Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting Information

Landscaping macrocyclic peptides: stapling hDM2-binding peptides for helicity, protein affinity, proteolytic stability and cell uptake

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1. SUPPORTING FIGURES

Peptide	IC_{50} (nM)	$pIC_{50} \pm$ SEM (M)
1	51	7.30 ± 0.09
2a	49	7.31 ± 0.06
2b	7.0	8.15 ± 0.03
2c	46	7.34 ± 0.03
2d	350	6.46 ± 0.04
2e	92	7.03 ± 0.04
2f	5.2	8.28 ± 0.02
2g	9.1	8.04 ± 0.03
2h	4749	5.32 ± 0.05
3a	42	7.37 ± 0.03
3b	40	7.39 ± 0.02
3c	37	7.43 ± 0.03
3d	67	7.18 ± 0.02
3e	48	7.32 ± 0.02
4a	45	7.34 ± 0.05
4b	134	6.87 ± 0.02
4c	95	7.02 ± 0.02
4d	420	6.38 ± 0.02
4e	107	6.97 ± 0.02
5a	2358	5.63 ± 0.05
5b	514	6.29 ± 0.04
5c	347	6.46 ± 0.03
5d	154	6.81 ± 0.03
5e	194	6.71 ± 0.06
ATSP-7041	25	7.60 ± 0.01

Figure S1. Competitive inhibition of binding of hDM2 (25 nM) to FITC-labelled PDI (**1-F,** 10 nM) measured by fluorescence polarization assays (mean ± SEM). Compound ATSP-7041[1] was used as control.

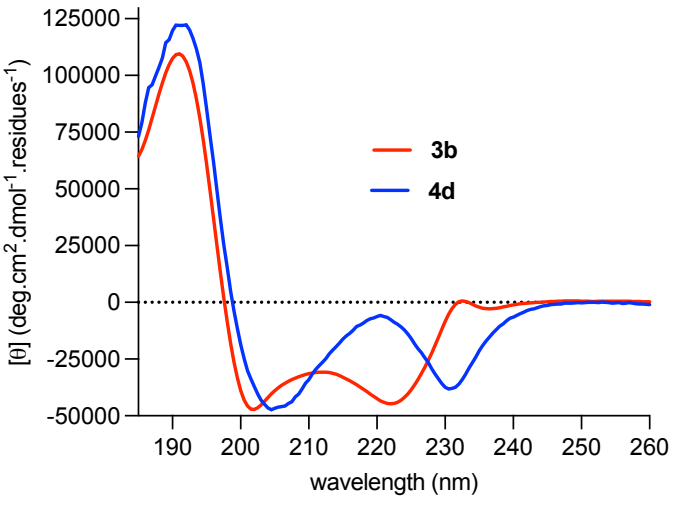


Figure S2. CD spectra for stapled peptides **3b** and **4d** (50 µM) in 10 mM sodium phosphate buffer pH 7.4.

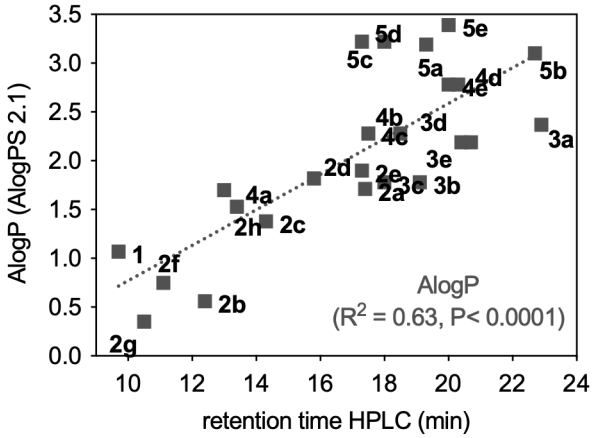


Figure S3. Correlation between experimental HPLC column retention time for each peptide and their calculated AlogP using AlogPS 2.1 application.

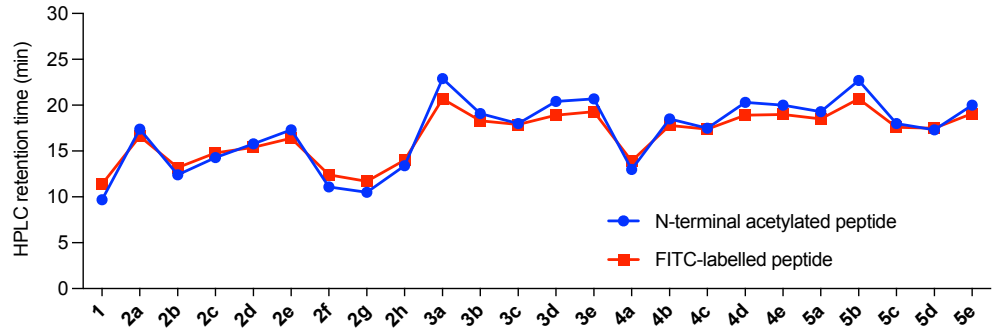


Figure S4. Comparison of HPLC retention times of the N-acetylated and FITC-labelled analogues of the peptides.

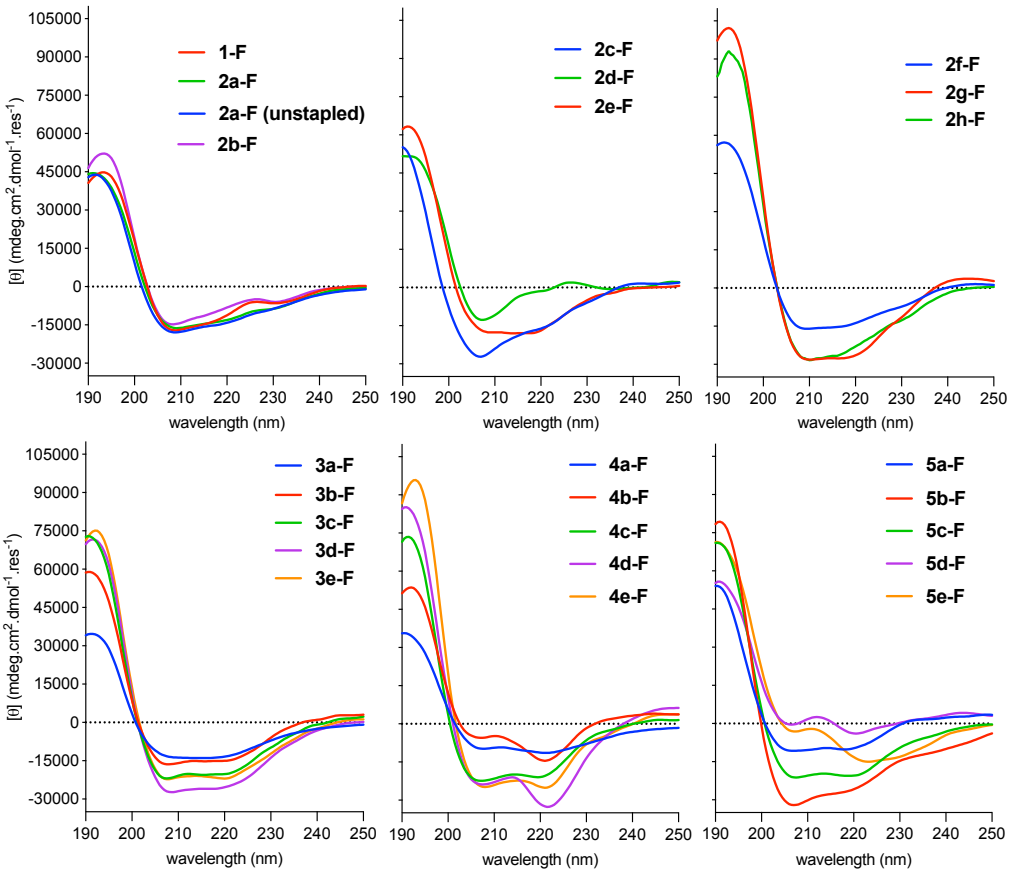


Figure S5. CD spectra of the FITC-derived stapled peptides (50 µM) in 20 mM SDS, 10 mM sodium phosphate, 150 mM sodium fluoride buffer pH 7.4.

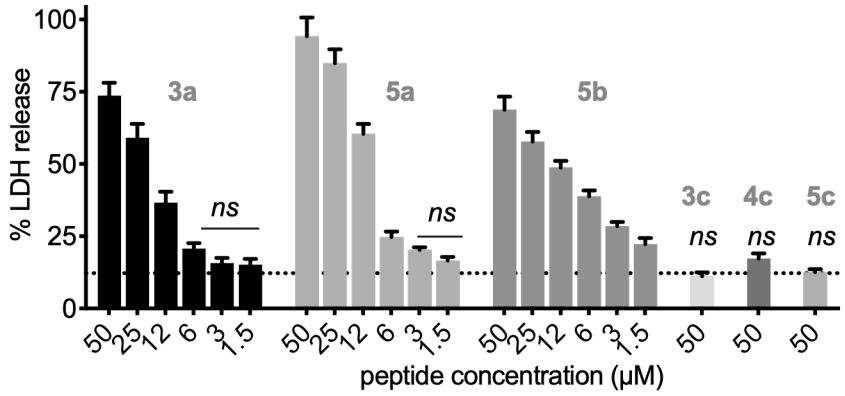


Figure S6. LDH release after incubation of stapled peptides at different concentrations in HeLa cells for 1h.

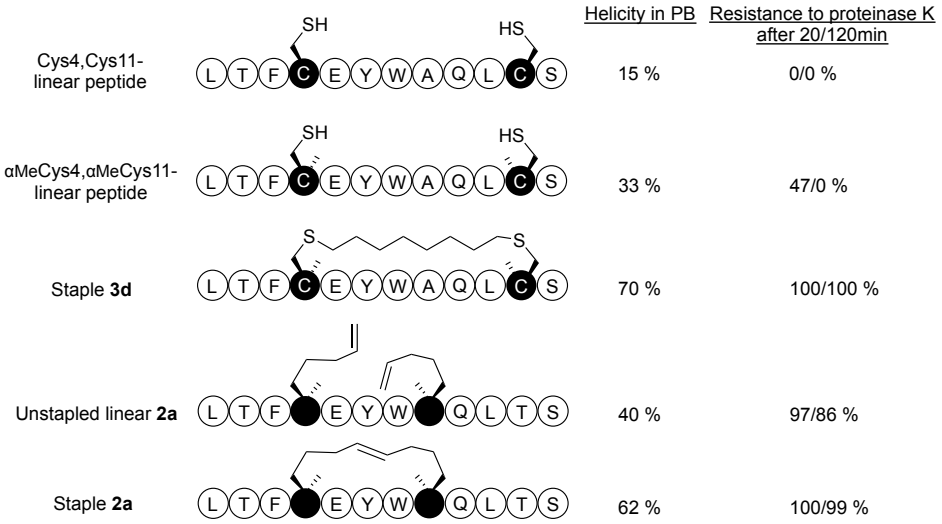


Figure S7. Effect of α-methyl substitution on helical configuration in 10 mM phosphate buffer pH 7.4 (PB) and on proteolytic degradation by proteinase K.

