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Tuning the binding affinity and selectivity of perfluoroaryl-stapled peptides by cysteine-editing

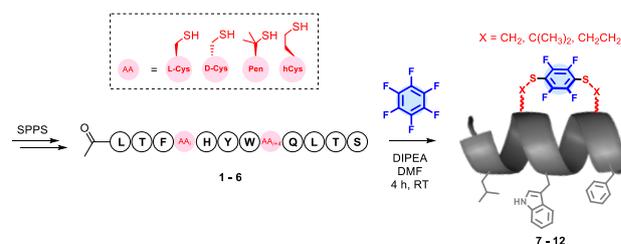
Sanne J.M. Verhoorck,^[a] Claire E. Jennings,^[b] Neshat Rozatian,^[c] Judith Reeks,^[b] Jieman Meng,^[b] Emily K. Corlett,^[c] Fazila Bunglawala,^[a] Martin E.M. Noble,^[b] Andrew G. Leach,^[a] and Christopher R. Coxon*^[a]

Abstract: A growing number of approaches to 'staple' α -helical peptides into a bioactive conformation using cysteine cross-linking are emerging. Here we explore the replacement of L-cysteine with 'cysteine analogues' in combinations of different stereochemistry, side chain length and beta-carbon substitution, to examine the influence that the thiol-containing residue(s) has on target protein-binding affinity in a well explored model system, p53-MDM2/MDMX. In some cases, replacement of one or more L-cysteine residues afforded significant changes in the measured binding affinity and target selectivity of the peptide. Computationally constructed homology models indicate that some modifications, such as incorporating two D-cysteines favourably alter the positions of key functional amino acid side chains, which is likely to cause changes in binding affinity, in agreement with measured SPR data.

Linear, unstructured peptide sequences often suffer from low proteolytic stability when excised from their parent proteins, limiting their development as potential therapeutics. Stapled α -helical peptides (SAHs) are a highly-promising class of therapeutic agent, which are designed to mimic an α -helical motif of a protein, and have superior proteolytic stability *in vivo* over the equivalent unconstrained peptide.^[1] The most common method of peptide stapling employs the use of the all-hydrocarbon (alkene) linker developed by Grubbs and Blackwell,^[2-4] and pioneered by the Verdine Group. This strategy is used to stabilise a peptide α -helix and can often deliver impressive biological activity through steric constraint of a bio-active conformation.^[5] Using the alkene metathesis approach requires the incorporation of α,α' -disubstituted alkene-containing amino acids into the peptide sequence. Typically, these building blocks are either purchased at significant expense or can be obtained by multistep synthesis using, for example, nucleophilic glycine equivalents.^[6] The standard all-hydrocarbon stapling approach typically incorporates a combination of *R,S*- or *S,R*- α,α' -disubstituted alkenyl amino acids with optimised chain length.^[7,8] It is well known that linker length, linker orientation and linker type of the stapled peptides

can affect the binding properties.^[9, 10] Typically, the rationale for inclusion of an additional α -methyl group was to overcome the perceived destabilising effect upon helical conformation by introducing D-amino acids.^[7] However, mono-substituted α -alkenyl amino acids have been shown to be similarly effective.^[11] The importance of stereochemical effects on the helical character and, thus, biological activity have been clearly demonstrated in the alkene metathesis *i,i+4* peptide stapling approach.^[12]

The recent literature has shown a significant surge in interest in the two-component chemoselective cross-linking of peptides *via* e.g., 'double-click' CuAAC chemistry.^[13-15] Most often, however, cysteine thiol residues have provided an excellent handle for peptide stapling, driven mainly by the ease and relatively-low cost of obtaining the linear pre-stapled peptide. This topic has been recently reviewed by Fairlie.^[16] Example thiol cross-linkers include, the use of dibromomaleimide,^[17] dichloroacetone,^[18] 1-,4-dichlorotetrazine,^[19] 1,2,4,5-tetrabromodurene,^[20] α, α' -dibromo-*m*-xylene,^[21] *trans*-1,4-dibromo-2-butene and *cis*-1,4-dichloro-2-butene^[22] and perfluoroaryl reagents.^[23] Whilst significant attention has focussed on the nature of the crosslinking electrophile, comparatively little, if any attention has focused on the cysteines, with the single exception of introducing homocysteine.^[17] The distance between the cysteine residues has been explored and optimised, albeit in non-helical systems,^[24] yet the stereochemistry of cysteine has not been taken into account, in terms of the consequences on biological activity and the position of key amino acid side chain residues. By drawing analogy with the traditional all-hydrocarbon approach, we investigated the replacement of L-cysteine (cysteine-editing) with selected combinations of D-cysteine, homocysteine and penicillamine to examine the effect of i) stereochemistry, ii) cysteine homologation, and iii) beta-carbon substitution. We considered that the outcomes will be directly important to the cysteine-stapling work of other groups as highlighted above.



