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Macrocyclisation and functionalisation of unprotected peptides *via* divinyltriazine cysteine stapling†

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We report a novel divinyltriazine linker for the stapling of two cysteine residues to form macrocyclic peptides from their unprotected linear counterparts. The stapling reaction occurred rapidly under mild conditions on a range of unprotected peptide sequences. The resulting constrained peptides displayed greater stability in a serum stability assay when compared to their linear counterparts.

Many vital cellular processes are governed by protein–protein interactions (PPIs). In addition, aberrant PPIs have also been linked to numerous disease states including Alzheimer's disease and cancer. A notable example is the p53/MDM2 interaction, which has been implicated in numerous cancers and has been identified as a potentially druggable PPI. As such, PPIs have become important targets both therapeutically and as tools to investigate the role of specific proteins in a wider network.[1] A recognised problem with targeting PPIs is that they generally have a large binding area with little or no defined binding pockets, thus making small molecule binders difficult to design. An attractive approach for developing inhibitors of PPIs is the synthesis of mimetics based on the binding partner of the protein of interest. In this way, disruption of many PPIs has been reported using the native PPI interface as an initial template to design oligopeptide modulators of these interactions.[2]

In recent years, macrocyclised peptides have emerged as valuable tools for probing and inhibiting PPIs. However, many commonly employed macrocyclisation strategies rely on the incorporation of unnatural amino acids into a linear peptide sequence before forming a covalent linkage. An emerging class of therapeutics known as stapled peptides have shown considerable promise within this field. One-component peptide stapling (1C-PS) has been shown to be an effective stapling method through the covalent linkage of two natural or non-proteinogenic amino acids. In particular, ring-closing metathesis (RCM) has been extensively used to staple alkene-containing amino acids.[3] A complementary approach known as two-component peptide stapling (2C-PS) involves the use of an external linker to covalently staple two amino acids. A major advantage of this 2C-PS system over 1C-PS methodologies is the ability to modify the linker and thus allow a series of tailored peptides to be synthesised from one linear peptide sequence. We previously described the development of a 2C-PS system, where two natural amino acids in a peptide sequence are replaced with two azido amino acids, which then undergo two azide–alkyne click reactions with a bis-alkynyl linker to give the stapled peptide.[4] A drawback of this system is the need to synthesise the unnatural azido amino acids required for stapling.[5]

A number of 2C-PS techniques have also been developed that utilize proteinogenic amino acids. Cysteine stapling has emerged as an attractive stapling method due to the high nucleophilicity of the sulfhydryl group, which can readily react with electrophiles such as Michael acceptors.[6] Numerous cysteine-specific stapling strategies have been reported including *S*-alkylation or *S*-arylation *via* nucleophilic aromatic substitution or palladium-catalysed cross-coupling chemistry.[7] However, linker functionalisation has only been reported for a small selection of cysteine linkers such as dichlorotetrazines, *N*-phenyl-divinylsulfonamides, pyridazinediones and dibromomaleimide reagents.[8]

We recently reported the development of a divinylpyrimidine (DVP) linker system for site-selective modification of proteins to generate stable and functional antibody–drug conjugates (ADCs) *via* cysteine bridging chemistry.[9] We envisaged that this technology would also enable efficient peptide stapling, thus allowing facile alteration of the physical properties of a single linear peptide through functionalization of the linker. It was initially postulated that the stapling efficiency could be optimised by increasing the conjugation rate of the DVP linkers. To this end, a divinyltriazine (DVT) motif was

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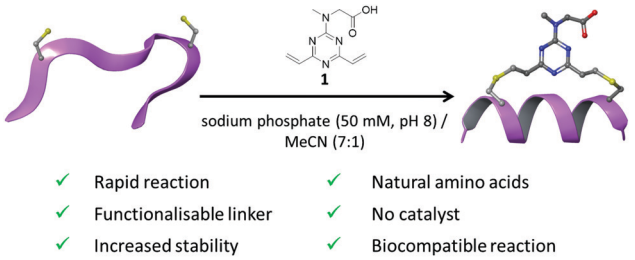


Fig. 1 This work involving two-component peptide stapling using a divinyltriazine linker.

hypothesised (**1**, Fig. 1). DVT **1** was designed and subsequently synthesised in a simple three step procedure from cyanuric chloride (ESI,† Scheme S1).

With DVT linker **1** in hand, we chose a model peptide, targeting the p53/MDM2 interaction to begin our stapling evaluation (Fig. 2). The chosen peptide had a similar amino acid sequence to other peptides previously investigated by our group as well as other p53/MDM2 inhibitors. 2C-PS of these sequences has resulted in helical peptides with enhanced bioactivity over their linear counterparts.[4a,10] The wild type peptide sequence (Ac-ETFSDLWRLLPEN-NH_2) was altered by mutations of the Ser20 and Pro27 residues to cysteines in an *i*, *i* + 7 configuration.