2. EXPERIMENTAL PROCEDURES

Abbreviations

Ac, acetyl; DCE, dichloroethane; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DTT, dithiolthreitol; EDT, 1,2-ethanedithiol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electron spray mass spectroscopy; Fmoc, 9-fluorenylmethyloxycarbonyl; ; FITC, fluorescein isothiocyanate; HATU, 2-(7-Aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HCTU, 2-(1*H*-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hcy, homocysteine; HPLC, high performance liquid chromatography; HR-MS, high-resolution mass spectroscopy; MBHA, 4-methyl-benzylhydrylamine; LysN3, azidolysine; Mtt, 4-methyltrityl; OPip, 2-phenylisopropyl ester; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, R8, (R)-2-(7'octenyl)alanine; RCM, ring-closing metathesis; S5, S-2-(4′-pentenyl) alanine; SDS, sodium dodecyl sulfate; TCE, trichloroethane; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIS, triisopropylsilane; UPLC, ultra-performance liquid chromatography.

2.1. General solid phase peptide synthesis

Materials

All solvents and reagents used during peptide chain assembly were peptide synthesis grade and purchased from commercial suppliers. Fmoc-αMe-Cys was prepared as described in sessions 2.5 and 2.6. All crosslinkers used were commercially available, except 4,4'-bis(bromomethyl)-2,2',3,3',5,5',6,6'-octafluoro-1,1'-biphenyl, prepared as reported in reference 2.

Solid-phase peptide synthesis (SPPS)

Linear peptides were assembled using Fmoc-based chemistry on a peptide synthesizer (Symphony, Protein Technologies) using Rink Amide MBHA resin (Novabiochem or Chemimpex). Peptides undergoing on-resin cyclization were assembled on a low-loading resin Rink-amide resin (Novabiochem). Standard Fmoc-protected amino acids were used for all peptides, unless otherwise stated (see procedure for each peptide). Usually, 4 equiv. of Fmoc-protected amino acid, 4 equiv. of HCTU and 4 equiv. of DIPEA were used in 2 x 30 min coupling cycles. Fmoc deprotection was achieved by treatment with 1:3 piperidine:DMF for 2 × 3 min. At the final stage, the *N*-terminus was acetylated with Ac_2O:DIPEA:DMF (0.87:0.47:15 mL) for 10 min.. For peptides featuring FITC, the *N*-terminus was first coupled to a Fmoc-β-alanine residue, followed by Fmoc-group removal and labeling with FITC by treating the free amine resin with FITC (2 equiv.) and DIPEA (4 equiv.) in DMF overnight.

Cleavage from solid support (TFA acidolysis)

Peptides were cleaved from the resin with TFA:TIS:H_2O (95:2.5:2.5) for 2.5 h. Peptides containing Cys were cleaved with TFA:TIS:EDT:H_2O (91:3:3:3) for 2.5 h. The crude peptides were precipitated and washed with cold Et_2O, redissolved in 75% acetonitrile/0.05% TFA in water and lyophilized. Incomplete decarboxylation of the Trp residue was observed in all crude material, characterized by the presence of a +44 Da adduct (+CO_2) in ESI-MS analysis. Because spontaneous decarboxylation occurred under basic conditions, crude mixtures containing the carboxylated adduct were directly used for subsequent thioether stapling reactions under basic conditions. For the other peptides, the crude was redissolved in 70% acetonitrile/10% acetic acid and let stand at room temperature to allow complete decarboxylation of the Trp residue (usually overnight), as monitored by ESI-MS. After that, the crude was again freeze-dried and submitted to HPLC purification.

Peptide purification by HPLC

Peptides were purified on a Shimadzu preparative HPLC System using a Phenomenex Luna C18 column eluting at a flow rate of 20 mL/min and a gradient of 20 to 80% buffer B (90% CH_3CN/10% H_2O/0.1% TFA in buffer A, 0.1% TFA in water) over 30 min. Purity and identity of collected peptide fractions were performed by analytical HPLC, UPLC-MS and/or HR-MS.

Peptide stock solutions

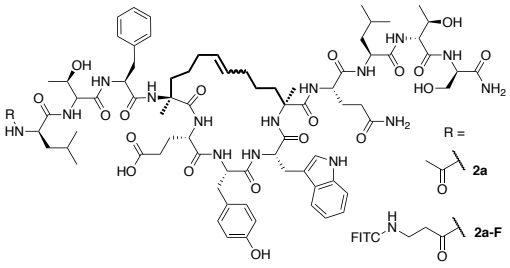
Pure peptides were dissolved in 75% deuterated acetonitrile-d3 in water and the concentration of the resulting solution was determined by NMR using Pulcon method.[3] 90° pulses were accurately determined and then 1D Spectra were acquired using the standard watergate sequence with a ns= 16-3, d1= 30s to ensure complete relaxation of proton signals. Integration of well resolved signals compared using the following equation:

$$c_u = c_R \frac{S_U T_U \vartheta_{360}^U n_R r g_R}{S_R T_R \vartheta_{360}^R n_U r g_U}$$

where *c* is the concentration, *S* is the integral (in absolute units)/number of protons, *T* is the temperature in Kelvin, θ_{360} is the 360° rf pulse, *n* is the number of scans, and *rg* is the receiver gain used for measuring the reference (R) and unknown (U) samples. Solutions were stored at -20°C or lyophilized and redissolved in DMSO (for a 10 mM final solution).

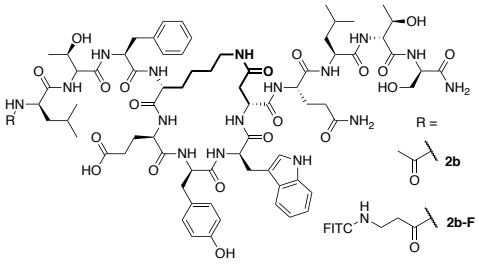
2.2. Synthesis of (i→i+4)-stapled peptides

Hydrocarbon staple 2a and 2a-F



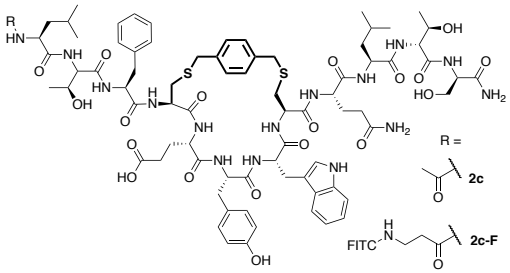
The sequence Fmoc-Leu-Thr(tBu)-Phe-**S_5**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-**S_5**-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled following the general SPPS procedure. The resin was then washed with DCM and dried under high vacuum overnight. The dry resin was then placed in the synthesizer apparatus and swollen in dry DCE under N_2 stream for 10 min and drained. The RCM reaction was performed by treating the resin with a 10 mM solution of Grubbs catalyst 1st generation in dry DCE (2 mL per 50 µmol resin) under N_2 bubbling for 2h. The catalyst solution was drained and a fresh 10 mM Grubbs catalyst solution was added to the resin and mixed for 2h. After that, the resin was washed with DCE and DMF. Next, the Fmoc group was removed and the N-terminus was acetylated (**2a**) or coupled to β-alanine and FITC (**2a-F**). Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC. A major stapled peptide product peak was isolated exhibiting the expected mass. The *E/Z* ratio of the double bond was not determined. About 30 % peptide remained unstapled after the RCM step, and the linear peptide Ac-Leu-Thr-Phe-**S_5**-Glu-Tyr-Trp-**S_5**-Leu-Thr-Ser-$CONH_2$ was also isolated.

Lactam staple 2b and 2b-F



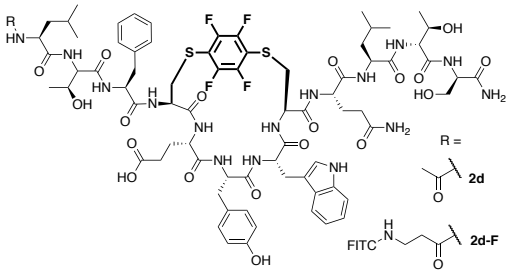
The sequence Fmoc-Leu-Thr(tBu)-Phe-**Lys(Mtt)-**Glu(tBu)-Tyr(tBu)-Trp(Boc)-**Asp(OPip)**-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled using the SPPS procedure. The resin was washed with DCM and treated repeatedly with 3% TFA in DCM (10 x 1 min). After washing with DCM and DMF, a solution of PyBOP (4 equiv) and DIPEA (8 equiv) in DMF was added to the resin and the reaction was agitated overnight. Subsequently, the Fmoc group was removed and the N-terminus was acetylated (**2b**) or coupled to β-alanine and FITC (**2a-F**). Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC.

Thioether staple 2c and 2c-F



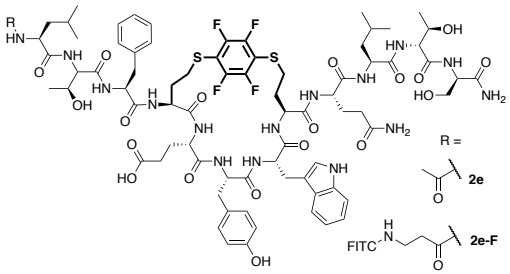
The sequence Fmoc-Leu-Thr(tBu)-Phe-**Cys(Trt)**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-**Cys(Trt)-**Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled using the SPPS procedure, cleaved by TFA acidolysis and lyophilized. The crude linear di-Cys peptide (15 mg) was then combined to 1,4-Bis(bromomethyl)benzene (1.5 eq) in 100 mM TRIS base (5 mL). After 30 min, the mixture was acidified by addition of 1 % TFA and the product **2c** was purified by HPLC. Compound **2c-F** was synthetized similarly from the linear sequence FITC-βAla-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Cys(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu).

Thioether staple 2d and 2d-F



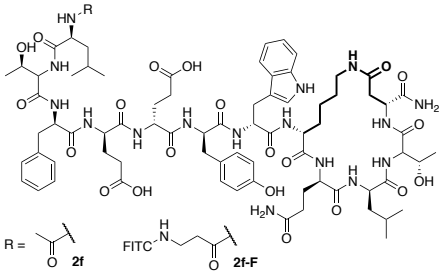
The sequence Ac-Leu-Thr(tBu)-Phe-**Cys(Trt)**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-**Cys(Trt)-**Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled using the SPPS procedure, cleaved by TFA acidolysis and lyophilized. The crude linear di-Cys peptide (16 mg, 10.3 µmol) was then combined to hexafluorobenzene (12 µL, 10 eq) in 100 mM TRIS base (10 mL). After 3h, the mixture was acidified by addition of 1 % TFA and the product (**2d**) was purified by HPLC. Compound **2d-F** was synthetized similarly from the linear sequence FITC-βAla-Leu-Thr(tBu)-Phe-Cys(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Cys(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu).