Scheme 1. Synthesis of perfluoroaryl-stapled peptides.

The p53-MDM2 and p53-MDMX protein-protein interactions were selected as a model system in which to study cysteine-editing,

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due to its well characterised interaction and the availability of published known stapled α -helical peptide inhibitors.^[13, 14, 25-27] The p53 tumour suppressor is a major regulator of the cell cycle and is activated in response to genotoxic stress resulting from oncogenic signalling and exposure to, for example, ionising radiation and carcinogenic agents.^[28] Through its role as a transcription factor, p53 induces cell cycle arrest and apoptosis in afflicted cells,^[29] fulfilling a critical role in the maintenance of healthy functioning of cells and the avoidance of malignancy. A 12 amino acid peptide LTFEHYWAQLTS (PDI peptide) identified by phage display^[30] was reported to disrupt the p53-MDM2 protein-protein interaction and has previously been stapled using non-hydrocarbon techniques.^[31,32] This served as a test-bed for diversification in our studies. The key features of this peptide (and indeed, the p53 protein) that promote the biological activity are the three amino acids, Phe, Trp and Leu, with the positions being important for activity. These residues were retained and other selected residues that were previously reported to be tolerant to substitution^[31] were replaced with cysteine analogues in the PDI sequence with the standard relative spacing of four (*i*, *i*+4) amino acids, corresponding to equivalent positions at neighbouring turns on the α -helix. Six different combinations were synthesised using solid phase peptide synthesis on Rink amide resin to afford the C-terminal amide of the form Ac-LTF(AA_{*i*})HYW(AA_{*i*+4})QLTS (Table 1). Stapling was performed using the cross-linking reagent hexafluorobenzene (Scheme 1) as demonstrated by Pentelute and co-workers^[23] due to an ongoing interest in related reactions in our laboratory. In each case the thiol-crosslinking occurred cleanly under relatively mild conditions (25 mM DIPEA in DMF, room temperature, <4.5 h).^[33, 34]

The binding affinity of the synthesised p53-mimicking peptides **1** and **7-12** were examined by measuring dissociation constants (K_d) for their interactions with GST-MDM2 (17-125) and GST-MDMX (22-111) constructs using surface plasmon resonance (SPR) (Table 1 and supporting information). Binding affinities were measured for the PDI peptide and the *cis*-imidazole small molecule, nutlin-3a as positive binding controls and to validate the SPR approach against a biochemical HTRF assay (see supporting information). SPR evaluation identified a number of highly potent tetrafluorobenzene-cross-linked SAHs with low-to-moderate micromolar affinities for MDM2 and MDMX as measured by K_d values. In general, the perfluoroaryl-stapled peptides had higher K_d (lower affinity) than the phage display PDI peptide; however, the additional proteolytic stability gained from this modification^[23] (see supporting information) may offset the sacrifice in affinity. In fact, introduction of L-cysteine residues into the non-stapled PDI analogue (**1**) decreased equally binding affinity for both MDM2 and MDMX compared with PDI. In any case, the primary purpose of this study was for comparison of the parent **7** (L-Cys, L-Cys) with peptides with different cysteine analogues comprising the cross-link. One particularly interesting outcome is that most of the stapled peptides (**7** (> 2-fold), **9** (> 4-fold), **10** (> 3.5-fold)) had a generally higher affinity for MDMX compared with MDM2, whereas, PDI and **1** were equipotent for each isoform, albeit with lower K_d values. In general, changing a single cysteine stereochemistry from L- to D- at *i* or *i*+4 positions was well tolerated by both MDM2 and MDMX, however, MDMX appeared generally more tolerant to 'cysteine-editing' than MDM2. Inversion of *both* L- *i* and *i*+4 α -carbon substituents to the D-configuration (**10**) significantly enhanced the affinity for both

MDMX (~7-fold) and MDM2 (~5 fold) compared with **7**. This has particular importance as peptides comprising D-amino acids are typically more resistant to proteolytic degradation than their canonical counterparts.

