Supporting Information

Bridged β^3-Peptide Inhibitors of p53-hDM2 Complexation – Correlation Between Affinity and Cell Permeability

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I. General information. Fmoc-protected α-amino acids, Fmoc-OSu and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), *N*-methylmorpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). PyAOP was purchased from Oakwood Products, Inc. (West Columbia, SC). HOAt was purchased from ChemPep, Inc. (Miami, FL). *N,N*-diisopropylethylamine (DIEA), 3,6-dioxa-1,8-octanedithiol (DODT), Boc-Ser-OH, benzyl chloride, allyl bromide, 5-bromo-1-pentene, triisopropylsilane (TIPS) and first generation Grubbs Catalyst were purchased from Sigma-Aldrich. Sodium hydride was purchased from Alfa Aesar (Ward Hill, MA). (*S*)-proline was purchased from VWR (West Chester, PA). 2-aminobenzophenone was purchased from Acros Organics USA (Morris Plains, NJ). Nickel (II) nitrate hexahydrate was purchased from Strem Chemicals, Inc. (Newburyport, MA). Glycine (molecular biology grade) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). 5-(-and-

6)-carboxyfluorescein succinimidyl ester, Alexa Fluor 647 conjugate of transferrin from human serum, Hoescht 33342 trihydrochloride trihydrate, phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, CA). EDTA and EGTA were purchased from Mallinckrodt Baker (Phillipsburg, NJ). *N*-Fmoc-(*S*)-3-amino-4-(3-trifluoromethyl-phenyl)-butyric acid was purchased from Anaspec, Inc. (San Jose, CA). Mass spectra were acquired with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA). Reverse-phase HPLC was performed using a Varian HPLC and Vydac analytical (C8, 300 Å, 5 μm, 4.6 mm x 150 mm) or semi-preparative (C8, 300 Å, 5 μm, 10 mm x 250 mm) columns, using water/acetonitrile gradients with 0.1% TFA. β-peptides were synthesized using a MARS Microwave Assisted Reaction System (CEM Corporation, Matthews, NC). Fluorescence polarization experiments were performed with an Analyst AD (Molecular Devices, Sunnyvale, CA) spectrofluorimeter. MALDI-MS/MS analyses were performed at the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University).

II. Synthesis of *N*-Fmoc-O-allyl-L-β-homoserine

***N*-Boc-O-allyl-L-serine.** *N*-Boc-O-allyl-L-serine was synthesized as described by Boal *et al.*[1] ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.85 (1H, m), 5.40 (1H, d, *J* = 7.7 Hz), 5.31 (1H, dq, *J* = 17.3, 1.5 Hz), 5.20 (1H, d, *J* = 10.4 Hz), 4.46 (1H, d, *J* = 7.5 Hz), 4.02 (2H, dt, *J* = 5.6, 1.2 Hz), 3.91 (1H, dd, 9.4, 2.7 Hz), 3.68 (1H, dd, *J* = 9.5, 3.9 Hz), 1.46 (9H, s). Calculated Mass $[M+H]^+$ 246.1342 observed 268.20 (+Na).

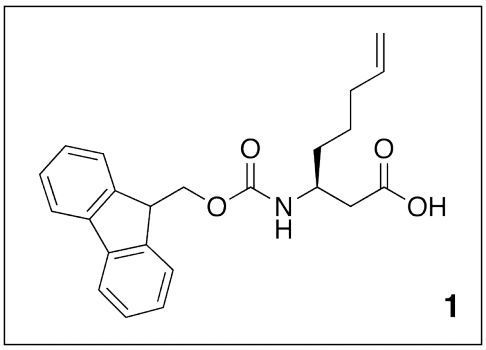
***N*-Fmoc-O-allyl-L-serine.** *N*-Boc-O-allyl-L-serine (0.25 g, 1.02 mmol) was dissolved in 2.04 mL CH_2Cl_2 (total concentration: 500 mM) and cooled to 0°C. To this solution was added 2.04

mL trifluoroacetic acid and the reaction was stirred for 30 min. Solvents were then removed by rotary evaporation until fewer than 2 equiv. TFA remained by weight. The residue was reconstituted in 3.4 mL of 1:1 water/acetone to create a 300 mM solution. Sodium carbonate (0.32 g, 3.06 mmol) and Fmoc-OSu (0.36 g, 1.07 mmol) were added and the reaction was stirred at RT for 17 h. The mixture was then acidified to pH 3 with 3 M HCl and extracted 3x with ethyl acetate. The combined organic layers were dried over sodium sulfate and concentrated to yield a light yellow solid, which was purified via flash chromatography[2] using MeOH/CH_2Cl_2/AcOH (3:96:1). The purified compound was brought up in water/acetonitrile (1:1) and dried under vacuum to yield 0.19 g (51%) of a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (2H, d, *J* = 7.5 Hz), 7.61 (2H, m), 7.40 (2H, t, *J* = 7.4 Hz), 7.32 (2H, t, *J* = 7.5 Hz), 5.87 (1H, m), 5.67 (1H, d, *J* = 8.1 Hz), 5.29 (1H, d, *J* = 17.4 Hz), 5.22 (1H, d, *J* = 10.3 Hz), 4.55 (1H d, *J* = 7.9 Hz), 4.41 (2H, m), 4.24 (1H, t, *J* = 7.0 Hz), 4.04 (2H, d, 5.4 Hz), 3.96 (1H, dd, *J* = 9.2, 2.9 Hz), 3.71 (1H, dd, *J* = 9.1, 3.6 Hz). ^{13}C NMR (125 MHz, MeOD): δ 174.00, 158.97, 145.68, 142.99, 136.16, 129.20, 128.60, 126.72, 121.33, 117.92, 73.57, 71.15, 68.58, 56.22, 48.78. Calculated mass $[M+H]^+$ 368.1499 observed 390.70 (+Na).