Thioether staple 2e and 2e-F



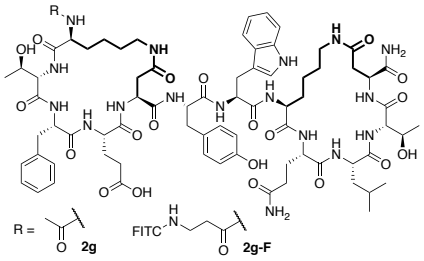
The sequence Ac-Leu-Thr(tBu)-Phe-**Hcy(Trt)**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-**Hcy(Trt)-**Gln(Trt)-Leu-Thr(tBu)-Ser(tBu) was assembled using the SPPS procedure, cleaved by TFA acidolysis and lyophilized. The crude linear di-Cys peptide (21 mg, 13.2 µmol) was then combined to hexafluorobenzene (16 µL, 100 eq) in 100 mM TRIS base (10 mL). After overnight reaction, the mixture was acidified by addition of 1 % TFA and the product (**2e**) was purified by HPLC. Compound **2e-F** was synthetized similarly from the linear sequence FITC-βAla-Leu-Thr(tBu)-Phe-Hcy(Trt)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Hcy(Trt)-Gln(Trt)-Leu-Thr(tBu)-Ser(tBu).

Lactam staple 2f and 2f-F



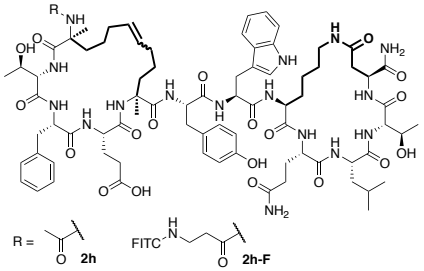
The sequence Fmoc-Leu-Thr(tBu)-Phe-Glu(tBu)-Glu(tBu)-Tyr(tBu)-Trp(Boc)-**Lys(Mtt)**-Gln(Trt)-Leu-Thr(tBu)-**Asp(OPip)** was assembled by SPPS. The resin was washed with DCM and treated repeatedly with 3% TFA in DCM (10 x 1 min). After washing with DCM and DMF, a solution of PyBOP (4 equiv) and DIPEA (4 equiv) in DMF was added to the resin and the reaction was agitated overnight. Next, the Fmoc group was removed and the N-terminus was acetylated (**2f**) or coupled to β-alanine and FITC (**2f-F**). Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC.

Double lactam staple 2g and 2g-F



The sequence Fmoc-Tyr(tBu)-Trp(Boc)-**Lys(Mtt)**-Gln(Trt)-Leu-Thr(tBu)-**Asp(OPip)** was assembled by SPPS. The resin was then washed with DCM and treated repeatedly with 3% TFA in DCM (10 x 1 min). After washing with DCM and DMF, a solution of PyBOP (4 equiv) and DIPEA (4 equiv) in DMF was added to the resin and the first lactam bridge was formed after 6h. Next, the Fmoc group was removed and peptide chain was elongated to: Fmoc-**Lys(Mtt)**-Thr(tBu)-Phe-Glu(tBu)-**Asp(OPip)**-Tyr(tBu)-Trp(Boc)-[Lys-Gln(Trt)-Leu-Thr(tBu)-Asp]. The second lactam bridge was then constructed by treating the resin with 3% TFA in DCM followed by PyBOP-assisted lactamization. Next, the Fmoc group was removed and the N-terminus was acetylated (**2g**) or coupled to β-alanine and FITC (**2g-F**). Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC.

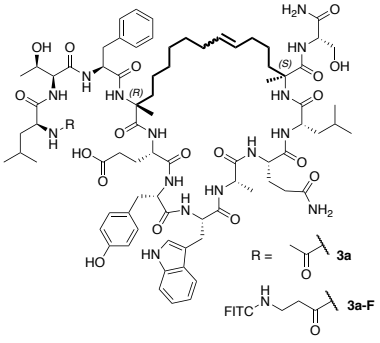
Dual hydrocarbon/lactam staple 2h adn 2h-F



The sequence Fmoc-**R8**-Thr(tBu)-Phe-Glu(tBu)-**S5**-Tyr(tBu)-Trp(Boc)-Lys(Mtt)-Gln(Trt)-Leu-Thr(tBu)-Asp(OPip) was assembled by SPPS. The resin was then washed with DCM and treated repeatedly with 3% TFA in DCM (10 x 1 min). After washing with DCM and DMF, a solution of PyBOP (4 equiv) and DIPEA (4 equiv) in DMF was added to the resin and the reaction was agitated overnight. The resulting resin was washed with DCM and dried under high vacuum overnight. The dry resin was then placed in the synthesizer apparatus and swollen in dry DCE under N_2 stream for 10 min and drained. The RCM reaction was performed by treating the resin with a 10 mM solution of Grubbs catalyst 1st generation in dry DCE under N_2 bubbling for 2h. The catalyst solution was drained and a fresh 10 mM Grubbs catalyst solution was added to the resin and reacted for 2h. After that, the resin was washed with DCE and DMF. Next, the Fmoc group was removed and the N-terminus was acetylated (**2h**) or coupled to β-alanine and FITC (**2h-F**). Finally, the peptide was cleaved by TFA acidolysis and purified by HPLC. The *cis*/*trans* configuration of the double bond was not determined.

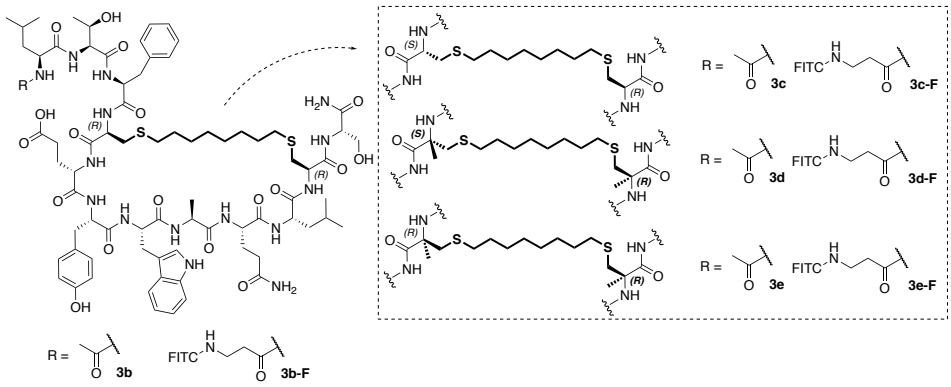
2.3. Synthesis of aliphatic (i→i+7)-stapled peptides

Hydrocarbon staples 3a and 3a-F



The sequence Ac-Leu-Thr(tBu)-Phe-***R8***-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-***S5***-Ser(tBu) was assembled by SPPS. The resulting resin was washed with DCM and dried under high vacuum overnight. The dry resin was then placed inside an oven-dried microwave vessel, sealed and flushed with N_2. A solution of 10 mM Hoveyda-Grubbs catalyst 2nd generation in dry DCE was added via syringe and the system flushed with N_2 for 5 min. The reaction was submitted to microwave heating at 100 ^{0}C for 10 min using a Biotage microwave reactor. After that, the resin was washed with DCE, DMF and DCM; Next, and peptide **3a** was finally cleaved by TFA acidolysis and purified by HPLC. For peptide **3a-F**, Fmoc-βAla-Leu-Thr(tBu)-Phe-***R8***-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-***S5***-Ser(tBu) was assembled by SPPS and the RCM step was performed as for **3a**. After that, the Fmoc group was removed and the FITC label was attached to the N-terminus. Finally, **3a-F** peptide was cleaved from the resin and purified by HPLC. A major product peak was isolated exhibiting the expected mass. The *E/Z* ratio of the linker double bond was not determined. To note, a report by Wallbrecher *et al* revealed that E/Z-stereoisomers of a similar (i, i+7)-hydrocarbon stapled PDI sequence showed comparable binding and cell activity.[4]

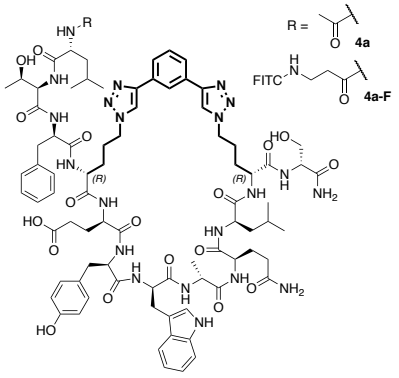
Thioether staples 3b,c,d,e and 3b,c,d,e-F



The sequences Ac-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) -where X1, X2 = L-Cys (**3b**); X1= D-Cys, X2 = L-Cys (**3c**); X1, X2 = αMe-L-Cys (**3d**); or X1 = αMe-D-Cys, X2 = αMe-L-Cys (**3f**) – and FITC-βAla-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**3b-F**); X1= D-Cys, X2 = L-Cys (**3c-F**); X1, X2 = αMe-L-Cys (**3d-F**); or X1 = αMe-D-Cys, X2 = αMe-L-Cys (**3e-F**) – were assembled on solid support as described in the general SPPS procedure. Subsequently, the linear peptide was cleaved by TFA acidolysis and purified by HPLC. The purified linear di-cysteine peptide was dissolved in DMF to a 2mM concentration inside a microwave flask equipped with a stirring bar. 1,8-dibromoctane (15 equiv) and $Zn(OAc)_2$ (250 mM in 0.1 % TFA, 10 equiv) were added and the system degassed with argon for 5 min. The reaction vessel was sealed and heated inside a microwave reactor (Biotage) at 100 ^{0}C for 1 h. After that, the stapled peptide was purified by HPLC.

