A two-component 'double-click' approach to peptide stapling

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Peptide cyclization is a useful strategy for the stabilization of short flexible peptides into well-defined bioactive conformations, thereby enhancing their ability to interact with proteins and other important biomolecules. We present an optimized procedure for the stabilization of linear diazido peptides in an α-helical conformation upon reaction with dialkynyl linkers under Cu(I) catalysis. As this procedure generates side chain–cyclized peptides bearing a bis-triazole linkage, it is referred to as 'double-click' stapling. Double-click stapling can enhance the binding affinity, proteolytic stability and cellular activity of a peptide inhibitor. A distinguishing feature of double-click stapling is the efficiency with which peptides bearing different staple linkages can be synthesized, thus allowing for modular control over peptide bioactivity. This protocol describes the double-click reaction between a 1,3-dialkynylbenzene linker and peptides that contain azidoornithine. Subsequent peptide purification and confirmation steps are also described. The entire double-click stapling protocol can be completed in ~48 h, including two overnight lyophilization steps.

INTRODUCTION

Background

Peptide stapling is a macrocyclization strategy for the generation of α-helical peptides. By mimicking α-helical binding motifs that are found in many proteins, stapled peptides can act as competitive inhibitors of protein-protein interactions (PPIs)[1]. Stapled peptide inhibitors have found applications as potential therapeutic agents and as biochemical tools for studying PPI networks.

The stapling process is performed on linear peptides containing two non-native amino acid residues bearing side chains that can be covalently linked together, which constrain the peptide into the desired α-helical conformation. Compared with unmodified peptides, stapled peptides can have improved biological properties, including resistance to proteolytic degradation, enhanced cell permeability and higher-affinity binding toward its intended biological target[2]. Numerous macrocyclization chemistries can be used to carry out the stapling process, each using different non-native amino acids and giving rise to stapled peptides with different staple linkages[3–14].

Development and applications of the protocol

We have developed a peptide stapling approach that capitalizes on the robust nature of the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction[9,15], the archetypal click reaction as described by both Sharpless and Meldal[16,17] and based on the original 1,3-dipolar cycloaddition chemistry developed by Huisgen[18] in the 1960s . This stapling technique involves reacting linear *i,i+7* diazido peptides (i.e., containing two azido amino acids that are seven residues apart) with dialkynyl stapling linkers under Cu(I) catalysis (**Fig. 1**). As this reaction produces peptides bearing a bis-triazole linkage, this process is referred to as double-click stapling. The reason for choosing to use a two-component stapling approach (i.e., the linear peptide and the staple linkage are separate moieties) is that different stapled peptides can be efficiently generated by reacting a single linear diazido peptide with a collection of different dialkynyl stapling linkers in a divergent manner. The excellent functional group tolerance of the CuAAC reaction ensures that stapling is compatible with a wide variety of stapling linkers, and it can occur in one simple step without the need for protecting groups.

We recently reported an example of the use of double-click stapling for synthesizing inhibitors of the p53–MDM2 interaction[9], which is an important PPI target for cancer therapy[19]. Starting from a single p53-based diazido peptide, we were able to efficiently generate stapled peptides with improved cellular uptake and p53-activating capability in cells, simply by introducing differently functionalized staple linkages (**Fig. 2**). This proof-of-principle study illustrates one of the various possible applications that are made possible by having efficient synthetic control over the staple itself.

We are currently developing more functional linkers that not only affect the binding and cellular activity of a stapled peptide but also contain motifs with properties that can be exploited in biochemical and biophysical studies. Through this protocol, we endeavor to make double-click stapling accessible to other research groups who are looking to apply different stapling techniques toward the inhibition of their PPI of interest. Arora and Jochim[20] have estimated that there are over 1,500 PPI interfaces in the Protein Data Bank that contain helices. In principle, our double-click stapling protocol could be applied to any of these PPIs, although the feasibility and success of the approach depends highly on the specifics of each individual interaction.

Comparison with other methods

Out of the various stapling techniques that are available, hydrocarbon peptide stapling by ring-closing metathesis is currently the most widely used method. This approach was developed by the work of Grubbs, Verdine, Walensky and colleagues[3,4], and it has now been successfully applied to a range of PPIs by a number of research groups[21], although there have also been reports of difficulties associated with optimizing peptides for their desired applications[22–24]. Lactamization between aspartate/glutamate and lysine residues is another common stapling technique that has been extensively studied[25,26].