***N*-Fmoc-O-allyl-L-β-homoserine.** The Fmoc-protected β-amino acid was synthesized from *N*-Fmoc-O-allyl-L-serine (0.50 g, 1.36 mmol) using the Arndt-Eistert procedure.[3] This procedure resulted in 0.24 g (47%) of *N*-Fmoc-O-allyl-L-β-homoserine. ^{1}H NMR (400 MHz, CD_3OD) δ 7.82 (2H, d, *J* = 7.5 Hz), 7.68 (2H, d, *J* = 7.2 Hz), 7.41 (2H, t, *J* = 7.4 Hz), 7.33 (2H, t, *J* = 7.5 Hz), 5.92 (1H, m), 5.30 (1H, d, *J* =17.5 Hz), 5.17 (1H, dq, *J* = 10.5, 1.2 Hz), 4.35 (2H, d, *J* = 7.0 Hz), 4.24 (1H, t, *J* = 6.6 Hz), 4.17 (1H, t, *J* = 6.0 Hz), 4.02 (2H, d, *J* = 5.2 Hz), 3.49 (2H, m), 2.57 (2H, m). ^{13}C NMR (125 MHz, $CDCl_3$): 176.23, 155.91, 143.85, 141.33, 134.19, 127.71,

127.07, 125.05, 119.99, 117.41, 72.18, 70.66, 66.89, 47.69, 47.23, 35.90. Calculated mass $[M+H]^+$ 382.1655 observed 405.30 (+Na).

III. Synthesis of *N*-Fmoc-(*S*)-3-aminooct-7-enoic acid. β^3-amino acid **1** was prepared according to procedures for analogous compounds, as described by Belokon[4,5], Ueki[6] and Qiu[7].



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Gly-Ni-BPB. The Nickel (II)-complex of Schiff base of glycine and (*S*)-2-[*N*-(*N*'-benzylprolyl)amino]-benzophenone (hereafter referred to as Gly-Ni-BPB) was synthesized according to the aforementioned procedures,[4-6] with no modifications. Overall yield (over 3 steps): 39%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (1H, dd, *J* = 8.7, 0.9 Hz), 8.07 (2H, d, *J* = 7.1 Hz), 7.48-7.56 (3H, m), 7.43 (2H, t, *J* = 7.4 Hz), 7.31 (1H, tt, *J* = 7.4, 1.1 Hz), 7.21 (1H, m), 7.10 (1H, d, *J* = 7.3 Hz), 6.98 (1H, m), 6.80 (1H, dd, *J* = 8.2, 1.6 Hz), 6.71 (1H, m), 4.49 (2H, d, *J* = 12.7 Hz), 3.68 (2H, d, *J* = 12.7 Hz), 3.66-3.81 (3H, m), 3.48 (1H, dd, *J* = 10.8, 5.4 Hz), 3.36 (1H, m), 2.58 (1H, m), 2.43 (1H, m), 2.03-2.20 (2H, m). $[\alpha]_D^{25}$ = +2014 (c = 0.1, MeOH). Calculated mass $[M+H]^+$ 498.1328 observed 498.07.

Alkylation of Gly-Ni-BPB with 5-bromo-1-pentene. (*Note:* This step is analogous to that carried out by Qiu *et al.* in the synthesis of Nickel (II)-complex of Schiff base of (*S*)-*trans*-2-cinnamylglycine and (*S*)-2-[*N*-(N'-benzylprolyl)amino]-benzophenone).[7] Gly-Ni-BPB (4.00 g, 8.05 mmol) was dissolved in 36 mL dry DMF and cooled to 4°C in an ice-water bath. Potassium hydroxide powder (4.51 g, 80.46 mmol) was added with stirring and allowed to dissolve completely. 5-bromo-1-pentene (0.95 mL, 1.19 g, 8.05 mmol, dissolved in 3.2 mL DMF) was added dropwise by syringe and the reaction continued until the starting material was no longer