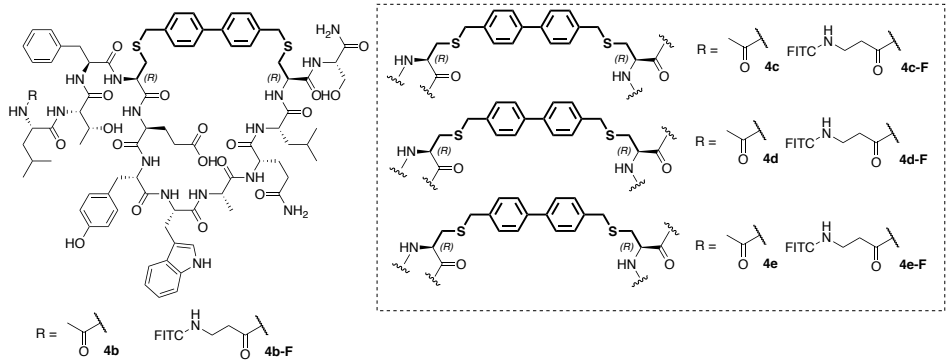
2.4. Synthesis of aromatic (i→i+7)-stapled peptides

Triazole staple 4a and 4a-F



The sequences Ac-Leu-Thr(tBu)-Phe-**Lys(N$_3$)**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**Lys(N$_3$)**-Ser(tBu) (for **4a**) and FITC-βAla-Leu-Thr(tBu)-Phe-**Lys(N$_3$)**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**Lys(N$_3$)**-Ser(tBu) (for **4a-F**) were assembled by SPPS, cleaved by TFA treatment and purified by HPLC. To solution of purified diazido peptide and 1,3-diethynylbenzene (1.1 equiv) in *tert*-butanol:water (2:1, to a 1 mL/mg peptide) degassed with N_2, $CuSO_4.5H_20$ (1.0 equiv), tris(3-hydroxypropyltriazolylmethyl)amine (1.0 equiv) and sodium ascorbate (3.0 equiv) were added. After 2h reaction under N_2 atmosphere, the macrocycle product was isolated by HPLC purification.

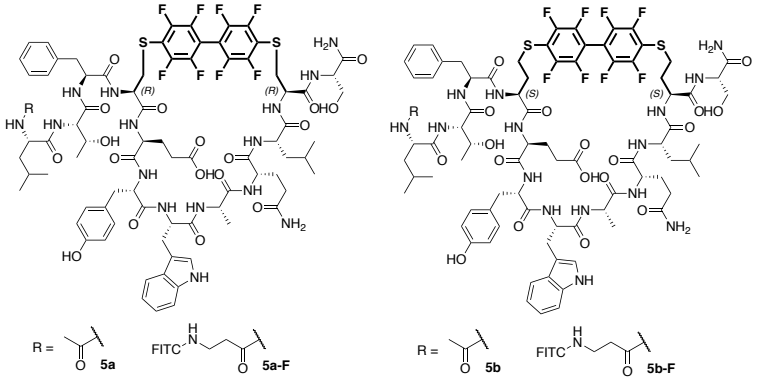
Thioether staples 4b,c,d,e and 4b,c,d,e-F



The sequences Ac-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**4b**); X1= D-Cys, X2 = L-Cys (**4c**); X1, X2 = αMe-L-Cys (**4d**); X1 = αMe-D-Cys, X2 = αMe-L-Cys (**4d**) – and FITC-βAla-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**4b-F**); X1= D-Cys, X2 = L-Cys (**4c-F**); X1, X2 = αMe-L-Cys (**4d-F**); X1 = αMe-D-Cys, X2 = αMe-L-Cys (**4e-F**)– were assembled on solid support as described in the general SPPS procedure. The crude lyophilized linear di-cysteine peptide was then combined to 1.5 equiv of 4,4′-

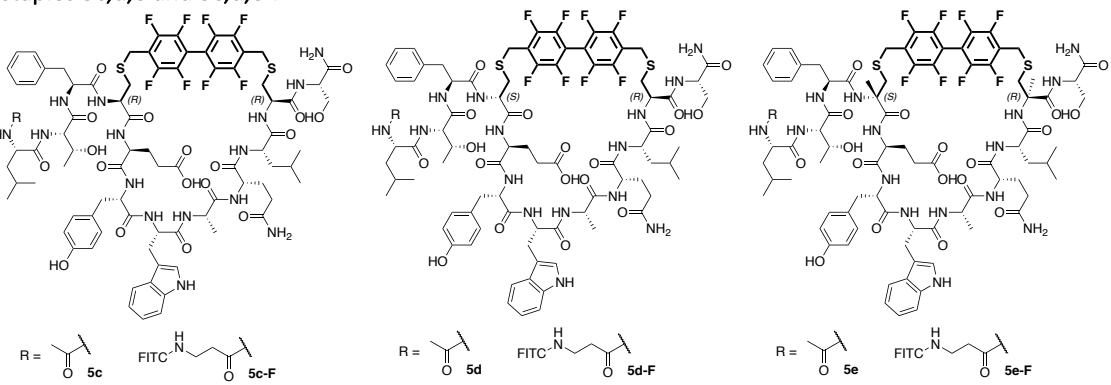
bis(bromomethyl)biphenyl in a solution of 0.1M $NaHCO_3$:DMF (1:1, to a 1 mg/mL peptide concentration). After 1-2h reaction, the stapled peptide was isolated by HPLC purification.

Thioether staples 5a,b and 5a,b-F



The sequences Ac-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**5a**) or X1, X2 = L-Hcy (**5b**) – and FITC-βAla-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**5a-F**) or X1, X2 = L-Hcy (**5b-F**) – were assembled on solid support as described in the general SPPS procedure. The crude lyophilized linear di-cysteine peptide was then combined to 2.0 equiv of decafluorobiphenyl (in a solution of 50 mM Tris base) (to a 2 mg/mL peptide concentration). After 1-2h reaction, the stapled peptide was isolated by HPLC purification.

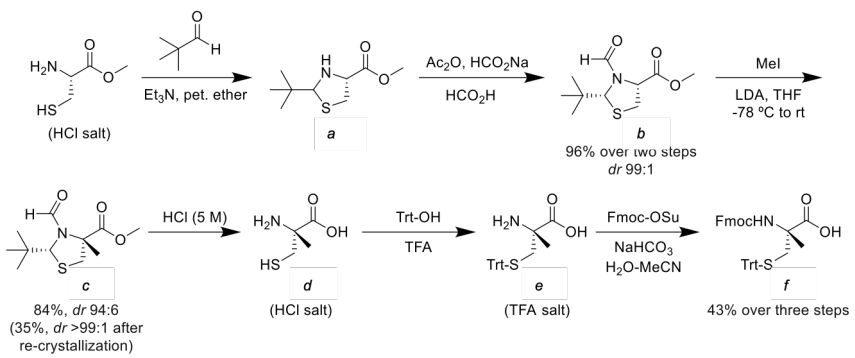
Thioether staples 5c,d,e and 5c,d,e-F



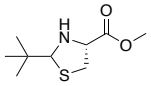
The sequences Ac-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**5c**); X1= D-Cys, X2 = L-Cys (**5d**); X1 = αMe-D-Cys, X2 = αMe-L-Cys (**5e** – and FITC-βAla-Leu-Thr(tBu)-Phe-**X1**-Glu(tBu)-Tyr(tBu)-Trp(Boc)-Ala-Gln(Trt)-Leu-**X2**-Ser(tBu) - where X1, X2 = L-Cys (**5c-F**); X1= D-Cys, X2 = L-Cys (**5d-F**); X1 = αMe-D-Cys, X2 = αMe-L-Cys (**5e-F**)– were assembled on solid support as described in the general SPPS procedure. The crude lyophilized linear di-cysteine peptide was then combined to 1.5 equiv of 4,4'-bis(bromomethyl)-2,2',3,3',5,5',6,6'-octafluoro-1,1'-biphenyl in a solution of 0.1M $NaHCO_3$:DMF (1:1, to a 1 mg/mL peptide concentration). After 1-2h reaction, the stapled peptide was isolated by HPLC purification.

2.5. Synthesis of (*R*)-N-Fmoc-S-Trityl-2-methylcysteine

The synthesis of the Fmoc-αMe-L-Cys(Trt)-OH building block was adapted from reported procedures.[5-8]

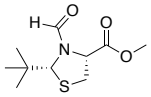


(*4R*)-Methyl 2-(*tert*-butyl)thiazolidine-4-carboxylate (*a*)[5]



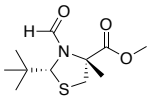
A suspension of L-cysteine methyl ester hydrochloride (13.7 g, 80.0 mmol), trimethylacetaldehyde (13.5 mL, 0.12 mol), triethylamine (17.0 mL, 0.12 mol) and petroleum ether (160 mL) was heated to reflux with continuous removal of water via a Dean-Stark condenser for 1 d. The reaction mixture was then cooled to rt and filtered. The solid residue was washed with Et_2O. The combined filtrates were concentrated *in vacuo* to give the product as a pale yellow oil (16.2 g, 79.8 mmol, >99%). A 2:1 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. The diastereomeric mixture was used without further purification. TLC (10:90 EtOAc–petroleum ether) R_f = 0.2 (two spots). TLC (10:90 EtOAc–petroleum ether) R_f = 0.2 (two spots). Mixture of diastereomers (2:1) (1.6:1 reported in literature): ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.53 (s, 1H, $H2_{thiazolidine}$, minor), 4.46 (s, 1H, $H2_{thiazolidine}$, major), 4.14 (t, *J* = 6.0 Hz, 1H, $H4_{thiazolidine}$, minor), 3.81 (dd, *J* = 9.7, 6.7 Hz, 1H, $H5_{thiazolidine}$, major), 3.77 (s, 3H, CO_2CH_3, major), 3.75 (s, 3H, CO_2CH_3, minor), 3.25 (dd, *J* = 10.2, 6.8 Hz, 1H, $H5_{thiazolidine}$, major), 3.11 (dd, *J* = 10.6, 6.4 Hz, 1H, $H5_{thiazolidine}$, minor), 3.02 (dd, *J* = 10.6, 5.6 Hz, 1H, $H5_{thiazolidine}$, minor), 2.68 (t, *J* = 10.0 Hz, 1H, $H4_{thiazolidine}$, major), 2.29 (br s, 1H, NH, single diastereomeric signal), 1.07 (s, 9H, $CH(\underline{CH_3})_3$, major), 0.97 (s, 9H, $CH(\underline{CH_3})_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.6 (CO, minor), 172.0 (CO, major), 82.0 ($C2_{thiazolidine}$, major), 80.0 ($C2_{thiazolidine}$, minor), 65.6 ($C4_{thiazolidine}$, major), 65.2 ($C4_{thiazolidine}$, minor), 52.6 (CO_2CH_3, single conformer signal), 37.6 ($C5_{thiazolidine}$, major), 37.2 ($C5_{thiazolidine}$, major), 36.1 ($\underline{C}(CH_3)_3$, minor), 34.1 ($\underline{C}(CH_3)_3$, minor), 27.1 ($C(\underline{CH_3})_3$, major), 26.7 ($C(\underline{CH_3})_3$, minor). UPLC–MS t_R = 3.4 min, *m/z*: calcd for $[M+H]^+$ $C_9H_{18}NO_2S^+$ 204.3, found: 204.3.