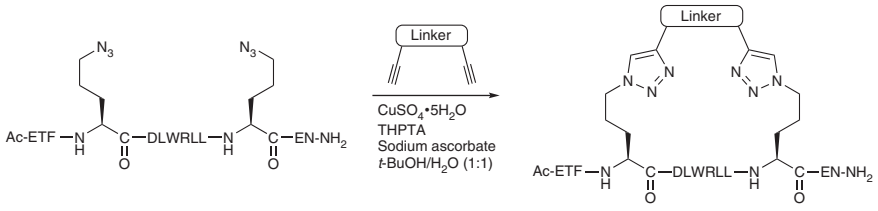


Figure 1 | Double-click stapling is a two-component reaction, in which the peptide and linker are separate moieties. THPTA, tris(3-hydroxypropyltriazolylmethyl)amine.

Hydrocarbon stapling and lactamization are one-component reactions, with the staple pre-encoded by the choice of stapling amino acids during solid-phase peptide synthesis. As a consequence, each variation in the staple component requires a completely new peptide to be synthesized. This limitation applies to all one-component stapling approaches, but it does not apply to two-component methods such as double-click stapling. By using double-click stapling, a collection of different stapled peptides can be divergently synthesized by stapling different linkers to a single peptide sequence.

There are several other two-component stapling techniques in the literature, which primarily use chemistry based on cysteine and lysine residues[10–12,14,27]. As these amino acids are proteogenic amino acids, their synthesis and incorporation into peptides is a standard procedure. The azido amino acids required for double-click stapling are nonproteogenic, but they are simple to synthesize[28], and they are compatible with solid-phase peptide synthesis. The advantage of using azido amino acids is that they are not prone to cross-reactivity with other unprotected functional groups. Double-click stapling exploits the specificity of the CuAAC to ensure maximum compatibility with different dialkynyl stapling linkers, regardless of their chemical functionality. Cysteine and lysine residues may cross-react if unprotected electrophilic functionalities are present elsewhere, or if there is competition with other nucleophilic groups in the system.

In terms of alternative double-click stapling techniques, Bong and co-workers[29] were the first group to investigate double-click chemistry, using an *i,i+4* helix-dimer system based on leucine zipper GCN4 (ref. 29). Helix dimerization was restored by using azidoalanine as the non-native amino acid and 1,5-hexadiyne as the optimal staple linker. This work provides an *i,i+4* stapling technique that is complementary to our *i,i+7* double-click protocol, and it uses different optimized reaction conditions. In contrast to our double-click protocol, however, the 1,5-hexadiyne linker provides no chemical handle to which further functional motifs can be attached. Both the stapling techniques may be useful in different scenarios, depending on the desired spacing of the non-native amino acids.

Apart from synthetic concerns, the biological activity of stapled peptides can vary depending on what stapling technique is used. Various stapling techniques have been used to create biologically active peptide inhibitors, but few studies provide a comparative assessment of different stapling techniques in biological assays for the same PPI system[5,13,30,31]. At this stage, it is difficult to rationalize which stapling technique will give the best results in biological systems, and this is likely to be highly dependent on the PPI itself. Therefore, we would suggest that a variety of stapling techniques be trialed when attempting to inhibit any given PPI. For a more in-depth discussion, we refer the reader to a recent review of different peptide stapling techniques[32].

In a broader context, there are other peptide macrocyclization techniques that are related to peptide stapling. Notable examples include the hydrogen bond surrogate approach, a backbone cyclization technique for helix formation used by Arora and co-workers[33] and other methods for generating cyclic peptides developed by several groups including Fasan and co-workers[34], Timmerman *et al.*[35], Pentelute and co-workers[36] and Suga and co-workers[37].

Experimental design

As with any other stapling approach, the first step to designing a stapled peptide inhibitor for a given PPI is to gain structural information about the α-helix to be mimicked. If available, crystallographic data are highly useful for identifying which residues are less important for target binding, and hence they can be replaced by non-native amino acids. Mutational studies such as alanine scanning are also useful for this purpose. Such an analysis can be applied to both the native peptide sequence of the α-helix and the alternative sequences derived from phage display.

A panel of different peptides can then be synthesized, containing amino acids with different stapling positions, by finding all the possible *i,i+7* pairs of amino acid residues that can be substituted, as has previously been described for hydrocarbon staples. It may be possible to narrow down the number of possibilities using computer simulations such as molecular dynamics, which may be able to predict which positions are favorable. At this stage, we would recommend choosing one type of dialkynyl linker to screen the different staple positions, using a primary high-throughput *in vitro* screening assay such as fluorescence polarization to identify the optimal position. Subsequently, this single peptide sequence can then be stapled with a range of different dialkynyl linkers to ascertain which combination gives the optimal result in follow-up biological assays[38].