detectable by TLC (circa 10 min). The reaction was worked up by pouring into 160 mL of an icy 5% acetic acid aqueous solution. The mixture was then stirred with a glass rod and allowed to stand 4 h at 4°C. The crude product was filtered and washed several times with water, then dried and purified via flash chromatography[7] using acetone/ethyl acetate (15:85) to yield 3.42 g (75%) of a deep red-orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (1H, dd, *J* = 8.6, 0.7 Hz), 8.05 (2H, d, *J* = 8.0 Hz), 7.42-7.53 (3H, m), 7.35 (2H, t, *J* = 7.6 Hz), 7.19 (1H, tt, *J* = 7.5, 1.0 Hz), 7.14 (1H, m), 5.73 (1H, m), 4.93-5.01 (2H, m), 4.44 (1H, d, *J* = 12.7 Hz), 3.91 (1H, dd, *J* = 8.1, 3.4 Hz), 3.45-3.61 (4H, m), 2.77 (1H, m), 2.53 (1H, m), 1.85-2.29 (6H, m), 1.58-1.73 (2H, m). ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.42, 179.40, 170.40, 142.27, 137.76, 133.83, 133.24, 132.14, 131.59, 129.72, 128.89 127.63, 127.20, 126.53, 123.71, 120.74, 115.26, 70.41, 70.30, 63.12, 56.99, 34.84, 33.31, 30.76, 24.64, 23.63. $[\alpha]_D^{25}$ = +2172 (c = 0.1, MeOH). Calculated mass $[M+H]^+$ 566.1954 observed 566.13.

(*S*)-2-aminohept-6-enoic acid. Alkylated Gly-Ni-BPB from the previous step (100 mg, 0.18 mmol) was dissolved in 265 μL methanol and added dropwise to a stirred solution of 1.25 mL 3N HCl/methanol (1:1) at 70°C. The reaction was stirred until the red color of the nickel complex faded to a transparent yellow-green and the starting material was no longer detectable by TLC. After 10 min, the reaction was removed from heat and solvents evaporated by rotary evaporation. The residue was dissolved in 800 μL concentrated $NH_3 \cdot H_2O$ and extracted 3x with chloroform. The combined organic fractions were dried over sodium sulfate and evaporated to recover (*S*)-2-[*N*-(N'-benzylprolyl)amino]-benzophenone. The aqueous layer was subsequently evaporated, brought up in water/ethanol (1:1) and loaded onto Dowex 50X2 100 cation exchange resin for removal of residual nickel. The product was eluted from the column using 10% $NH_3 \cdot H_2O$/ethanol (1:1) and evaporated to yield 13.8 mg (54.5%) of a white solid. ^{1}H NMR (400

MHz, CD_3OD): δ 5.84 (1H, m), 5.08 (1H, d, *J* = 17.3 Hz), 5.02 (1H, d, *J* = 10.2 Hz), 3.71 (1H, t, *J* = 6.1 Hz), 2.12 (2H, m), 1.86 (2H, m), 1.49 (2H, m). ^{13}C NMR (125 MHz, CD_3OD): δ 174.56, 138.28, 114.88, 32.40, 29.79, 23.48 (*Note:* The presence of an additional peak obscured by the methanol complex at δ ~ 47 was confirmed by heteronuclear multiple quantum coherence (HMQC); Figure S4). Calculated mass $[M+H]^+$ 144.10 observed 146.03.

***N*-Fmoc-(*S*)-2-aminohept-6-enoic acid.** (*S*)-2-aminohept-6-enoic acid (0.040 g, 0.280 mmol) and sodium carbonate (0.059 g, 0.559 mmol) were dissolved in 500 μL water and cooled to 4ºC with stirring. A solution of Fmoc-OSu (0.141 g, 0.419 mmol) in *para*-dioxane was also cooled to 4ºC and added dropwise to the stirring amino acid solution. The reaction was continued at 0ºC for 1 h and then allowed to come to room temperature overnight with stirring. An excess volume of water was added and the mixture extracted 2x with ethyl acetate. The combined organic fractions were back-extracted 2x with saturated sodium bicarbonate solution and the aqueous layers acidified to pH 1 with 3N hydrochloric acid. The aqueous fractions were then extracted 3x with ethyl acetate, and the organic fractions combined, dried over sodium sulfate and removed by rotary evaporation. The crude product was purified via flash chromatography using MeOH/CH_2Cl_2/AcOH (3:96:1) to yield 0.065 g (63%) of a white solid. ^1H NMR (400 MHz, CD_3OD): δ 7.81 (2H, d, *J* = 7.5 Hz), 7.70 (2H, t, *J* = 7.0 Hz), 7.41 (2H, t, *J* = 7.5 Hz), 7.33 (2H, t, *J* = 7.5 Hz), 5.84 (1H, m), 5.05 (1H, d, *J* = 17.1 Hz), 4.99 (1H, d, *J* = 10.2 Hz), 4.37 (2H, d, *J* = 6.9 Hz), 4.25 (1H, t, *J* = 7.0 Hz), 4.17 (1H, m), 2.11 (2H, m), 1.88 (1H, m), 1.71 (1H, m), 1.52 (2H, m). ^{13}C NMR (125 MHz, CD_3OD): δ 176.03, 158.70, 145.25, 142.56, 139.36, 128.75, 128.13, 126.24, 120.88, 115.37, 67.91, 55.17, 48.39, 34.23, 32.11, 26.25. Calculated mass $[M+H]^+$ 366.1706 observed 366.11.