An initial screen of the stapling reaction in a mixture of sodium phosphate (50 mM, pH 8) and four different organic co-solvents (acetonitrile, *N*,*N*-dimethylformamide (DMF), hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE)) was conducted (ESI,† Fig. S2 and S3). HFIP and TFE are known to induce helix formation of helical peptides in solution, which would potentially increase the rate of stapling due to the target residues being in a favourable orientation. No significant difference was seen between any of the co-solvents with all stapling reactions being complete after one hour with just 1.1 molar equivalents of **1** required. Acetonitrile was used as the co-solvent of choice for further test reactions to allow direct HPLC purification without the need for a workup first. Increasing the organic solvent percentage in the reaction (7 : 1, 1 : 1 and 1 : 7 sodium phosphate/MeCN) resulted in a slower conversion to the desired stapled peptide but all reactions went to conversion within one hour (ESI,† Fig. S4). A similar pattern was observed for the other three solvents investigated (ESI,† Fig. S4). We believe that the conjugation reaction follows a general base catalysis mechanism, where buffer is required for the reaction to proceed efficiently. The reaction concentration was subsequently investigated, with concentrations ranging from 0.5–5 mg mL^{-1} of peptide (ESI,† Fig. S5 and S6). With 1.1 molar equivalents of **1**, macrocylisation at 5 mg mL^{-1} reached complete conversion in just 15 minutes. At low peptide concentration (0.5 mg mL^{-1}), with still only 1.1 molar equivalents of **1**, complete conversion to the stapled product was achieved in 60 minutes. Importantly, no evidence of double labelling was observed under the conditions investigated. Significantly, the stapled peptide was obtained for all reaction conditions tried, demonstrating the compatibility of the coupling.

One drawback with currently used 2C-PS techniques is the need for multiple purification steps,[4a] which is inefficient and time-consuming. After examining the DVT stapling reaction with both crude and purified peptides, we found the efficiency of the two reactions to be almost identical thus alleviating the need for multiple purification steps (ESI,† Fig. S7).

The effect of stapling on peptide helicity was investigated next. Circular dichroism (CD) spectroscopy showed that the *i*, *i* + 7 DVT-stapled peptide **P1-C-1** had an increased α-helical conformation over its linear serine (**P1-S**) counterpart, which displayed a random coil conformation (Fig. 3). Introduction of a conformational constraint into peptides with an inherently helical bioactive conformation can increase their biophysical properties relative to the linear counterpart.[11] To determine the effect of DVT stapling on the binding affinity of **P1** peptides to their endogenous MDM2 protein, competitive fluorescence polarisation (FP) assays were carried out. The binding affinity of the stapled peptide **P1-C-1** (K_i = 13 nM) was a 100-fold improvement over the analogous cysteine linear peptide **P1-C** (Fig. 4), and comparable to the previously reported binding

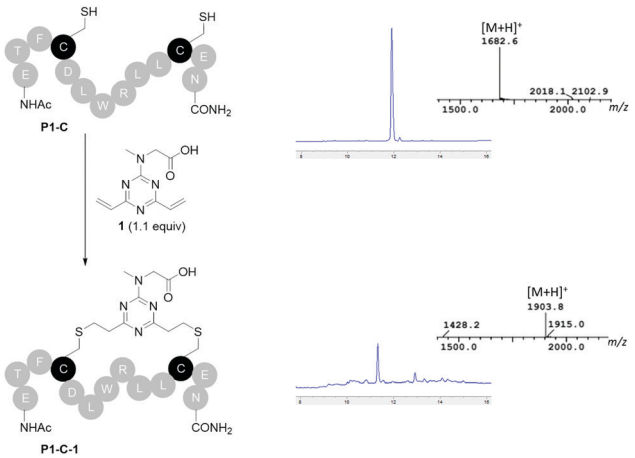


Fig. 2 Stapling of peptide **P1-C** with divinyltriazine (DVT) linker **1**. HPLC traces and mass spectrometry data are shown for purified **P1-C** and the crude stapling reaction at 60 mins (at 2 mg mL^{-1} peptide concentration).