When conducting the double-click reaction itself, we find that there are several factors that are important for efficient conversion. Deoxygenating the solvent is essential to avoid catalyst oxidation, which otherwise leads to incomplete reaction. The presence of the tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) ligand is also important for increasing the reaction rate. We also recommend preforming the Cu(I) ligand–catalyst complex, instead of simply adding the reagents separately, as this also improves the efficiency of the reaction. We have also noticed that some peptide

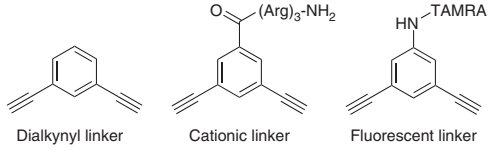


Figure 2 | Dialkynyl staple linkers bearing different functionalities. The cationic linker can help with cell uptake, whereas the fluorescent linker is useful for biophysical studies. TAMRA, carboxytetramethylrhodamine.

sequences or linkers can chelate copper, which leads to an extra broad peak on the HPLC chromatograph. This phenomenon is most common with stapled peptides that contain multiple polar residues on the linker such as lysine or arginine, but the peptide sequence also appears to influence the likelihood of chelation. We recommend checking the peak shape on the HPLC chromatograph each time a new stapled peptide is synthesized. To avoid contamination of the stapled peptide, copper can be removed by the addition of EDTA before purification.

Limitations

Although we have successfully used the double-click stapling protocol on a range of peptide sequences, the current system is only optimized for an *i*,*i*+7 staple spacing. Work is currently being done to find optimal double-click staples for *i*,*i*+4 and *i*,*i*+11 spacings. Another limitation of double-click stapling is the necessary formation of the bis-triazole motif in every case. It is uncertain whether the triazoles can form constructive interactions with protein surface or whether they instead clash sterically or electronically; however, this is likely to depend on the specific characteristics of a given PPI interface. Indeed, this limitation applies to all stapling techniques, as the functional groups involved in the macrocyclization will be present in the final structure of the staple linkage.

The presence of copper is also a potential limitation, as there is the possibility of unwanted oxidation of residues such as histidine and methionine. In our studies so far, we have not attempted to staple any peptides that contain methionine or cysteine. However, we have used peptides that contain all other proteogenic amino acids, and we have not observed any copper-promoted side reactions when applying our stapling protocol to histidine-containing peptides.

A general limitation for two-component reactions that uses the same ligation reaction at both ends of the linker (i.e., CuAAC in the case of double-click stapling) is that nonsymmetrical linkers give rise to two different stapled products that may be hard to separate, thus restricting the range of linkers that are available for structure-function studies. Finally, stapling a peptide will not always lead to a superior inhibitor[22–24]. In some cases, the binding affinity of a stapled peptide may be weaker than the corresponding native peptide, although through extensive optimization of peptide sequence and linker a more potent binder can usually be found. Stapling also does not guarantee improved cellular uptake. For the double-click stapling of p53 peptides[9], we have found that using cationic-functionalized staple linkers can enhance uptake, but the success of this strategy is likely to depend on the PPI involved. Although for most stapling techniques changes in the peptide sequence are often explored to achieve cell permeability[39], double-click stapling allows both the staple and the peptide sequence to be changed, thus increasing the chance of finding a stapled peptide with the desired uptake properties[15].

MATERIALS

REAGENTS

! CAUTION Exercise care when handling all organic materials. A laboratory coat, gloves and safety goggles should be worn. Synthetic operations should be performed in a chemical fume hood whenever possible.

- Copper(II) sulfate pentahydrate (Sigma-Aldrich, cat. no. 203165)
- Distilled water
- Dialkynyl linker: 1,3-diethynylbenzene (Sigma-Aldrich, cat. no. 632104)
- EDTA (Sigma-Aldrich, cat. no. 431788)
- *i*,*i*+7 diazido peptide (Mimotopes; can also be synthesized by standard Fmoc solid-phase peptide synthesis[40] using Fmoc-Orn(N_3)-OH; AnaSpec, cat. no. AS-65518)
- Sodium ascorbate (Sigma-Aldrich, cat. no. A7631)
- *tert*-Butanol (Sigma-Aldrich, cat. no. 360538)
- Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA; Sigma-Aldrich, cat. no. 762342)

HPLC and LC-MS reagents:

- Acetonitrile (Rathburn Chemicals, cat. no. RH1015)
- Ammonium acetate (Sigma-Aldrich, cat. no. 73594)
- Formic acid (Sigma-Aldrich, cat. no. 56302)
- Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. 91707)
- Water (Rathburn Chemicals, cat. no. RH1020)