***N*-Fmoc-(*S*)-3-aminooct-7-enoic acid.** Fmoc-protected β-amino acid **1** was synthesized from *N*-Fmoc-(*S*)-2-aminohept-6-enoic acid (400 mg, 1.10 mmol) using the Arndt-Eistert procedure.[3] This procedure resulted in 203 mg (36%) of *N*-Fmoc-(*S*)-3-aminooct-7-enoic acid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.81 (2H, d, *J* = 7.5 Hz), 7.67 (2H, dd, *J* = 7.4, 2.9 Hz), 7.40 (2H, t, *J* = 7.4 Hz), 7.32 (2H, t, *J* = 7.4 Hz), 7.07 (1H, d, *J* = 8.9 Hz), 5.82 (1H, m), 5.02 (1H, d, *J* = 17.1 Hz), 4.96 (1H, d, *J* = 10.2 Hz), 4.36 (2H, d, *J* = 6.9 Hz), 4.22 (1H, t, *J* = 6.9 Hz), 3.97 (1H, m), 2.47 (2H, m), 2.09 (2H, m), 1.36-1.63 (4H, m). ^{13}C NMR (125 MHz, CD_3OD): δ 175.04, 158.30, 145.32, 142.58, 139.66, 128.73, 128.11, 126.20, 120.87, 115.13, 67.51, 49.46, 48.50, 40.90, 35.14, 34.47, 26.40. Calculated mass $[M+H]^+$ 380.1863 observed 380.11.

IV. Preparation of β^3-peptides. Fmoc-protected β^3-amino acids (with the exception of *N*-Fmoc-O-allyl-L-β-homoserine and *N*-Fmoc-(*S*)-3-aminooct-7-enoic acid, which were synthesized as above, and *N*-Fmoc-(*S*)-3-amino-4-(3-trifluoromethylphenyl)-butyric acid, which was purchased from Anaspec, Inc.) were prepared according to methods described by Seebach.[3] β-peptides were synthesized on a 25 μmol scale in a CEM MARS microwave reactor, using standard Fmoc chemistry and Wang resin loaded with β^3-homoglutamic acid as previously described.[8] Microwave irradiation was conducted at a maximum power of 400 W and monitored via fiber optic temperature sensor. Reactions were agitated by magnetic stirring during irradiation. One cycle of peptide elongation consisted of the following steps: First, the loaded resin was washed manually with dimethylformamide (DMF) and the terminal Fmoc group removed with 20% piperidine in DMF (50% power at 400 W maximum, 70 °C, ramp 2 min, hold 4 min). The deprotected resin was washed with DMF and treated with a cocktail containing 3 equiv. of the appropriate β^3-amino acid, 3 equiv. PyAOP, 3 equiv. HOAt, and 8 equiv. DIEA (50% power at

400 W maximum, 60 °C, ramp 2 min, hold 6 min). The coupled resin was then washed extensively with DMF. Following removal of the final Fmoc protecting group, the resin was washed alternately with DMF and methylene chloride for a total of 16 washes and dried 20 min under N_2.

Labeling of β^3-Peptides with 5-(and-6-)-carboxyfluorescein, succinimidyl ester. Unmetathesized and resin-bound β^3-peptides were extended by two β-alanine (β-homoglycine) residues to provide a suitable linker for the fluorophore. Following removal of the final Fmoc protecting group, the resin was washed alternately with DMF and methylene chloride for a total of 16 washes and dried 20 min under N_2. Applicable peptides were then metathesized according to the protocol described below. All peptides were treated o/n with a cocktail comprised of 5-(and-6-)-carboxyfluorescein, succinimidyl ester (8 mg, 0,017 mmol) and DIEA (24 μL, 17.8 mg, 0.138 mmol) in 1.5 mL DMF.

On-resin ring-closing metathesis protocol.[2] Bis(tricyclohexylphosphine)benzylidene ruthenium (IV) dichloride (First Generation Grubbs Catalyst; 20.5 mg, 0.025 mmol) was dissolved in 2.5 mL 1,2-dichloroethane to create a 10 mM solution. This was stirred under N_2 gas for 30 min before addition to a fritted peptide vessel containing 12.5 μmol *N*-terminally deprotected resin-bound β-peptide. This reaction was agitated for 2 h at RT, then the solvent drained and replaced with 2.5 mL fresh catalyst. Peptides were then cleaved and purified as described below.