Most notably, branching of L-cysteine at the β -position with geminal-dimethyl groups (L-penicillamine, **12**) exhibited a significantly higher K_d than all other analogues, indicating lower affinity for both MDM2 (>16-fold vs *i*-L-Cys, *i*+4-L-Cys, **7**) and MDMX (~22-fold vs *i*-L-Cys, *i*+4-L-Cys, **7**). This may suggest that the L-Pen-containing peptide is significantly distorted from a well-defined α -helix or presents destabilising interactions. In stark contrast, homologation of the *i*, *i*+4 cross-linker through incorporation of homocysteine (**11**) appeared to be much better tolerated than L-cysteine, affording around a 7-fold lower K_d (7-fold higher affinity) for MDM2 and 3-fold lower for MDMX versus the parent **7**. This was around equipotent with the non-stapled **1**, which itself was also non-selective for either MDM2 or MDMX. Overall, the observed SARs may be related to the geometric constraints imposed by the cysteine analogue and the cross-linker, and the resulting impact upon the helicity of the peptide and the relative positions of key amino acid side chains, Phe, Trp and Leu.

Table 1. Dissociation constants (K_d) for peptides (**1**, **7-12**) binding to MDM2/MDMX attained from an SPR assay and comparison with control inhibitor nutlin 3a and PDI peptide. Note: hCys = homocysteine, Pen = penicillamine.

Peptide	AA _{<i>i</i>}	AA _{<i>i</i>+4}	Cross-linker	K_d / μ M		Chi ² (RU ²)	
				MDM2	MDMX	MDM2	MDMX
PDI	L-Glu	L-Ala	-	0.04	0.02	1.36	0.82
Nutlin 3	-	-	-	0.17	ND	0.24	ND
1	L-Cys	L-Cys	-	0.18	0.18	1.49	0.40
7	L-Cys	L-Cys	-	1.02	0.44	2.37	0.81
8	L-Cys	D-Cys	-	0.41	0.37	3.08	0.99
9	D-Cys	L-Cys	-	1.70	0.40	3.69	6.49
10	D-Cys	D-Cys	-	0.22	0.06	1.71	0.86
11	L-hCys	L-hCys	-	0.14	0.15	4.07	2.91
12	L-Pen	L-Pen	-	16.90	9.89	10	8.17

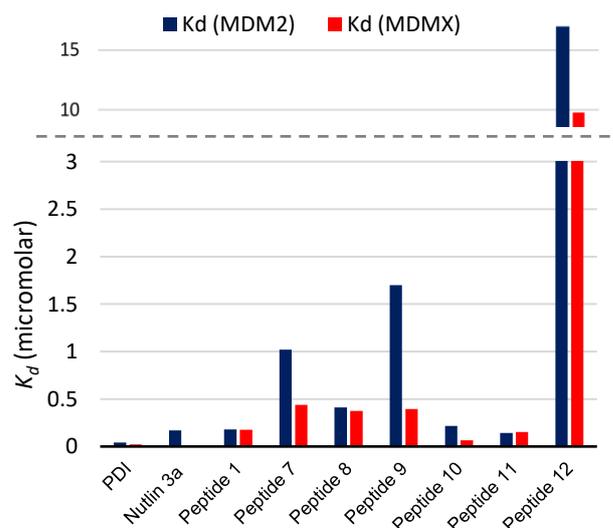


Chart 1. Comparison of binding affinities (K_d) for stapled peptides containing alternative cysteine analogues.

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In light of these observations, we employed an *in silico* modelling approach (Figure 1) to understand the structural and conformational consequences of cysteine-replacement. The apparent α -helicity of **7** was initially measured using circular dichroism but produced poor results (see supporting information) due to the absorbance of the fluoroaryl moiety at 222 nm, which was consistent with previous literature reports.^[17] Molecular dynamics simulations were performed starting from homology models for the free peptides **1** - **6**. The homology model used the sequence and chain B from the structure of human MDM2 in complex with the reported high affinity PDI peptide (PDB code: 3G03), where the Phe, Trp and Leu residues provide key points of interaction with MDM2 (Figure 1A). In order to benchmark simulations of the cysteine replacement peptides, this native ligand was also simulated in the same way as described below for the cross-linked complexes. In each simulation, the resulting geometries were assessed for their ability to place the three key binding residues in appropriate positions for MDM2 interaction. This used an analogy to pharmacophore triplets, a commonly used description in cheminformatics that uses the three-dimensional positioning of three pharmacophoric points (usually key interactions such as hydrogen bonding groups or hydrophobic groups) as a descriptor and benefits from the ease of analysis and understanding of the geometry of triangles. In this case, two triangles have been used to describe how modification of the peptide alters the positions of Phe, Trp and Leu sidechains: one triangle formed from the three C_α atoms and the second from the three C_β atoms, shown as dashed yellow lines in Figure 1A. This initial analysis reveals that the two triangles are rather similar because the C_α - C_β bonds are all pointed in approximately the same direction and this is clearly an important part of how the peptide forms tight interactions with the receptor.