EQUIPMENT

- Analytical HPLC: Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 µm)
- Centrifuge (Eppendorf MiniSpin)
- Circular dichroism spectrometer (Chirascan)
- Disposable 21-gauge needles
- Disposable plastic syringes, slip tip (various sizes)
- Dual nitrogen-vacuum manifold with high vacuum line
- Microcentrifuge tubes (Eppendorf tubes)
- Lyophilizer (Freeze dryer; Scanvac Coolsafe 100-9 Pro)
- Glass vials (various sizes)
- Liquid chromatography-mass spectrometry (LC-MS): Agilent 1200 series LC with an ESCi multi-mode ionization Waters ZQ spectrometer using MassLynx 4.1
- LC-MS vials
- Magnetic stirrer with temperature probe (Heidolph MR 3001 K and IKA WERKE RCT basic)
- Magnetic stir bar retriever
- Pipette tips (various sizes)
- Pipettes (Gilson Pipette Pipetman, various sizes)
- Quartz cuvette (Hellma 110 QD, 1 mm path length)
- Round-bottom flasks (various sizes)
- Rubber septa (various sizes)
- Screw-cap lids for glass vials
- Semipreparative HPLC: Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (250 mm × 21.22 mm, 5 µm)
- Snap-cap lids for LC-MS vials
- Syringe filters (Whatman)
- Teflon-coated magnetic stir bars for round-bottom flasks
- Vortexer (IKA MS1 Minishaker)

REAGENT SETUP

Sample preparation for analytical HPLC and LC-MS analysis Dissolve peptide reagents and products at a concentration of ~0.1 mg ml^{-1} in acetonitrile:water (1:1) and filter into an LC-MS vial through a small pad of cotton wool contained in a glass pipette to remove any insoluble material. For monitoring a reaction, remove a small aliquot of the reaction solution (~10 µl) and add it to a microcentrifuge tube containing 200 µl of acetonitrile:water (1:1). Centrifuge the microcentrifuge tube at 12,100*g* for 2 min at 25 °C to pellet any insoluble material, and then transfer the supernatant into an LC-MS vial.

EQUIPMENT SETUP

LC-MS LC-MS is run on an Agilent 1200 series LC using a Supelcosil ABZ+PLUS column (33 mm × 4.6 mm, 3 µm), together with an

ESCi multi-mode ionization Waters ZQ spectrometer using MassLynx 4.1 software. Samples are eluted using the conditions shown below:

Solvent A	10 mM ammonium acetate + 0.1% formic acid in water
Solvent B	95% acetonitrile + 5% water + 0.05% formic acid
Flow rate	1 ml min^{-1}
Injection volume	10 µl
Detection wavelengths	190–600 nm

Time (min)	Percentage of solvent B
0–0.7	Isocratic 0%
0.7–4.2	Linear gradient from 0–100%
4.2–7.7	Isocratic 100%
7.7–8.5	Linear gradient from 100–0%

Analytical HPLC
Analytical HPLC is run on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (150 mm × 4.6 mm, 3 µm) eluting with a linear gradient system (solvent A: 0.05% (vol/vol) TFA in water, solvent B: 0.05% (vol/vol) TFA in acetonitrile) at a flow rate of 1 ml min^{-1}. Typical gradient for stapled peptide analysis is 30–60% solvent B over 15 min, but it is dependent on the polarity of the peptide. HPLC is monitored by UV absorbance at 220 and 254 nm. Dye-labeled peptides can also be monitored by absorbance at their excitation wavelength (e.g., 550 nm for 5-TAMRA dye).

Semipreparative HPLC
Semipreparative HPLC is run on an Agilent 1260 Infinity using a Supelcosil ABZ+PLUS column (250 mm × 21.2 mm, 5 µm) eluting with a linear gradient system (solvent A: 0.1% (vol/vol) TFA in water, solvent B: 0.05% (vol/vol) TFA in acetonitrile) at a flow rate of 20 ml min^{-1}. Typical gradient for stapled peptide purification is 30–60% solvent B over 20 min, but it is dependent on the polarity of the peptide. HPLC is monitored by UV absorbance at 220 and 254 nm. Dye-labeled peptides can also be monitored by absorbance at their excitation wavelength (e.g., 550 nm for 5-TAMRA dye).

PROCEDURE

Solvent preparation ● TIMING 5 min

1| Combine 10 ml of *tert*-butanol and 10 ml of distilled water in a 50-ml round-bottom flask (flask A). Cap the flask with a rubber septum.
? TROUBLESHOOTING

2| Attach the flask to a dual nitrogen-vacuum manifold with vacuum line using a needle piercing through the septum. Submerge the tip of this needle into the solution. Pierce a second needle through the septum to provide a vent. Turn on the flow of nitrogen gas to begin deoxygenating the solvent.
▲ **CRITICAL STEP** Deoxygenating the solvent is essential to avoid catalyst oxidation, which otherwise leads to incomplete reaction.