(*2R,4R*)-Methyl 2-(*tert*-butyl)-3-formylthiazolidine-4-carboxylate (*b*)[8]



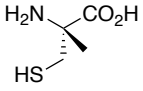
Acetic anhydride (22.7 mL, 0.24 mol) was added dropwise over 1 h via a syringe pump to a solution of (*4R*)-methyl 2-(*tert*-butyl)thiazolidine-4-carboxylate (16.2 g, 79.8 mmol), sodium formate (6.53 g, 96.0 mmol) in formic acid (160 mL) while stirring at 0 °C. The reaction mixture was then stirred for 18 h at rt and concentrated *in vacuo*. The residue was cooled in a water bath and saturated $NaHCO_3$ (150 mL) was slowly added. At this point, the pH of the mixture was 8. The mixture was extracted with Et_2O (3 × 100 mL). The combined organic layers were washed with saturated $NaHCO_3$ (50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give the product as a pale yellow oil the solidifies upon standing to give a colourless solid (17.7 g, 76.6 mmol, 96%). A 99:1 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. The solid was used without further purification. TLC (40:60 EtOAc–petroleum ether) R_f = 0.4. Mixture of conformers (7:1): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.35 (s, 1H, CHO, major), 8.29 (s, 1H, CHO, major), 5.27 (s, 1H, $H2_{thiazolidine}$, minor), 4.89 (t, *J* = 8.7 Hz, 1H, $H4_{thiazolidine}$, major), 4.78 (dd, *J* = 8.8, 7.3 Hz, 1H, $H4_{thiazolidine}$, minor), 4.74 (s, 1H, $H2_{thiazolidine}$, major), 3.81 (s, 3H, CO_2CH_3, minor), 3.77 (s, 3H, CO_2CH_3, major), 3.44 (dd, *J* = 12.0, 7.2 Hz, 2H, $H5_{thiazolidine}$, minor), 3.35 – 3.23 (m, 2H, $H5_{thiazolidine}$, major), 1.03 (s, 9H, $C(\underline{CH_3})_3$, major), 0.96 (s, 9H, $C(\underline{CH_3})_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.7 (CO, minor), 170.1 (CO, major), 164.0 (CHO, minor), 162.8 (CHO, major), 75.3 ($C2_{thiazolidine}$, major), 71.6 ($C2_{thiazolidine}$, minor), 63.9 ($C4_{thiazolidine}$, minor), 61.6 ($C4_{thiazolidine}$, major), 53.2 ($CO_2\underline{CH_3}$, minor), 52.8 ($CO_2\underline{CH_3}$, major), 39.1 ($\underline{C}(CH_3)_3$, minor), 38.7 ($\underline{C}(CH_3)_3$, major), 33.0 ($C5_{thiazolidine}$, major), 32.1 ($C5_{thiazolidine}$, minor), 26.9 ($C(\underline{CH_3})_3$, minor), 26.4 ($C(\underline{CH_3})_3$, major). UPLC–MS t_R = 3.6 min, *m/z*: calcd for $[M+H]^+$ $C_{10}H_{18}NO_3S^+$: 232.1, found: 232.1.

(*2R,4R*)-Methyl 2-(*tert*-butyl)-3-formyl-4-methylthiazolidine-4-carboxylate (*c*)[8]



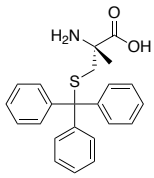
(*2R,4R*)-methyl 2-(*tert*-butyl)-3-formylthiazolidine-4-carboxylate (2.54 g, 11.0 mmol) was placed in a dried round-bottomed flask and subjected to three cycles of vacuum/N_2. Anhydrous THF (50 mL) was added by a syringe. The solution was cooled to −78 °C. Methyl iodide (1.00 mL, 16.1 mmol) was added by a syringe. Lithium diisopropylamide (2.0 M in THF/*n*-heptane/ethylbenzene, 11.0 mL) was added dropwise. The reaction mixture was stirred at –78 °C under N_2 for 0.75 h and then heated to rt. The reaction mixture was stirred for an additional 0.75 h and then concentrated *in vacuo* to give a yellow oil which was partitioned between H_2O-brine (75 mL, 2:1) and EtOAc (50 mL). The aqueous layer was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous $MgSO_4$ and concentrated in *vacuo*. The resulting yellow oil was purified by flash chromatography (0–20% EtOAc in petroleum ether to give the product as a pale yellow oil (2.26 g, 9.21 mmol, 84%). A 94:6 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. The oil was dissolved in Et_2O–*n*-heptane (5 mL, 1:4). The solution was heated gently with a heat gun for roughly 1 min. The solution was then cooled to rt and then placed in a fridge. Colorless crystals emerged within hours. After standing for 1 d, the mother liquor was removed by decantation. The crystals were suspended in petroleum ether (3 × 10 mL) and isolated via decantation. The crystals were then concentrated *in vacuo*. Yield: 948 mg (3.86 mmol, 35%, *dr* > 99:1). TLC (40:60 EtOAc–petroleum ether) R_f = 0.5. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.41 (s, 1H, CHO, minor), 8.28 (s, 1H, CHO, major), 5.31 (s, 1H, $H2_{thiazolidine}$, minor), 4.66 (s, 1H, $H2_{thiazolidine}$, major), 3.82 (s, 3H, CO_2CH_3, minor), 3.77 (s, 3H, CO_2CH_3, major), 3.65 (d, *J* = 12.7 Hz, 1H, $H5_{thiazolidine}$, minor), 3.33 (d, *J* = 11.6Hz, 1H, $H5_{thiazolidine}$, major), 2.86 (d, *J* = 12.3 Hz, 1H, $H5_{thiazolidine}$, minor),2.73 (d, *J* = 11.6 Hz, 1H, $H5_{thiazolidine}$, major), 1.79 (s, 3H, CH_3, minor), 1.76 (s, 3H, CH_3, major), 1.07 (s, 9H, $C(CH_3)_3$, major), 0.96 (s, 9H, $C(CH_3)_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.3 (CO, minor), 172.2 (CO, major), 163.0 (CHO, minor), 161.3 (CHO, major), 74.5 ($C2_{thiazolidine}$, major), 72.0 ($C4_{thiazolidine}$, major), 70.2 ($C2_{thiazolidine}$ or $C4_{thiazolidine}$, minor), 70.0 ($C2_{thiazolidine}$ or $C4_{thiazolidine}$, minor), 53.4 ($CO_2\underline{CH_3}$, minor), 52.9 ($CO_2\underline{CH_3}$, major), 42.3 ($C5_{thiazolidine}$, minor), 41.7 ($C5_{thiazolidine}$, major), 40.4 ($\underline{C}(CH_3)_3$, minor), 39.6 ($\underline{C}(CH_3)_3$, major), 28.3 (CH_3, minor), 27.3 ($C(\underline{CH_3})_3$, minor), 26.8 ($C(\underline{CH_3})_3$, major), 20.8 (CH_3, major). UPLC–MS t_R = 3.9 min, *m/z*: calcd for $[M+H]^+$ $C_{11}H_{20}NO_3S^+$: 246.1, found: 246.1.

(*R*)-2-Methylcysteine (*d*)[8]



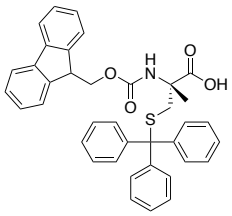
A suspension of (*2R*,*4R*)-methyl 2-(*tert*-butyl)-3-formyl-4-methylthiazolidine-4-carboxylate (944 mg, 3.85 mmol) in 5 M HCl (40 mL) was heated to reflux under a N_2 atmosphere for 3 d. At this time, the reaction mixture had become a pale yellow solution. The reaction mixture was cooled to rt and washed with EtOAc (3 × 20 mL). The aqueous layer was concentrated *in vacuo* to give the hydrochloride salt of the target compound as an off-white solid. The solid was used without further purification. UPLC–MS t_R = 0.4 min, *m*/*z*: calcd for $[M+H]^+$ $C_4H_{10}NO_2S^+$: 136.2, found: 136.1.

(*R*)-S-Trityl-2-methyl cysteine (*e*)



(*R*)-2-methylcysteine was suspended in TFA (40 mL). Triphenylmethanol (1.20 g, 4.61 mmol) was then added. An orange color emerged immediately. The reaction mixture was stirred at rt for 6 h and was then concentrated *in vacuo*. At 0 °C, 3 M NaOH was added under stirring until pH was above 7. The pH was then adjusted to 0-2 by 2 M HCl. A saturated solution of NaOAc was then added. The resulting suspension was filtered. The solid was washed with 1:1 H_2O–saturated NaOAc and then dissolved in MeOH. The solution was concentrated *in vacuo* to leave the target compound as a colorless solid. The solid was used without further purification. UPLC–MS t_R = 3.5 min, *m*/*z*: calcd for $[M+H]^+$ $C_{23}H_{24}NO_2S^+$: 378.2, found: 378.1.

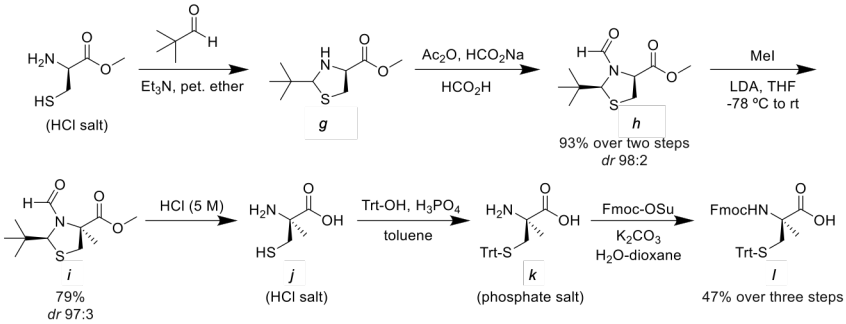
(*R*)-N-Fmoc-S-Trityl-2-methylcysteine (*f*)[6,7]