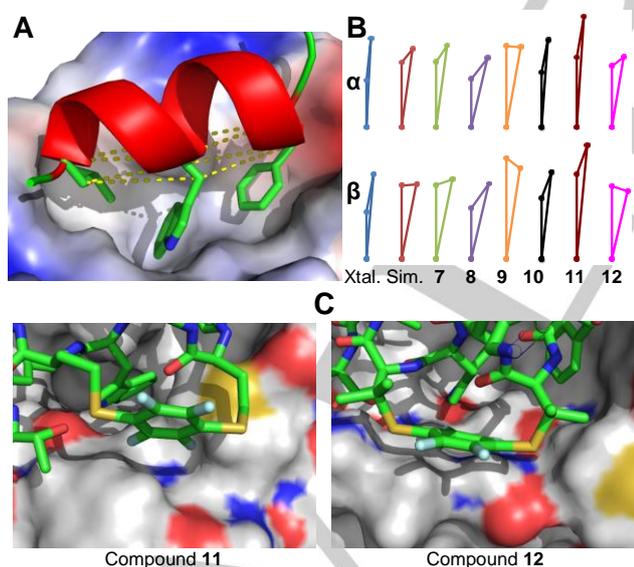


Figure 1. A) X-ray structure of MDM2-PDI peptide complex (PDB: 3G03) with C_α and C_β triangles shown with dotted yellow lines. B) Comparison of simulated cross-linked peptide Phe, Trp and Leu side chain positions. Note: Xtal = from X-ray structure 3G03, Sim = simulated PDI peptide. C) Simulated models of **11** and **12**, providing possible interactions with MDM2.

The simulations of the cross-linked peptides involved two stages: 1) the peptide with the two cysteines in their free thiol form was simulated (in MOE using default settings) starting from each of the nine possible rotamers of the cysteine (arising from rotation about the C_α - C_β bond in each of the cysteines); 2) the rotamer that positioned the two sulfur atoms closest to the separation between the two atoms in the cyclised adduct (6.37 Å) was selected and the linker was introduced by editing the molecule in MOE. The edited structure was energy minimised and a second simulation performed. In both stages, the default settings in MOE were employed. This entails use of the NPA algorithm, using the AMBER10 forcefield with implicit solvation (with interior dielectric of 1 and exterior dielectric of 80). An initial 100 ps of equilibration was followed by 500 ps of production of which the second half (last 250 ps) is used in the analysis (reported every 0.5 ps giving a total of 500 data points from each simulation). The simulations can be summarised succinctly by considering the average values of each side of the triangle equivalent to those shown in Figure 1B.

When the average distances are compared with those observed in the published MDM2 complex, an RMSD can be computed to permit an overall comparison of how well the free stapled peptide retains the geometry required for complex formation. This suggests that the double D-Cys-containing stapled peptide **10** (RMSD = 1.0 Å) will retain the required pharmacophoric arrangement better than even the native peptide, which adopts a slightly different geometry when free from the receptor. The next best is predicted to be compound **8** (RMSD = 1.2 Å), followed by **12** and **7**. The simulations correctly identify **10** as the best of the analogues in which only stereochemistry is varied, whereas, compound **9** can be considered the least suitable by this measure. Whilst this is in good agreement with the SPR data for stereoisomeric peptides, the approach does not appear able to correctly rank the structural variations in which methylation or homologation have been introduced, indicating that other factors also govern their interaction and affinity with the protein. In order to provide insight into these two structures, molecular editing in MOE was used to convert the native complex to the stapled form for compounds **11** and **12**. The complex was then simulated to investigate any extra contacts made by these two linkers that could explain the observed binding. The final snapshot is shown in Figure 1C and reveals that **11** is able to lay its linker on a hydrophobic part of the receptor surface. While compound **12** is also able to form some hydrophobic interactions, the shape of this linker is not amenable to making continuous contact because of the protrusion of one of the methyl groups in the penicillamine. These are particularly close to the sidechain of Met62 which is in a more constrained environment when compound **12** is bound. Overall, these insights help to explain the differential measured binding affinities following cysteine editing.