3| Begin deoxygenating 20 ml of distilled water in a separate round-bottom flask (flask B), according to Steps 1 and 2.

Reaction setup ● TIMING 2 h

4| Weigh out ~5 mg of diazido peptide into a 10-ml round-bottom flask (flask C). Take note of the exact weight.
▲ **CRITICAL STEP** If the peptide is fluorescently labeled, flask C should be kept wrapped in foil hereafter.
? TROUBLESHOOTING

5| Place a Teflon-coated magnetic stir bar into flask C. Cap the flask with a rubber septum. Attach the flask to a dual nitrogen-vacuum manifold with vacuum line using a needle piercing through the septum. Clamp the flask over a magnetic stirrer.

6| Calculate the amount of reagents (copper(II) sulfate pentahydrate, sodium ascorbate, THPTA and dialkynyl linker) needed on the basis of the peptide weight. First, divide the weighed amount of peptide in milligrams by its molecular weight (include the mass of the trifluoroacetate counterion for each arginine, lysine and histidine residue in the sequence) to calculate how many millimoles of peptide are present. Multiply by the molecular weight of each reagent to obtain the mass of 1 equivalent in milligrams. For the copper(II) sulfate and THPTA, this is the amount required. For the linker, multiply the mass by 1.1 to give the amount required (1.1 equivalents) in milligrams. For the sodium ascorbate, multiply the mass by 3 to give the amount required (3 equivalents) in milligrams.

7| For each reagent, weigh approximately ten times the amount required, as calculated in Step 6, into a separate glass vial. Take note of the exact weighed mass of each reagent.

8| Calculate the volume of the solvent required such that 100 µl of the resultant stock solution will contain the appropriate amount of reagent, as calculated in Step 6. This can be done by dividing the weighed mass of each reagent in Step 7 by the calculated amount required from Step 6, and then multiplying by 100. This volume should be close to 1,000 µl.

9| By using a disposable syringe and needle, transfer 5 ml of the deoxygenated *tert*-butanol/water mixture from flask A into flask C containing the weighed peptide and stir bar.
▲ **CRITICAL STEP** The *tert*-butanol/water mixture in flask A should have been deoxygenating for at least 15 min before addition into flask C. Do not leave the solvent deoxygenating for several hours, as this can change the ratio of solvents present.

10| Immediately place Flask C under vacuum, wait for 1 s and then return the flow to nitrogen. Repeat this process a second time and then maintain the vessel under a positive pressure of nitrogen.

11| Switch on the magnetic stirrer to begin stirring the peptide solution. See **Figure 3** for a picture of the entire experimental setup at this stage.

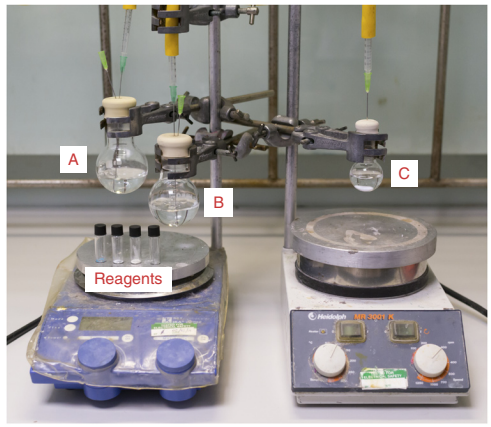


Figure 3 | The experimental setup for double-click stapling (shown at Step 11). A, B and C refer to the corresponding flasks.

12| Remove the rubber septum from flask A but keep the nitrogen bubbling through the solvent (**Fig. 4**). Pipette the appropriate amount of the deoxygenated *tert*-butanol/water in flask A (calculated in Step 8) to the glass vial containing the dialkynyl linker (prepared in Step 7). Immediately cap the vial and invert back and forth until the linker is dissolved.
▲ **CRITICAL STEP** The rubber septum should only be removed from flask A for a brief period, and the nitrogen flow through the solvent mixture must be maintained.

13| Switch off the magnetic stirring for flask C. Remove the rubber septum from flask C and pipette in 100 µl of the linker solution (prepared in Step 12). Immediately after addition, reseal flask C with the rubber septum, place it under vacuum, wait for 1 s and then return the flow to nitrogen. Repeat this vacuum/nitrogen process two more times, and then maintain the vessel under a positive pressure of nitrogen. Switch on the magnetic stirrer again.
▲ **CRITICAL STEP** The rubber septum should only be removed from flask C for a brief period, and the reaction vessel should be placed under a nitrogen atmosphere as soon as possible after the addition process.

