *J. Am. Chem. Soc.* **142**, 16285-16291 (2020).

2. Zha, M. R., Lin, P., Yao, H. W., Zhao, Y. B. & Wu, C. L. A phage display-based strategy for the de novo creation of disulfide-constrained and isomer-free bicyclic peptide affinity reagents. *Chem. Commun.* **54**, 4029-4032 (2018).

3. Zheng, X. et al. Condensation of 2-((alkylthio)(aryl)methylene)malononitrile with 1,2-aminethiol as a novel bioorthogonal reaction for site-specific protein modification and peptide cyclization. *J. Am. Chem. Soc.* **142**, 5097-5103 (2020).

4. Rebollo, I. R. & Heinis, C. Phage selection of bicyclic peptides. *Methods* **60**, 46-54 (2013).

5. Zheng, X. J., Liu, W. D., Liu, Z. Y., Zhao, Y. B. & Wu, C. L. Biocompatible and rapid cyclization of peptides with 2,4-difluoro-6-hydroxy-1,3,5-benzenetricarbonitrile for the development of cyclic peptide libraries. *Bioconjugate Chem.* **31**, 2085-2091 (2020).

6. Chen, Y. Q. et al. The interplay of disulfide bonds, alpha-helicity, and hydrophobic interactions leads to ultrahigh proteolytic stability of peptides. *Biomacromolecules* **16**, 2347-2355 (2015).

7. Delaglio, F. et al. NMRPipe - a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277-293 (1995).

8. Lee, W., Tonelli, M. & Markley, J. L. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics* **31**, 1325-1327 (2015).

9. Shen, Y. & Bax, A. Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *J. Biomol. NMR* **56**, 227-241 (2013).

10. Rieping, W. et al. ARIA2: Automated NOE assignment and data integration in NMR structure calculation. *Bioinformatics* **23**, 381-382 (2007).

11. Brunger, A. T. et al. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54**, 905-921 (1998).

12. Laskowski, R. A., Rullmann, J. A. C., MacArthur, M. W., Kaptein, R. & Thornton, J. M. AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**, 477-486 (1996).