Cleavage of peptides from resin. Completed peptides were treated with a cleavage cocktail of 2.5% v/v DODT, 2.5% v/v H_2O, 2.5% v/v TIPS in TFA (50% power at 400 W maximum, 38 °C, ramp 2 min, hold 30 min; followed by treatment with fresh cocktail at 50% power, 400 W

maximum, 38 °C, ramp 2 min, hold 5 min). Crude peptides were concentrated by rotary evaporation and reconstituted in acetonitrile/water (1:1) for purification by RP-HPLC.

β-peptide purification and analysis. The efficiency of each synthesis was assessed by MALDI-TOF analysis of the crude reaction mixture. β-peptides were then purified to homogeneity by reverse-phase HPLC. The presence of the RCM-generated staple was confirmed by a lack of fragmentation within the stapled region, as judged by MALDI-TOF MS/MS. All other purified peptides were evaluated by analytical HPLC and mass spectrometry (Section IX). Following purification, β-peptides were lyophilized and stored at -20 °C.

V. Computational modeling of β-peptides

Initial placement of the olefin-containing side-chains within the context of a β-peptide 3_{14}-helix was evaluated using the Spartan '04 molecular modeling program (Wavefunction, Inc., Irvine, CA). The coordinates for a single, highly 3_{14}-helical β^3-peptide 12-mer was obtained from the crystal structure of Zwit-1F as a basis for the generated models. This 3_{14}-helix was truncated to an 11-mer in accordance with the length of the host-guest peptide, **2**.[9] This peptide was represented as a homopolymer of β^3-hAla. (O-allyl)-β^3-L-Ser and (*S*)-3-aminooct-7-enoic acid were then individually substituted into the helix at positions 4 and 6 (*i* and *i+2*), 4 and 7 (*i* and *i+3*) and 4 and 8 (*i* and *i+4*), independently. These side-chains were then linked to generate both the *cis*- and *trans*-alkene staple, resulting in 12 individual models. Starting from this initial helical conformation, the models were minimized (MMFF) to determine plausible equilibrium geometries. While the constructs were generally helical, a larger deviation from 3_{14}-helicity was observed for the staples at positions *i* and *i+2*, as well as *i* and *i+4* versus the staple at positions *i* and *i+3*, regardless of the identity of the side chain (all-hydrocarbon or ether-containing).

To evaluate whether the stapled β^3-peptides could be accommodated in the p53-hDM2 binding site, a previous Monte Carlo simulation of β53-12 in complex with hDM2 was analyzed.[10] Representative snapshots of β53-12 were extracted from the trajectory and selected side chains replaced to generate the *cis* or *trans* alkene staple. The resulting complexes were energy minimized using the OPLS 2001 force field,[11] as implemented in the molecular modeling program Macromodel (Version 9.5; Schrodinger LLC, New York, NY).

VI. Circular dichroism (CD) spectroscopy. Compounds were dissolved in TBST (Peptides **2**, **2(3-6)**, **2(3-6)s**; 10 mM Tris, 100 mM NaCl, 0.01% Tween-20, pH 7.4) or TBS buffer (Peptides **β53-12**, 25 series and 47 series; 10 mM Tris, 100 mM NaCl, pH 7.4) to concentrations ranging from 20 to 80 μM. The spectra between 190 and 300 nm were acquired on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD) using an 0.2 cm path length quartz cuvette (Hellma, Plainview, NY). Three scans of each 300 μL peptide solution were taken, with 4 s averaging times and a 1 nm bandwidth. These scans were averaged, and a blank buffer spectrum was subtracted to generate the corrected spectra. This full procedure was performed three independent times to ensure accuracy and reproducibility, and these three spectra were averaged to generate the final spectrum for each peptide at each concentration. No data smoothing was used at any step. CD signal was converted into mean residue ellipticity (MRE; [Θ], deg $cm^2 \bullet dmol^{-1}$) using the equation:

$$[\Theta] = \Psi / (100 \bullet n \bullet l \bullet c)$$

where Ψ is raw ellipticity in degrees, *n* is the number of residues, *l* is path length in decimeters, and *c* is molar concentration.

VII. Fluorescence polarization assays. Fluorescence polarization experiments were performed at RT in Corning 384-well low flange black flat-bottom polystyrene non-binding microplates (Product No. 3575; Fisher Scientific, Pittsburgh, PA). $hDM2_{1-188}$, expressed and purified as described,[12] was stored at 178 μM in Buffer A (0.5 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM EDTA, 2 mM DTT, pH 7.4). To begin the binding experiment, serial dilutions of this stock solution were made in phosphate-buffered saline (PBS; 138 mM NaCl, 2 mM KCl, 13 mM Na_2HPO_4, 1.9 mM KH_2PO_4, pH 7.4), to final concentrations ranging from 0.001 to 15 μM. To these dilutions were added 2 μL aliquots of fluorescently labeled peptide (250 nM stock solution in PBS) to establish a final concentration of 25 nM (total volume: 20 μL per well). Thirty minutes was adequate to establish binding equilibrium, as judged by an absence of additional change in observed polarization values after 1 h and 90 min. The equilibrium dissociation constant (K_d) of a β-peptide·hDM2 complex (L·P) was determined by fitting the fluorescence polarization (FP) data to the equation:

$$F = F_L + ((F_{LP} - F_L)/(2[L]_T))*([L]_T + [P]_T + K_d - (([L]_T + [P]_T + K_d)^2 - 4[L]_T[P]_T))^{0.5}$$

where K_d = equilibrium dissociation constant of the L·P complex; F_L = fluorescence polarization of free ligand L; F_{LP} = observed fluorescence polarization of the L·P complex; $[L]_T$ = total concentration of ligand L; and $[P]_T$ = total concentration of protein P.