Figure 4 | Pipetting deoxygenated solvent from flask A into reagent vials.

14| Pipette appropriate volumes (calculated in Step 8) of deoxygenated water from flask B into the vials containing copper(II) sulfate pentahydrate, sodium ascorbate and THPTA (prepared in Step 7). Cap the vials immediately and vortex until all the solids are dissolved.

15| Pipette 100 µl each of both copper(II) sulfate pentahydrate and THPTA solutions into an microcentrifuge tube, followed by 100 µl of sodium ascorbate solution. The mixture should turn from blue to colorless upon addition of the sodium ascorbate.

16| Immediately after mixing the solutions in Step 15, transfer all 300 µl of the mixture into flask C using a disposable syringe and needle. Place flask C under vacuum, wait for 1 s and then return the flow to nitrogen. Repeat this vacuum/nitrogen process two more times and then maintain the vessel under a positive pressure of nitrogen.

17| Leave the solution in flask C stirring at room temperature (25 °C) for 1 h.
■ **PAUSE POINT** The reaction can be left to stir overnight at room temperature at this stage if desired.

Reaction monitoring ● **TIMING 45 min**

18| After 1 h, withdraw a small amount (~10 µl) of the reaction mixture using a disposable syringe and needle, and analyze by LC-MS and analytical HPLC (compared with the starting diazido peptide) to check the progress of the reaction. To do this, add 10 µl to a microcentrifuge tube containing 200 µl of acetonitrile:water (1:1). Centrifuge the microcentrifuge tube at 12,100*g* for 2 min at 25 °C to pellet any insoluble material, and transfer the supernatant into an LC-MS vial. Analyze by LC-MS, as described in Equipment Setup. See the ANTICIPATED RESULTS section for example analytical HPLC chromatographs (see also **Figs. 5** and **6**).

? TROUBLESHOOTING

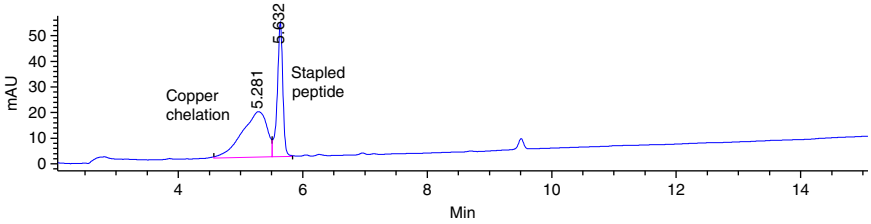


Figure 5 | HPLC chromatograph showing an example of copper chelation in the crude reaction mixture.

Reaction workup ● **TIMING 18 h**

19| When the reaction is complete, unclamp flask C and remove the needles, septum and magnetic stir bar from the flask.

20| Freeze the reaction mixture by submerging the flask in liquid nitrogen, and then lyophilize it overnight.

■ **PAUSE POINT** The lyophilized crude reaction mixture can be kept sealed in the freezer at –20 °C for up to several weeks if it cannot be purified immediately.

HPLC purification ● **TIMING 2 h**

21| Dissolve the crude lyophilized mixture in 800 µl of a 1:1 mixture of acetonitrile and water.

22| Transfer the contents to a microcentrifuge tube and centrifuge the tube to pellet any solids.

23| Remove the supernatant from the microcentrifuge tube using a pipette. Purify the supernatant by semipreparative HPLC (see Equipment Setup).

Solvent removal ● **TIMING 24 h**

24| Pool the fractions containing the stapled peptide product and remove most of the organic solvents under a stream of nitrogen.

25| Freeze the remaining peptide solution/suspension with liquid nitrogen, and then lyophilize it overnight or until it is dry.

Confirmation of stapling ● **TIMING 30 min**

26| Take an IR spectrum of the stapled peptide product, and compare it with the starting linear diazido peptide. The linear peptide should have a clear peak at ~2,100 cm^{-1} corresponding to the azide functional group, which should be absent in the stapled peptide product.

27| Obtain a high-resolution mass spectrum of the peptide product that shows the isotope pattern to confirm that the peptide is a monomer rather than higher-order oligomers.

Quantification of helicity ● **TIMING 30 min**

28| Weigh out ~1 mg of peptide. Dissolve it in an appropriate aqueous solvent in which the peptide is soluble (typically buffer, although a small amount of acetonitrile can be added to aid solubility if necessary), aiming for a concentration of ~50 µM.