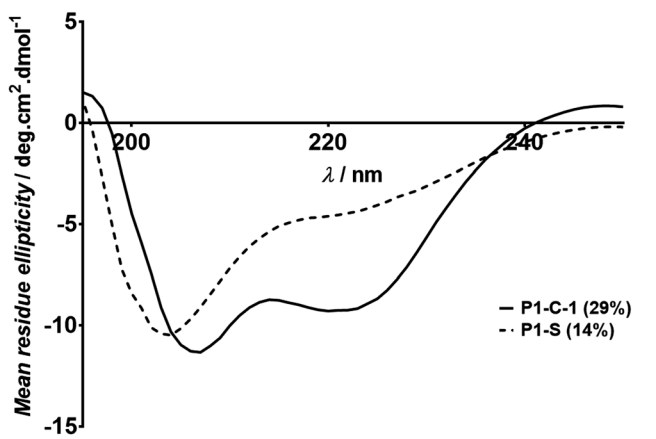
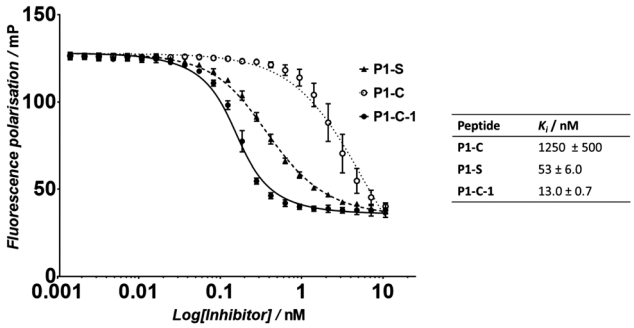


Fig. 3 Circular dichroism spectra of linear serine model peptide **P1-S** and stapled cysteine peptide **P1-C-1**.



Peptide	K_i / nM
P1-C	1250 ± 500
P1-S	53 ± 6.0
P1-C-1	13.0 ± 0.7

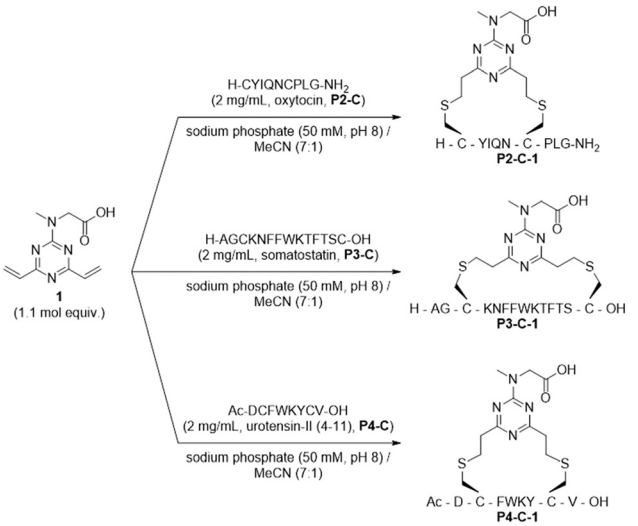
Fig. 4 Competitive fluorescence polarisation (FP) measurements of linear peptides **P1-C** (○) and **P1-S** (▲), and stapled peptide **P1-C-1** (●) to determine binding towards MDM2.

affinity for the same peptide using the copper-catalysed azide–alkyne click (CuAAC) 2C-PS system (3–16 nM depending on functionalisation).[4a] Only a modest 4-fold improvement in binding affinity was observed when compared with the linear serine mutant **P1-S** (Fig. 4). A relatively high binding affinity for the serine linear peptide is also comparable with the binding affinity for the linear azidoornithine peptide reported by Lau *et al.*[4a] and is consistent with findings that mutation of Pro27 to Ser results in an increase in binding affinity for MDM2.[12]

To investigate the generality of the stapling methodology we explored the coupling efficiently of DVT **1** with a range of biologically relevant peptide sequences, with different stapling residue spacing and various charges. The peptides chosen all contain natural disulfide bonds: oxytocin **P2-C**, a peptide hormone; somatostatin **P3-C**, a growth hormone-inhibiting peptide that regulates the endocrine system, neurotransmission and cell proliferation; and urotensin-II (4–11) **P4-C**, a vasoconstrictor, were identified as natural peptides containing disulfide bonds. Stapling of **P2-C**, **P3-C** and **P4-C** was conducted under the previously optimised conditions of 7 : 1 sodium phosphate (50 mM, pH 8)/MeCN with 1.1 molar equivalents of DVT **1** (Scheme 1). In all cases, the reactions proceeded efficiently with >95% conversion within one hour.