VIII. Confocal microscopy. Approximately 2000 HeLa cells were seeded in 200 μL Dulbecco's Modified Eagle Medium (DMEM; Catalog No. 11995; Invitrogen Corporation, Carlsbad, CA) supplemented with 10% Fetal Calf Serum (Catalog No. 26140; Invitrogen Corporation, Carlsbad, CA) and 1x penicillin/streptomycin (100 units penicillin, 100 μg/ml streptomycin; Catalog No. 15140-122; Invitrogen Corporation, Carlsbad, CA) in 96-well glass bottom MicroWell plates (Catalog No. MGB096-1-2-LG; MatriCal Bioscience, Spokane, WA). After allowing the cells to adhere for 48 h, the media was removed by aspiration and the cells were washed twice with 200 μL 37 ºC Hanks' Balanced Salt Solution (HBSS; Catalog No. 14025; Invitrogen Corporation, Carlsbad, CA; 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.41 mM $MgSO_4 7 \cdot H_2O$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.34 mM Na_2HPO_4, 5.56 mM dextrose, pH 7.4). The media was replaced with 100 μL of HBSS containing 20 μM fluorescein-labeled β-peptide and the cells and incubated for 2.5 h. 30 min prior to the end of the incubation, 1 μL of an aqueous solution containing 500 μg/ml of Alexa Fluor 647-labeled transferrin and 1.5 μM Hoescht 33342 dye was added. At the end of the incubation, the cells were washed with 4°C HBSS (2 x 200 mL) and imaged on a Zeiss 510 NLO Meta confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY). Fluorescein was detected via a 488 nm Ar laser line and a 525/50 nm emission filter. Alexa Fluor 647 was detected via a 633 nm He/Ne laser line and a 680 nM long pass filter. Hoescht 33324 was detected using two-photon excitation (Ti:Sapphire laser mode, locked at 905 nm) and a 445/50 nm emission filter.

IX. Flow cytometry. HeLa cells were grown to ~80% confluency in T-75 culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum in a humidified environment with 5% CO_2. The cells were then washed twice with 15 mL

37 ºC PBS and treated with 1 mL 37 ºC PBS containing 0.5 mM EDTA and 0.1 mM EGTA at 37°C for 15 min. Cells were collected in PBS, centrifuged at 500 g for 5 min, and counted by hemocytometer. This stock was diluted to ~2200 cells/μL with DMEM and aliquots of cells (92 μL) were added to fluorescein-labeled β-peptides (8 μL, 25 μM in PBS) in non-tissue culture treated round-bottom 96-well microplates (Catalog No. 351177; BD Biosciences, San Jose, CA). Cells were incubated with peptides for 4 h at 37 ºC and subsequently washed twice with 150 μL 37 ºC PBS to remove extracellular peptide. To degrade membrane proteins that might sequester the peptides, cells were incubated with 100 μL 0.25% trypsin at 37 ºC for 10 min, washed twice with 150 μL 4 ºC media and once with 150 μL 4 ºC PBS. Cells were suspended in 500 μL PBS containing 1 μg/mL propidium iodide and analyzed on a BD FACS Calibur (BD Biosciences, San Jose, CA) equipped with a 488 nm Argon laser. A total of 15,000 events were collected monitoring fluorescein and propidium iodide with 530/30 nm bandpass and 650 longpass filters, respectively. Events corresponding to cellular debris were removed by gating on forward and side scatter, while dead cells were identified and removed by propidium iodide staining.

IX. Tables and figures

Peptide	Calculated Mass (Da)	Observed Masses (Da)
2	1333.6	1332.7
2(3-6)	1445.8	1445.0
2(3-6)s	1417.7	1418.0
β53-12^{Flu}	1959.0	1959.5
β53-12SB3^{Flu}	2071.4	2071.9
25.O ^{Flu}	1987.0	1985.5
25.O-s ^{Flu}	1958.9	1958.5
47.O ^{Flu}	1970.0	1968.9
47.O-s ^{Flu}	1942.0	1940.7
25.C ^{Flu}	1983.0	1981.5
25.C-s ^{Flu}	1955.0	1954.2
47.C ^{Flu}	1966.0	1965.7
47.C-s ^{Flu}	1938.0	1936.2

Table S1: Mass spectrometry data for β-peptides used in this study.