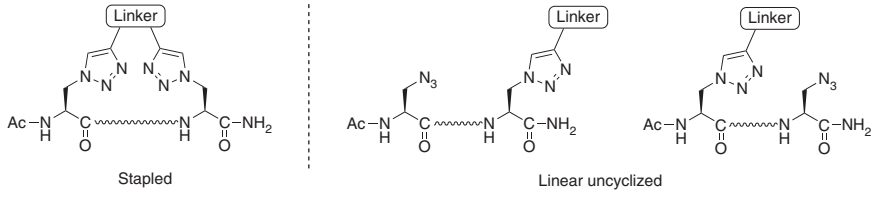


Figure 6 | General schematic of the stapled product and potential linear clicked but uncyclized side products arising from double-click stapling.

29| Filter the sample through a syringe filter. Transfer the filtrate into a quartz cuvette and obtain a circular dichroism (CD) spectrum using a CD spectrometer. In addition, make several dilutions of this filtrate and obtain their CD spectra to confirm that the spectral shape is not affected by aggregation.

30| Submit the filtrate for amino acid analysis to determine the exact concentration of the peptide in solution. Amino acid analysis is typically offered as a service by independent analytical laboratories.

31| Starting with the raw ellipticity data of the CD spectrum, divide the raw ellipticity values by the concentration determined by amino acid analysis (expressed in moles per liter, i.e., M), and then divide by the total number of amino acid residues present in the peptide. This gives the mean residue ellipticity in deg cm^2 $dmol^{-1}$.

32| Calculate the estimated theoretical mean residue ellipticity for a pure helix using the formula $-40{,}000 \times (1 - 2.5/n)$, where n is the total number of amino acid residues present in the peptide. Take the experimental mean residue ellipticity at 222 nm from Step 31, divide this by the calculated theoretical value and multiply by 100 to obtain the estimated percentage helicity.

? TROUBLESHOOTING

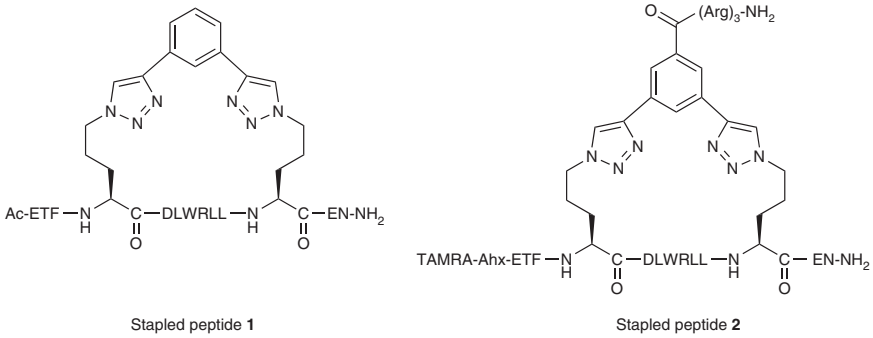
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	The *tert*-butanol is solid/partially solid and difficult to handle	*tert*-Butanol has a melting point of ~25 °C. Therefore, in colder laboratories, it may be solid	Place the bottle of *tert*-butanol in a warm water bath before use
4	Difficulties with accurately weighing the amount of peptide added to the flask	Lyophilized peptide is very light and prone to static electrical effects	Exercise extra care when weighing out material. Compensate as far as possible for environmental factors (e.g., air draughts, temperature, vibrations and levelness) and follow standard practices for dealing with compounds that are prone to static electrical effects (e.g., use a static gun to remove static electricity from the neck of the flask). An analytical balance accurate to ±0.01 mg should be used for small-scale weighing
18	The reaction has not proceeded to completion, and starting peptide still remains	The reaction is proceeding slowly	Leave the reaction stirring for an additional 2 h, and then monitor again (Step 18)
		The rubber septum may have leaked	Replace the septum if necessary
		The peptide or linker is a strong chelator for copper	Deoxygenate the reaction mixture with nitrogen for 10 min (as described in Steps 1 and 2), and then add a second round of reagents (Steps 15 and 16)
		There is insufficient linker owing to weighing errors	Deoxygenate the reaction mixture with nitrogen for 10 min (as described in Steps 1 and 2), and then add a second portion of linker (Step 13) followed by reagents (Steps 15 and 16)
	The analytical HPLC chromatograph displays a broad peak near the expected stapled peptide peak (**Fig. 5**)	The stapled peptide contains motifs that can chelate copper (multiple consecutive lysine residues for example) and thus form a complex that may not dissociate under purification conditions	Add EDTA to the lyophilized mixture in Step 21 until the broad copper complex peak disappears by analytical HPLC
	There are two peaks corresponding to the stapled peptide mass by LC-MS	The stapled peptide has the same mass as the corresponding linear clicked, but uncyclized, peptide (**Fig. 6**), so the reaction may not have gone to completion yet. The two products can be distinguished, as the stapled peptide typically has a shorter retention time than the linear peptide	Deoxygenate the reaction mixture with nitrogen for 10 min (as described in Steps 1 and 2), and then add a second round of reagents (Step 15 and 16)
	The stapled peptide peak appears very early or late in the HPLC chromatograph	The solvent gradient for HPLC is not appropriate and needs optimization	If the peak is too early, lower the overall percentage of solvent B (see Equipment Setup). If the peak is too late, increase the overall percentage of solvent B