This work has demonstrated that the conformational properties of a stapled peptide, and thus the biological activity, can be modified by the nature (size and stereochemistry) of the thiol groups to be cross-linked; and indeed, the combination of these with a suitable cross-linker. This has clear implications in the tuning of binding affinity and/or target selectivity in two-component disulfide-stapling of α -helical peptides and provides an important new tool in this rapidly growing area.

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Experimental Section

Materials. All Fmoc L- and D-amino acids (CEM), Rink Amide ProTide resin (CEM), diisopropylcarbodiimide (DIC; Apollo Scientific), Oxyma Pure™ (CEM), N,N'-dimethylformamide (DMF; Fisher Scientific), diisopropylethylamine (DIPEA; Merck Millipore) and piperidine (Merck Millipore) were purchased from commercial suppliers and used directly as indicated in the appropriate experimental procedures. All other reagents (hexafluorobenzene, trifluoroacetic acid (TFA), triisopropylsilane (TIPS) were purchased from Sigma Aldrich and solvents (HPLC grade) were purchased from Fisher Scientific. Nutlin-3a was purchased from NewChem Technologies Ltd, Newcastle upon Tyne, UK.

Solid phase peptide synthesis (SPPS) of peptides 1 – 6 and PDI peptide. Each linear thiol-containing peptide sequence was prepared using automated Fmoc-SPPS methods on a Liberty Blue microwave-assisted peptide synthesiser (CEM). Solid phase synthesis was conducted using Rink amide ProTide resin (180 mg, 0.56 mmol/g loading; 0.1 mmol), employing the required Fmoc amino acids (0.2 M in DMF; 5 eq.); with DIC (1M stock solution in DMF; 10 eq.), Oxyma Pure (1M stock solution, 5 eq) and piperidine (20% v/v in DMF; 587 eq., 4 mL) as activator, and deprotection, respectively. Standard coupling procedures employed double coupling of each amino acid (2.5 min, 90 °C). Amino acids bearing thermally-sensitive protecting groups e.g., Fmoc-L-Cys(Trt)-OH, Fmoc-D-Cys(Trt)-OH, Fmoc-Pen(Trt)-OH, Fmoc-hCys(Trt)-OH and Fmoc-His(Boc)-OH were coupled under milder conditions (50°C for 10 min). Following on-resin synthesis of the appropriate sequence, N-terminal capping was performed using Ac₂O/DMF (20% v/v, 2 x 15 min) with shaking at room temperature. Finally, peptides were cleaved from the resin as the C-terminal amide by treatment with a cleavage cocktail (5 mL; comprising TFA, TIPS and water (9 : 0.5 : 0.5 v/v) with regular shaking at room temperature for 4 h. Peptides were precipitated from cleavage solutions by dropwise addition into cold diethyl ether followed by centrifugation. The resulting pellet was successively suspended in cold diethyl ether and centrifuged twice further. The solids obtained were dissolved in water/MeCN (depending upon solubility), frozen and lyophilised. PDI and peptide 1 were purified by semi-preparative HPLC (see supporting information). The crude disulfide peptides 1 - 6 were used without further chromatographic purification.

Synthesis of perfluoroaryl-stapled peptides 7 - 12. To a centrifuge tube containing solid crude peptide 1 - 6 (approx. 50 mg) was added DIPEA stock solution (25 mM in DMF, 1.0 mL), followed by hexafluorobenzene (20 eq., 0.66 mmol). The resulting mixture was shaken at room temperature for 4.5 h. After precipitation in Et₂O (as above), the resulting crude stapled peptide was redissolved in a mixture of water/MeCN (exact amounts depended on solubility). Crude samples were purified using semi-preparative HPLC. Stapled peptides were characterised by analytical HPLC and high resolution mass spectrometry (see supporting information).

Protein Expression and Purification. PCR amplified DNA encoding MDM2 17-125 and MDMX 22-111 was cloned into pGEX 6P-1 to allow expression as GST fusion proteins. Recombinant DNA was transformed into *E. coli* BL21(DE3) pLysS for expression in LB media at 18°C and 160 rpm following IPTG induction (100 mM) at an OD₆₀₀ of 0.6-1.0. Following overnight incubation, cultures were harvested, sonicated and the fusion proteins were purified from the resulting cell lysate using GST-affinity chromatography and gel filtration, using a HiLoad® 26/60 Superdex® 75 column equilibrated in mHBS (20 mM HEPES, 100 mM NaCl, 5 mM DTT, pH 7.4). Fractions containing purified protein were pooled, concentrated and frozen at -80°C before performing SPR analysis.