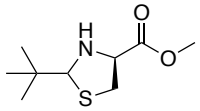
(*R*)-S-trityl-2-methyl cysteine was combined with H_2O-CH_3CN (75 mL, 1:4). $NaHCO_3$ (969 mg, 11.5 mmol) and Fmoc *N*-hydroxysuccinimide ester (1.56 g, 4.62 mmol) were then added. The suspension was stirred vigorously o/n at rt. The reaction mixture was then combined with H_2O-brine (60 mL, 1:1) and extracted with Et_2O (3 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and then concentrated *in vacuo* to give an off-white solid which was purified by flash chromatography (20–100% EtOAc in petroleum ether, then 0.05:100 AcOH–EtOAc to give the product as a colorless solid (991 mg, 1.65 mmol, 43%). TLC (8:92:1 MeOH-CH_2Cl_2-AcOH) R_f = 0.4. $[\alpha]^{21.9}_D$ = +33.6° (*c* 0.25, CH_3OH). ^{1}H NMR (600 MHz, CD_3OD) δ 7.80 (d, *J* = 7.6 Hz, 2H, $H4_{Fmoc}$, $H5_{Fmoc}$), 7.72 (d, *J* = 6.7 Hz, 2H, $H1_{Fmoc}$, $H8_{Fmoc}$), 7.40 – 7.33 (m, 8H, $H3_{Fmoc}$ [1H], $H6_{Fmoc}$ [1H] and H_{Trt} [6H]), 7.27 – 7.22 (m, 8H, $H2_{Fmoc}$ [1H], $H7_{Fmoc}$ [1H] and H_{Trt} [6H]), 7.18 (app. t, 3H, H_{Trt}), 4.35 – 4.25 (m, 3H, $H9_{Fmoc}$ [1H] and $CH_{2,Fmoc}$ [2H]), 3.12 (d, *J* = 11.7 Hz, 1H, SCH_2), 2.60 (d, *J* = 11.7 Hz, 1H, SCH_2), 1.32 (s, 3H, CH_3). ^{13}C NMR (151 MHz, CD_3OD) δ 176.8 (COOH), 157.0 (CO_{Fmoc}), 146.2 (C_{Trt}), 145.2 ($C8a_{Fmoc}$ and $C9a_{Fmoc}$), 142.5 ($C4a_{Fmoc}$ and $C4b_{Fmoc}$), 130.8 (C_{Trt}), 128.83 ($C3_{Fmoc}$ and $C6_{Fmoc}$), 128.76 ($C2_{Fmoc}$ and $C7_{Fmoc}$), 128.2 (C_{Trt}), 127.7 (C_{Trt}), 126.4 ($C1_{Fmoc}$ and $C8_{Fmoc}$), 120.9 ($C4_{Fmoc}$ and $C5_{Fmoc}$), 67.9 ($CH_{2,Fmoc}$), 67.2 ($S\underline{C}(Trt)_3$), 59.7 (C_α), 38.8 (CH_2S), 23.7 (CH_3). UPLC–MS t_R = 5.6 min, *m*/*z*: calcd for $[M+Na]^+$ $C_{38}H_{33}NO_4SNa^+$: 622.2, found: 622.2.

2.6. Synthesis of (*S*)-N-Fmoc-S-Trityl-2-methylcysteine

The synthesis of the Fmoc-αMe-D-Cys(Trt)-OH building block was adapted from reported procedures.[5-8]



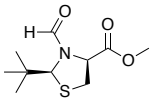
(*4S*)-Methyl 2-(*tert*-butyl)thiazolidine-4-carboxylate (*g*)[5]



A suspension of D-cysteine methyl ester hydrochloride (3.43 g, 20.0 mmol), trimethylacetaldehyde (3.90 mL, 35.5 mmol), triethylamine (4.20 mL, 30.1 mmol) and petroleum ether (40 mL) was heated to reflux with continuous removal of water via a Dean-Stark condenser for 4 h. The reaction mixture was then cooled to rt and filtered. The solid residue was washed with Et_2O. The combined filtrates were concentrated *in vacuo* to give the product as a pale yellow oil (4.06 g, 20.0 mmol, >99%). A 2:1 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. The mixture was was used without further purification. TLC (10:90 EtOAc–petroleum ether) R_f = 0.2 (two spots). Mixture of diastereomers (2:1): ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.53 (s, 1H, $H2_{thiazolidine}$, minor), 4.46 (s, 1H, $H2_{thiazolidine}$, major), 4.14 (t, *J* = 6.0 Hz, 1H, $H4_{thiazolidine}$, minor), 3.81 (dd, *J* = 9.8, 6.8 Hz, 1H, $H5_{thiazolidine}$, major), 3.78 (s, 3H, CO_2CH_3, major), 3.76 (s, 3H, CO_2CH_3, minor), 3.26 (dd, *J* = 10.2, 6.7 Hz, 1H, $H5_{thiazolidine}$, major), 3.12 (dd, J = 10.6, 6.4 Hz, 1H, $H5_{thiazolidine}$, minor), 3.03 (dd, J = 10.6, 5.6 Hz, 1H, $H5_{thiazolidine}$, minor), 2.68 (t, *J* = 10.0 Hz, 1H,

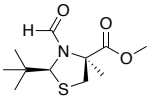
H4$_{thiazolidine}$, major), 2.31 (br s, 1H, NH, single diastereomeric signal), 1.07 (s, 9H, CH(C<u>H$_3$</u>)$_3$, major), 0.98 (s, 9H, CH(C<u>H$_3$</u>)$_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.6 (CO, minor), 172.0 (CO, major), 82.0 (C2$_{thiazolidine}$, major), 80.0 (C2$_{thiazolidine}$, minor), 65.6 (C4$_{thiazolidine}$, major), 65.2 (C4$_{thiazolidine}$, minor), 52.6 (CO_2CH_3, single conformer signal), 37.6 (C5$_{thiazolidine}$, major), 37.2 (C5$_{thiazolidine}$, minor), 34.2 (<u>C</u>(CH$_3$)$_3$, major), 29.8 (<u>C</u>(CH$_3$)$_3$, minor),27.1 (C(<u>CH$_3$</u>)$_3$, major), 26.7 (C(<u>CH$_3$</u>)$_3$, minor). UPLC–MS t_R = 3.4 min, *m*/*z*: calcd for $[M+H]^+$ $C_9H_{18}NO_2S^+$ 204.3, found: 204.3.

(*2S,4S*)-Methyl 2-(*tert*-butyl)-3-formylthiazolidine-4-carboxylat (*h*)[5]



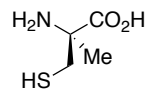
Acetic anhydride (6.0 mL, 63.5 mmol) was added dropwise over 1 h to a solution of ***g*** (4.06 g, 20.0 mmol) and sodium formate (1.63 g, 24.0 mmol) in formic acid (40 mL) at 0 °C. The reaction mixture was stirred for 18 h at rt and then concentrated *in vacuo*. The residue was taken up in saturated $NaHCO_3$ (50 mL) and Et_2O (50 mL). The aqueous layer was extracted with Et_2O (2 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give the product as a pale yellow oil which solidifies upon standing to give a colourless solid (4.28 g, 18.5 mmol, 93%). The solid was used without further purification. A 98:2 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. TLC (40:60 EtOAc–petroleum ether) R_f = 0.4. Mixture of conformers (7:1): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.33 (s, 1H, CHO, major), 8.26 (s, 1H, CHO, minor), 5.24 (s, 1H, H2$_{thiazolidine}$, minor), 4.86 (t, *J* = 8.7 Hz, 1H, H4$_{thiazolidine}$, major), 4.78 (dd, *J* = 8.8, 7.2 Hz, 1H, H4$_{thiazolidine}$, minor), 4.73 (s, 1H, H2$_{thiazolidine}$, major), 3.79 (s, 3H, CO_2CH_3, minor), 3.75 (s, 3H, CO_2CH_3, major), 3.45 (q, *J* = 7.0 Hz, 1H, H5$_{thiazolidine}$, minor), 3.41 (dd, *J* = 11.9, 7.2 Hz, 1H, H5$_{thiazolidine}$, minor), 3.33 – 3.21 (m, 2H, H5$_{thiazolidine}$, major), 1.01 (s, 9H, C(<u>CH$_3$</u>)$_3$, major), 0.94 (s, 9H, C(<u>CH$_3$</u>)$_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.7 (CO, minor), 170.1 (CO, major), 164.0 (CHO, minor), 162.7 (CHO, major), 75.2 (C2$_{thiazolidine}$, major), 71.5 (C2$_{thiazolidine}$, minor), 63.8 (C4$_{thiazolidine}$, minor), 61.5 (C4$_{thiazolidine}$, major), 53.1 (CO_2<u>CH$_3$</u>, minor), 52.7 (CO_2<u>CH$_3$</u>, major), 39.0 (<u>C</u>(CH$_3$)$_3$, minor), 38.6 (<u>C</u>(CH$_3$)$_3$, major), 32.9 (C5$_{thiazolidine}$, major), 32.1 (C5$_{thiazolidine}$, minor), 26.8 (<u>C</u>(CH$_3$)$_3$, minor), 26.4 (<u>C</u>(CH$_3$)$_3$, major). UPLC–MS t_R = 3.6 min, *m*/*z*: calcd for $[M+H]^+$ $C_{10}H_{18}NO_3S^+$: 232.1, found: 232.1.

(*2S,4S*)-Methyl 2-(*tert*-butyl)-3-formyl-4-methylthiazolidine-4-carboxylate (*i*)[5]



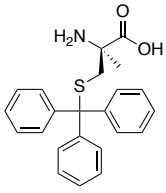
Compound ***h*** (3.96 g, 17.1 mmol) was placed in a dried round-bottomed flask and subjected to three cycles of vacuum/N_2. Anhydrous THF (68 mL) was added by syringe. The solution was cooled to –78 °C. Methyl iodide (1.30 mL, 20.9 mmol) was added by syringe. Lithium diisopropylamide (1.76 M in THF/*n*-heptane/ethylbenzene, 11.0 mL) was added dropwise. The reaction mixture was stirred at –78 °C under N_2 for 2 h and then allowed to reach rt. The reaction mixture was stirred for an additional 3 h and then concentrated *in vacuo* to give an orange oil which was partitioned between brine (50 mL) and EtOAc (50 mL). The aqueous layer was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over anhydrous $MgSO_4$ and concentrated in *vacuo* to give an orange oil which was purified by flash chromatography (0–40% EtOAc in petroleum ether) to give a pale yellow oil which solidifies upon standing to give an off-white solid (3.31 g, 13.48 mmol, 79%). A 97:3 diastereomeric mixture was obtained as determined by ^{1}H NMR spectroscopy. TLC (40:60 EtOAc–petroleum ether) R_f = 0.5. Mixture of conformers (2.6:1): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.40 (s, 1H, CHO, minor), 8.28 (s, 1H, CHO, major), 5.30 (s, 1H, H2$_{thiazolidine}$, minor), 4.66 (s, 1H, H2$_{thiazolidine}$, major), 3.82 (s, 3H, CO_2CH_3, minor), 3.77 (s, 3H, CO_2CH_3, major), 3.64 (d, *J* = 12.4 Hz, 1H, H5$_{thiazolidine}$, minor), 3.32 (dd, *J* = 11.6, 1.0 Hz, 1H, H5$_{thiazolidine}$, major), 2.86 (d, *J* = 12.3 Hz, 1H, H5$_{thiazolidine}$, minor), 2.72 (dd, *J* = 11.5, 0.9 Hz, 1H, H5$_{thiazolidine}$, major), 1.78 (s, 3H, CH_3, minor), 1.76 (s, 3H, CH_3, major), 1.07 (s, 9H, C(CH$_3$)$_3$, major), 0.96 (s, 9H, C(CH$_3$)$_3$, minor). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.3 (CO, minor), 172.2 (CO, major), 163.0 (CHO, minor), 161.3 (CHO, major), 74.5 (C2$_{thiazolidine}$, major), 72.0 (C4$_{thiazolidine}$, major), 70.2 (C2$_{thiazolidine}$ or C4$_{thiazolidine}$, minor), 70.0 (C2$_{thiazolidine}$ or C4$_{thiazolidine}$, minor), 53.4 (CO_2<u>CH$_3$</u>, minor),52.9 (CO_2<u>CH$_3$</u>, major), 42.3 (C5$_{thiazolidine}$, minor), 41.7 (C5$_{thiazolidine}$, major), 40.4 (<u>C</u>(CH$_3$)$_3$, minor), 39.7 (<u>C</u>(CH$_3$)$_3$, major), 28.3 (CH_3, minor), 27.2 (C(<u>CH$_3$</u>)$_3$, minor), 26.8 (C(<u>CH$_3$</u>)$_3$, major), 20.8 (CH_3, major). UPLC–MS t_R = 3.9 min, *m*/*z*: calcd for $[M+H]^+$ $C_{11}H_{20}NO_3S^+$: 246.1, found: 246.1.