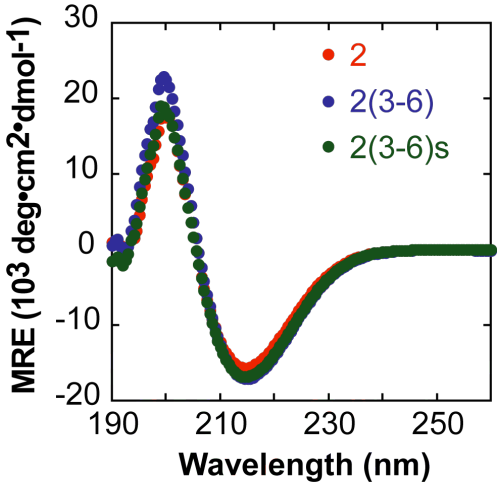

Figure S1: Circular dichroism of β-peptides **2**, **2(3-6)** and **2(3-6)s** at 80 μM in TBST (10 mM Tris, 100 mM NaCl, 0.01% Tween-20, pH 7.4) at 25°C.

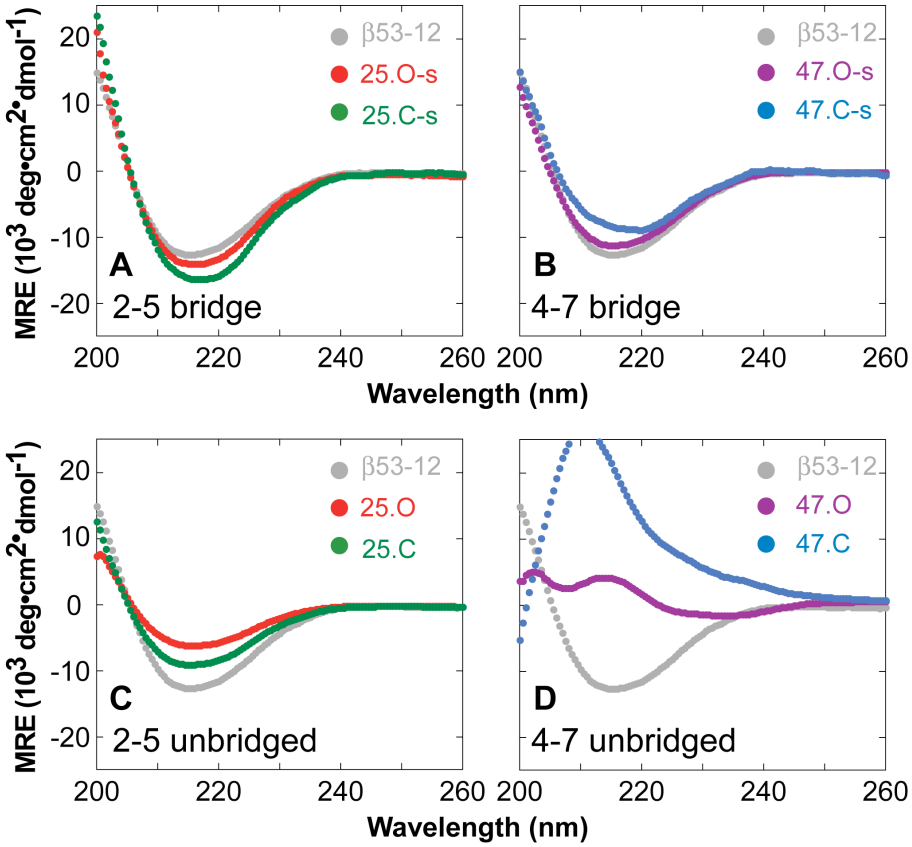

Figure S2: Circular dichroism of stapled (A, B) and unstapled (C, D) variants of **β53-12**, taken at 20-80 μM in TBS buffer (10 mM Tris, 100 mM NaCl, pH 7.4) at 25°C. Each β-peptide was labeled on the *N*-terminus with 5-(and-6-)-carboxyfluorescein, succinimidyl ester.