Surface Plasmon Resonance (SPR) binding experiments. SPR experiments were performed on a Biacore S200 instrument (GE Healthcare) at 25°C using a Series S carboxyl-derivatized sensor chip (CM5) prepared for capture of GST, GST-MDM2 and GST-MDMX

following immobilisation of anti-GST antibody using GST-capture and amine coupling kits (GE Healthcare). A running buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20 and 3 mM EDTA was used during sensor chip preparation. Polyclonal goat Anti-GST antibody (30 µg/mL) was prepared in immobilisation buffer (10 mM sodium acetate, pH 5.0) and was immobilised to the CM5 chip through amine coupling after injection of EDC/NHS (1:1) onto the sensor chip surface for a contact time of 840 s and at a flow rate of 5 µL/min. The antibody was injected onto the activated surface for 600 s at 5 µL/min and unreacted groups were then deactivated by injection of ethanolamine for 420 s at 10 µL/min. High affinity sites were blocked by injecting recombinant GST (5 µg/mL in running buffer) twice for 300 s at 5 µL/min prior to regenerating the sensor surface through injection of regeneration solution (10 mM glycine-HCl, pH 2.1) for 120 s at 10 µL/min.

Analytes were dissolved in 100% DMSO to 20 mM and an Echo 550 acoustic dispenser (Labcyte) was used to dispense 12 concentration-response points for each analyte, as 1 µL droplets into a 384 well microplate (Greiner). The droplets were diluted (1:100) with 99 µL of running buffer supplemented with 1 mM DTT prior to analysis. As for the analytes, 100% DMSO was also acoustically dispensed and diluted as a 12-point concentration series to allow for solvent correction during analysis. Freshly thawed GST or GST-fusion protein diluted in running buffer was applied to reference or test channels respectively for 600 s or until satisfactory response unit levels (>1000 RU) had been achieved. Binding experiments were performed at a flow rate of 30 µL/min using multi-cycle kinetics with injection of analytes over the captured ligand for a contact time of 60 s, followed by a dissociation period of 1500 s, with multi-channel data collection at 10 Hz. During multicycle analysis, running buffer was modified with 1 mM DTT and 1% (v/v) DMSO to match with analyte composition. Data evaluation was performed using Biacore S200 Evaluation software (Version 1.0, Build 20) with binding curves fit to a 1:1 (Langmuir) interaction model for evaluation of kinetic and affinity parameters, following solvent correction and reference channel subtraction.

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Keywords: cancer • computational modelling • cysteine • hexafluorobenzene • peptides

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- [33] Note: the reported method employed a 50 mM solution of TRIS (tris(hydroxymethyl)-aminomethane) base, yet we found that DIPEA performed equally well and offered the additional advantage of being volatile and, therefore, easily removed by evaporation and allowing direct analysis by electrospray mass spectrometry.
- [34] No disulfide reduction was required prior to the nucleophilic aromatic substitution reaction.

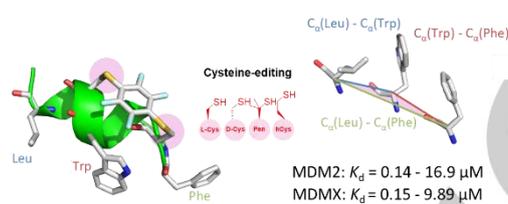
COMMUNICATION

Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Cysteine-editing: Stapled peptides can be prepared by cysteine cross-linking with hexafluorobenzene. Replacement of one or more L-cysteine residues with 'cysteine analogues' changes both its target affinity and selectivity for MDM2/MDMX based on surface plasmon resonance measurements.



Replacement of L-cysteine alters position of key side chains, target affinity and selectivity

Sanne J.M. Verhoorck, Claire E. Jennings, Neshat Rozatian, Judith Reeks, Jieman Meng, Emily K. Corlett, Fazila Bunglawala, Martin E.M. Noble, Andrew G. Leach, and Christopher R. Coxon*

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Tuning the binding affinity and selectivity of perfluoroaryl-stapled peptides by cysteine-editing