(*S*)-2-Methyl cysteine (*j*)



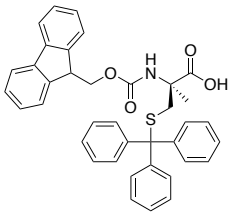
A suspension of compound ***i*** (503 mg, 2.05 mmol) in 5 M HCl (8 mL) was heated to reflux under N_2 atmosphere for 2 d. At this time, the reaction mixture had become a yellow solution. The reaction mixture was cooled to rt, diluted with H_2O (25 mL) and washed with EtOAc (3 × 10 mL). The yellow aqueous layer was concentrated *in vacuo* to give the hydrochloride salt of the target compound as a brown viscous oil. The oil was used without further purification. UPLC–MS t_R = 0.4 min, *m*/*z*: calcd for $[M+H]^+$ $C_4H_{10}NO_2S^+$: 136.2, found: 136.1.

(*S*)-S-Trityl-2-methyl cysteine (*k*)



(*S*)-2-Methyl cysteine was combined with toluene (20 mL), H_3PO_4 (85%, 0.8 mL) and triphenylmethanol (648 mg, 2.49 mmol). The biphasic mixture was heated to reflux under vigorous stirring. Additional H_3PO_4 (85%, 0.2 mL) and triphenylmethanol (160 mg, 0.61 mmol) was added after 2 h. The upper layer had obtained a faint yellow color after 1 d. At this time the reaction mixture was concentrated *in vacuo* to give a solid, yellow residue. This residue was used without further purification. UPLC–MS t_R = 3.5 min, *m*/*z*: calcd for $[M+H]^+$ $C_{23}H_{24}NO_2S^+$: 378.2, found: 378.1.

(*S*)-N-Fmoc-S-Trityl-2-methylcysteine (*l*)[6,7]



(*S*)-S-trityl-2-methyl cysteine was suspended in H_2O–dioxane (20 mL, 1:1). Potassium carbonate (2.84 g, 20.5 mmol) was added in portions. The pH of the reaction mixture was 8–9. Fmoc *N*-hydroxysuccinimide ester (830 mg, 2.46 mmol) was added. The off-white suspension was stirred at rt for 1 d and then diluted with H_2O (40 mL). This mixture was extracted with Et_2O (2 × 30 mL). The combined organic layers were concentrated *in vacuo*. The crude was purified by flash chromatography (0–100% EtOAc in petroleum ether, then 0.1:100 AcOH–EtOAc) to give the desired product as a colorless oil which solidified to form a colorless solid (579 mg, 0.97 mmol, 47% over three steps). TLC (8:92:1 MeOH-CH_2Cl_2-AcOH) R_f = 0.4. $[\alpha]^{rt}_D$ = –24.9° (*c* 0.15, MeOH). ^{1}H NMR (600 MHz, CD_3OD) δ 7.79 (d, *J* = 7.6 Hz, 2H, $H4_{Fmoc}$, $H5_{Fmoc}$), 7.71 (d, *J* = 7.5 Hz, 2H, $H1_{Fmoc}$, $H8_{Fmoc}$), 7.40 – 7.33 (m, 8H, $H3_{Fmoc}$ [1H], $H6_{Fmoc}$ [1H] and H_{Trt} [6H]), 7.26 – 7.20 (m, 8H, $H2_{Fmoc}$ [1H], $H7_{Fmoc}$ [1H] and H_{Trt} [6H]), 7.17 (app. T, 3H, H_{Trt}),4.32 – 4.25 (m, 3H, $H9_{Fmoc}$ [1H] and $CH_{2,Fmoc}$ [2H]), 3.04 (d, *J* = 11.5 Hz, 1H, SCH_2), 2.67 (d, *J* = 11.3 Hz, 1H, SCH_2), 1.34 (s, 3H, CH_3). ^{13}C NMR (151 MHz, CD_3OD) δ 177.7 (COOH), 156.8 (CO_{Fmoc}), 146.3 (C_{Trt}), 145.3 ($C8a_{Fmoc}$ and $C9a_{Fmoc}$), 142.5 ($C4a_{Fmoc}$ and $C4b_{Fmoc}$), 130.8 (C_{Trt}), 128.8 ($C3_{Fmoc}$ and $C6_{Fmoc}$), 128.7 ($C2_{Fmoc}$ and $C7_{Fmoc}$), 128.2 (C_{Trt}), 127.7 (C_{Trt}), 126.5 ($C1_{Fmoc}$ and $C8_{Fmoc}$), 120.9 ($C4_{Fmoc}$ and $C5_{Fmoc}$), 67.9 ($CH_{2,Fmoc}$), 67.0 ($S\underline{C}(Trt)_3$), 60.0 (C_α), 39.1 (CH_2S), 24.0 (CH_3). UPLC–MS t_R = 5.6 min, *m/z*: calcd for $[M+Na]^+$ $C_{38}H_{33}NO_4SNa^+$: 622.2, found: 622.2.

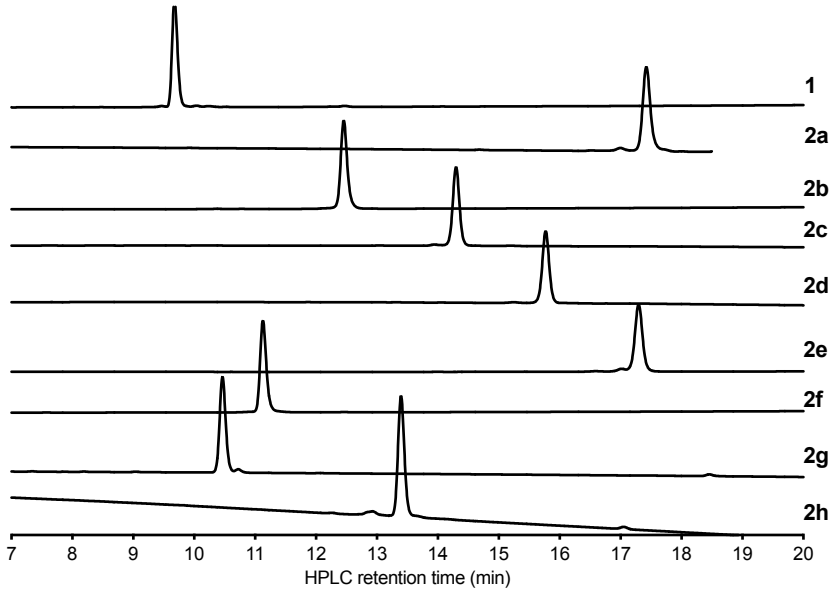
2.7. Analytical methods

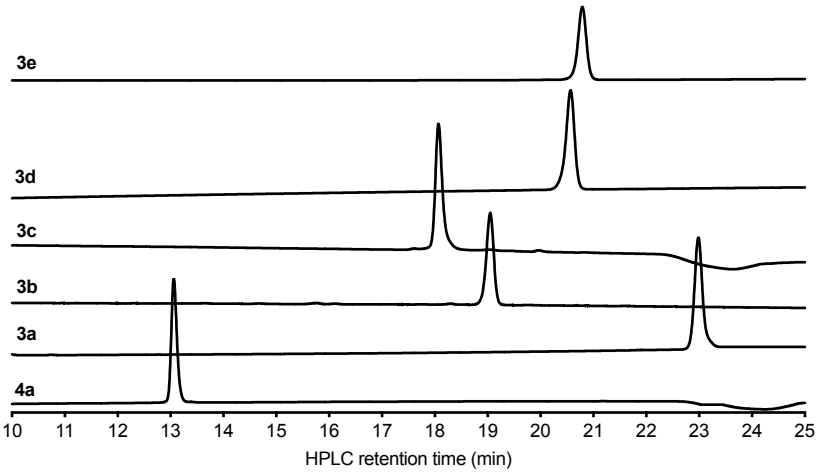
Analytical HPLC was performed on an Agilent system, using a Phenomenex Luna C18 5 µm (250 x 4.60 mm) column eluting at a flow rate of 1 mL/min and a gradient of 30 to 100 % buffer B (90% CH_3CN/10% H_2O/0.1% TFA) in buffer A (0.1% TFA in water) over 28 minutes (Figure S8). UHPLC-MS was performed on a Shimadzu Nexre using an Agilent Zorbax R-ODS III column 2.0 mm i.d x 75 mm 1.6 mm). ESI-MS was carried out on an Applied Biosystems QSTAR Elite time-of-flight mass spectrometer. Found masses of the purified compounds are listed in Table S1.

Table S1. Characterization of the peptides by mass spectrometry.