Extended peptide sequences, such as **P1-C**, can be flexible in nature until they reach their binding partner, and thus are easily degraded by circulating proteases. By constraining a peptide this inherent flexibility can be reduced and the proteolytic stability of the peptide increased. The stability of stapled peptide **P1-C-1**, its linear counterpart **P1-C** and the linear serine variant **P1-S** was monitored in human serum. After 24 hours incubation, 52% of the stapled p53 peptide **P1-C-1** remained intact, while 27% and 17% of the wild type and serine mutant remained, respectively (Fig. 5). Serum stability tests on the other synthesised macrocyclic peptides were also carried out (ESI,† Fig. S8). In all cases, the constrained peptide was significantly more stable than its linear counterpart, demonstrating the importance of stapling to increase peptide stability.§

Although two component cysteine macrocyclisation strategies have been reported, many of these do not allow efficient functionalisation of the linker directly but require the peptide itself to be modified. The DVT linker **1** enables further functionalisation by



Scheme 1 A series of naturally occurring peptide macrocycles containing disulfide bridges were rebridged using 1.1 molar equivalents of divinyltriazine (DVT) **1** at a final peptide concentration of 2 mg/mL.

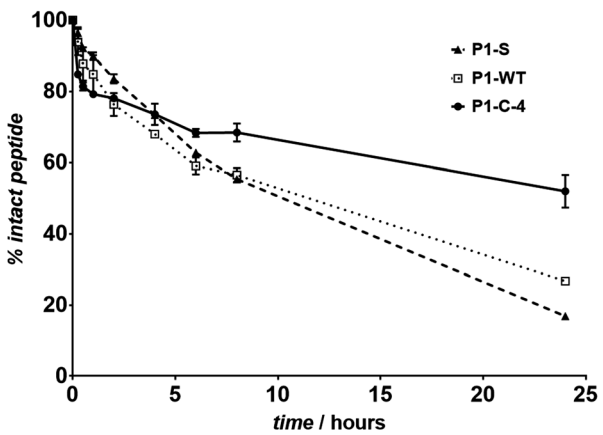
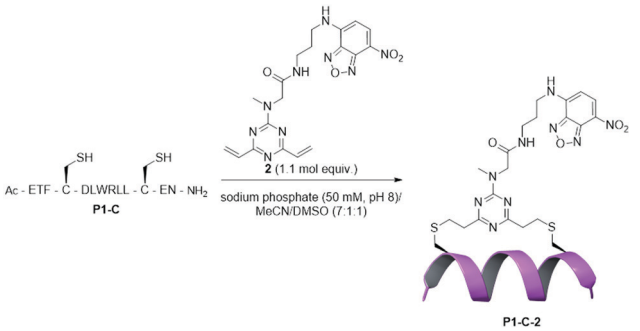


Fig. 5 Serum stability assay carried out on linear peptides **P1-S** (▲) and **P1-WT** (□) and on the stapled peptide **P1-C-1** (●), in human serum.

amide coupling of additional groups such as cell penetrating peptides or fluorophores to the terminal carboxylic acid. To exemplify this approach, DVT **1** was modified with a nitrobenzofurazan (NBD) fluorescent tag to yield functionalised DVT **2** (ESI,† Scheme 2). NBD tags have been previously reported for generating fluorescent bioconjugated proteins.[13] Using the further optimised conditions of 1.1 molar equivalents of DVT **2** in 7 : 1 : 1 sodium phosphate/MeCN/DMSO **P1-C** was efficiently stapled after one hour incubation at room temperature (Scheme 2).

In conclusion, we have described the development of a novel divinyltriazine linker for the macrocyclisation and functionalisation of peptides. The stapling reaction was shown to work well under a wide variety of different reaction conditions to accommodate the properties of the desired peptide and was successfully demonstrated on a range of different peptide substrates of different charge, length and stapling distance. The bioconjugation reaction generally proceeded rapidly and efficiently, even at low peptide concentrations (0.5 mg mL^{-1}). Functionalisation of



Scheme 2 Synthesis of fluorescently labelled stapled peptide **P1-C-2**, through the use of divinyltriazine linker **2** (1.1 equiv.) and linear peptide **P1-C** (2 mg/mL).

the linker with a biologically-relevant fluorescent tag was achieved and had no effect on the subsequent stapling reaction. Stapling of the *i*, *i* + 7 p53 peptide generated a more helical peptide than its linear variant with an enhanced binding affinity (13.0 ± 0.7 nM) for MDM2 over its linear variant (1.25 ± 0.5 μM). Having demonstrated the compatibility of the stapling methodology to efficiently staple and functionalise peptide scaffolds with a fluorescent imaging agent, we aim to further expand the utility of this technology to functionalise peptides with other modalities, such as affinity tags (*e.g.* biotin), cell-penetrating motifs and drug molecules to generate peptide-drug conjugates.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

§ Stapled peptide **P1-C-1** was found to be >90% stable in both TFA and 20% piperidine/DMF over 1 hour. As these range of deprotection conditions seem compatible with the staple, it should be compatible with orthogonal cysteine protection/deprotection strategies.

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