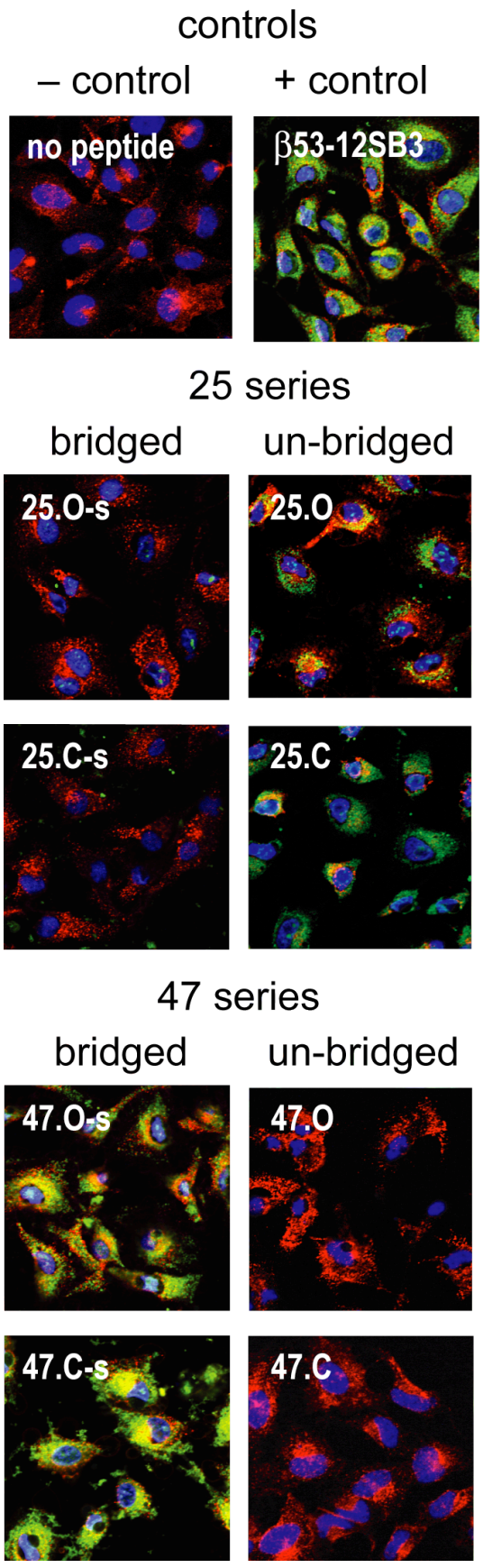

Figure S3. HeLa cell uptake and localization of fluorescein-tagged β-peptides. HeLa cells were treated with 20 μM of the indicated β-peptides (green), 5 mg•mL^{-1} Alexa Fluor 647-transferrin (red) and 150 nM Hoescht 33342 (blue).

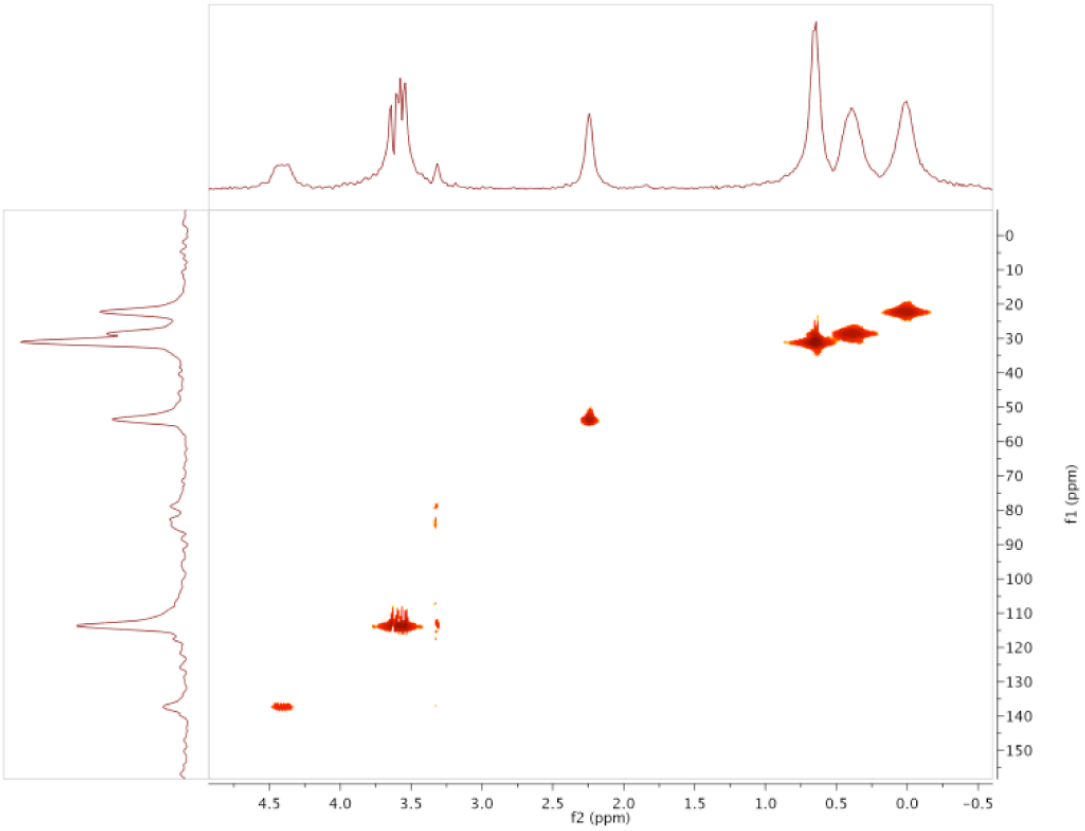

Figure S4: Heteronuclear Multiple Quantum Coherence (HMQC) spectrum of (*S*)-2-aminohept-6-enoic acid, taken to verify the presence of a ^{13}C peak within the 48-50 ppm range (obscured in the 1D ^{13}C NMR spectrum by methanol).

Expanded Reference

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