Acetylated-peptides	Expected mass	Found deconvoluted mass in ESI-MS
1	1536.71	1536.70
2a	1595.77	1595.78
2b	1553.74	1553.72
2c	1636.90	1636.70
2d	1680.81	1680.90
2e	1708.87	1708.90
2g	1578.75	1578.81
2h	1603.84	1603.82
3a	1578.83	1578.80
3a	1590.89	1590.87
3b	1614.94	1614.89
3c	1614.94	1614.90
3d	1643.00	1643.00
3e	1643.00	1643.00
4a	1704.91	1704.93
4b	1682.97	1682.96
4c	1682.97	1682.92
4d	1711.03	1711.19
4e	1711.03	1711.08
5a	1798.84	1798.75
5b	1826.89	1826.80
5c	1826.89	1826.80
5d	1826.89	1826.79
5e	1854.95	1855.03

FITC- peptides	Expected mass	Found deconvoluted mass in ESI-MS
1-F	1924.0	1923.2
2a-F	1995.9	1995.9
2b-F	1972.2	1971.7
2c-F	2055.3	2054.8
2d-F	2099.2	2098.6
2e-F	2127.3	2126.9
2f-F	2014.2	2013.6
2g-F	1997.2	1996.6
2h-F	2022.3	2021.7
3a-F	2009.3	2009.2
3b-F	2033.4	2033.4
3c-F	2033.4	2032.8
3d-F	2061.4	2061.0
3e-F	2061.4	2061.1
4a-F	2123.3	2123.3
4b-F	2101.4	2101.0
4c-F	2101.4	2101.3
4d-F	2129.4	2128.8
4e-F	2129.4	2129.1
5a-F	2217.3	2216.6
5b-F	2245.3	2244.7
5c-F	2245.3	2244.6
5d-F	2245.3	2244.6
5e-F	2273.4	2273.0





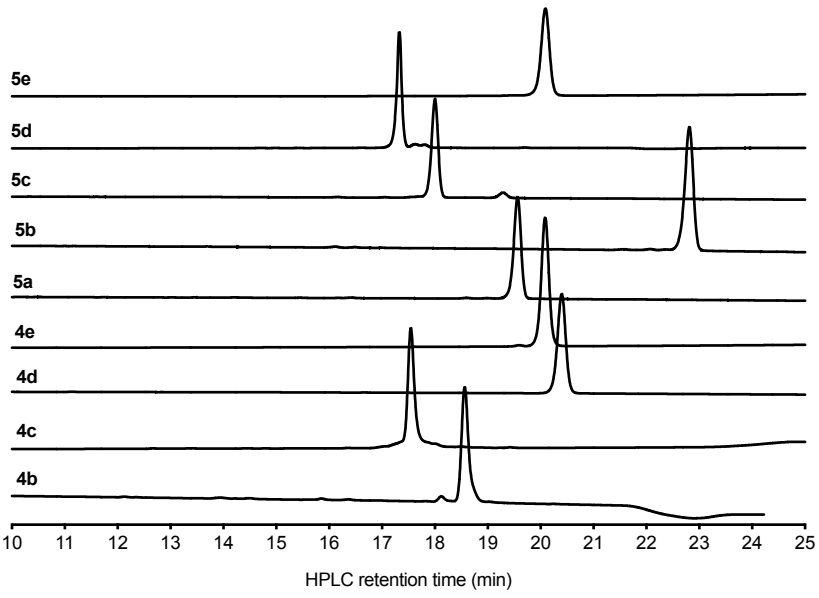


Figure S8. HPLC analysis of the N-acetylated peptides.

2.8. Biophysical methods

Circular Dichroism Spectroscopy

Peptide stock solutions were diluted in 10 mM phosphate buffer pH 7.4 (for acetylated peptides) or 20 mM SDS in 10 mM sodium phosphate, 100 mM sodium fluoride buffer pH 7.4 (for FITC-labeled peptides) to a final concentration of 50 or 20 µM. CD measurements were performed using a Jasco model J-710 spectropolarimeter which was routinely calibrated with (1*S*)-(+)-10-camphorsulfonic acid. Spectra were recorded at room temperature (298K), with a 0.1 cm Jasco quartz cell over the wavelength range 260-185 nm at 50 nm/min, with a bandwidth of 1.0 nm, response time of 1 s, resolution step width of 1 nm and sensitivity of 20-50 Mdeg. Each spectrum represents the average of 5 scans. Spectra were analyzed using the Spectra Analysis software and smoothed using 'adaptive smoothing' function. Percentage helicity of peptides was calculated from the residue-molar ellipticity found at 220 nm using reported procedures.[9]

Protease stability assay

Fresh protease solutions were prepared as: chymotrypsin (from bovine pancreas, 40 U/mg, Sigma) 0.5 mg/mL in 0.1 M Tris, 10 mM $CaCl_2$ buffer pH 8.0; pepsin (from porcine gastric mucosa, 3200-4500 U/mg, Sigma-Aldrich) 0.5 mg/mL in water; and proteinase K (fungal, > 40 U/mg, Invitrogen) 0.5 mg/mL in 0.1 M Tris, 10 mM $CaCl_2$ buffer pH 8.0. A 10 µM solution of each peptide (1 mL) in 0.1 M Tris, 10 mM $CaCl_2$ pH 8.0 buffer (for chymotrypsin and proteinase K digestions) or 10 mM HCl pH 2 (for pepsin digestion) was placed inside a HPLC glass vial. A 3 µL aliquot of this solution was analyzed by ESI-MS and the intensity of the $[M+2H]^{2+}$ peptide ion peak was quantified using Analyst software. After that, 1 µL protease solution was added (to a final protease concentration of 0.0005 mg/mL, or approximate ratio of (protease:peptide) of (1:30, mg/mg)) and the resulting solution was analyzed by ESI-MS after 20, 60 and 120 min incubation at 22^0C. The intensity of the $[M+2H]^{2+}$ peptide ion peak was used to quantify the ratio of peptide proteolytic degradation over the allocated time. The measurements were performed in duplicates. The site of proteolytic cleavage was assessed by MS analysis of the digested peptide fragments.

Fluorescence polarization assays

The K_d value for the interaction between fluorophore-labelled compound **1** (**1-F**) to hDM2 was determined by direct binding of **1-F** to hDM2. For this, **1-F** (10 nM) was incubated with different concentrations of recombinant human hDM2 (purchased from Abcam) (two-fold dilutions ranging from 1 to 1000 nM) in elution buffer (50 mM Tris, 150 mM NaCl, 1mM TCEP, 1mM EDTA, 0.01% pluronic F-127 buffer pH 7.4). Fluorescence anisotropy was measured after 1, 2 and 3 h incubation and K_d values for hDM2 binding to **1-F** were determined by nonlinear regression analysis using Prism software 7.0 (GraphPad). The experiment was repeated three times. The measured K_d value for **1-F** binding to hDM2 was 14.0 $\pm$ 0.4 nM.

The EC_{80} value calculated from the direct binding assay was then used to determine the hDM2 concentration for application in the competition assays. A 75 nM solution of recombinant human hDM2 was preincubated for 1h with a serial dilution of each peptide (ranging from 150 µM to 1.5 nM) in 50 mM Tris, 150 mM NaCl, 1mM TCEP, 1mM EDTA, 0.01% pluronic F-127 buffer pH 7.4. The peptide/hDM2 solution was then place into a 384-well FP plate and combined to a solution of **1-F** to a final concentration of 25 nM for hDM2 and 10 nM for **1-F**. After 1h incubation, fluorescence anisotropy was measured and IC_{50} values (Figure S1) were determined by nonlinear regression analysis using Prism software 7.0. The experiment was repeated at least three times (means + SEM). K_i values were then calculated from the observed competitive IC_{50} mean values, known K_d of **1-F**/hDM2 interaction and known concentrations of labelled peptide and protein using a formula described in http://www.umich.edu/~shaomengwanglab/software/calc_ki/index.html

2.9. Computational methods

clogP

clogP values were calculated using ChemDraw software version 20.0.

AlogP

AlogP values were calculated using online software AlogOS 2.1 (http://www.vcclab.org/lab/alogps/).

Total hydrophobic surface area (tHSA) calculations

Hydrophobic surface areas for all compounds were computed and characterised by Protein Surface analyser module in Biologics/Schrödinger program version 2020-2. The OPLS3e force field was used for the calculation protocol. All starting structures were prepared and passed through Protein Preparation Wizard function and energy minimisation prior for their surface calculations. The default parameters of 15 Å and 15 Å were used for negative and positive sizes cut-off. The hydrophobic cuoff was set to 50 Å. The potential energy for the negative, positive and hydrophobic areas were set to -0.05 eV, 0.05 eV respectively and sLogP = 0.05. The surface areas were analysed and displayed using Maestro 2020-2 and Pymol version 2.1 (for presentations).

Superimposition of peptides to hDM2 binding site

Three dimensional structures for cyclic α-helical peptides were build and modelled to α-helices using Protein Preparation/Build Biopolymer module in Schrödinger program version 2020-2. All structures were then energy minimized using the OPLS3e force filed. Peptides (**2a, 2b, 2c, 2e, 2f, 3a, 3c** and **4c)** were backbone superimposition to PDI peptide on hDM2 protein binding site (PDB 3G03) and displayed using Pymol (version 2.1) software.

2.10. Cell-based methods

Cell culture

Cell culture reagents were purchased from Invitrogen. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/mL of penicillin, 10 µg/mL streptomycin and 1x GlutaMax at 37°C in a 5 % CO_2 incubator

Flow cytometry

HeLa cells were plated into 12-well plates at a density of 3 x 10^5/well and incubated overnight. On the day of experiment, cells were washed with phosphate-buffered saline (PBS) then treated with 5 µM of peptide diluted in serum-free DMEM for 1 h at 37°C. After incubation, cells were washed twice with PBS and dissociated with TrypLE Express. Cells were collected by centrifugation at 500 g for 5 min at 4°C. Pelleted cells were then resuspended in ice-cold PBS supplemented with 1 % FBS and trypan blue solution (Sigma-Aldrich) prior to flow cytometric analysis. Median fluorescence intensity was measured using a CytoFLEX Flow Cytometer (Beckman Coulter) and analysed using CytExpert Software (Beckman Coulter). Cellular uptake of the peptides were then normalised relative to 5 µM TAT and expressed as percentage uptake.

LDH release assay

Hela cells were plated at a density of 4 x 10^4 /mL and allowed to adhere overnight. Peptides were incubated with cells at indicated concentrations in serum-free DMEM for 1 h at 37°C. LDH release from cells were measured using CytoTox Non-Radioactive Cytotoxic Assay (Promega) according to manufacturer's instruction. Absorbance was measured using a Pherastar FS (BMG Labtech) at 490 nm. Values are expressed as mean ± sem of at least three independent experiments. Data were normalized relative to Triton X-100 (Sigma-Aldrich) and analyzed using GraphPad Prism 9 for Mac OSX.

Confocal microscopy

Hela cells were seeded overnight into 35 mm glass bottom dish (MatTek Corp.) at a density of 1 x 10^5 /mL in complete DMEM. On the day of the experiment, medium was replaced with serum free DMEM containing peptides at 5 µM and incubated for 3 h at 37°C. Nuclei were counterstained with Hoechst for 10 min at 1 µg/mL then washed thrice with PBS. Serum-free DMEM was added and cells were placed into a humidified, temperature and atmosphere controlled microscope stage at 37°C with CO_2. Imaging was performed with confocal microscope (Zeiss LSM 880 Fast Airyscan, X63 oil objective), serial Z-sections were acquired with 0.31 µm intervals. Acquired images were adjusted using ImageJ software (NIH).

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