● TIMING

Steps 1–3, solvent preparation: 5 min
Steps 4–17, reaction setup: 2 h
Step 18, reaction monitoring: 45 min
Steps 19 and 20, reaction workup: 18 h
Steps 21–23, HPLC purification: 2 h
Steps 24 and 25, solvent removal: 24 h
Steps 26 and 27, confirmation of stapling: 30 min
Steps 28–32, quantification of helicity: 30 min



Stapled peptide **1**: MS (ESI+) (*m/z*): $[M+2H]_2^+$ calcd for $C_{88}H_{123}N_{25}O_{22}^{2+}$ 943.0, found 943.0
Stapled peptide **2**: MS (ESI+) (*m/z*): $[M+3H]_3^+$ calcd for $C_{136}H_{192}N_{41}O_{30}^{3+}$ 960.5, found 960.5

Figure 7 | Structures and LC-MS data for example stapled peptides **1** and **2**.

ANTICIPATED RESULTS

Appearance of the stapled peptide product will be accompanied by the disappearance of the starting unstapled peptide peak, as monitored by analytical HPLC. Usually, the stapled peptide product has a shorter retention time than the unstapled peptide (**Figs. 7** and **8**). The reagents (Cu complex, sodium ascorbate) will appear very early in the chromatograph, away from the peptide peaks.

The IR spectrum of the stapled peptide should not have a peak corresponding to the azide at ~2,100 cm^{-1} (**Fig. 9**). A high-resolution mass spectrum should reveal the expected isotopic pattern for the monomeric species (**Fig. 10**).

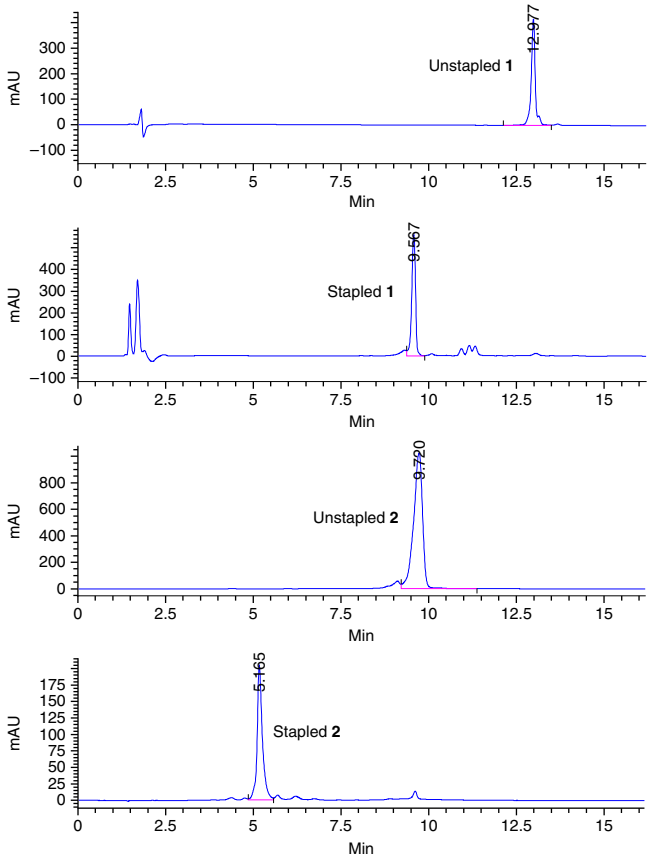


Figure 8 | HPLC chromatographs of unstapled starting peptides and corresponding crude reaction mixtures for stapled peptides **1** and **2**. Unstapled and stapled peptide **1** are run at 30–60% solvent B over 15 min and monitored at 220 nm. Unstapled and stapled peptide **2** are run at 40–70% solvent B over 15 min and monitored at 550 nm.

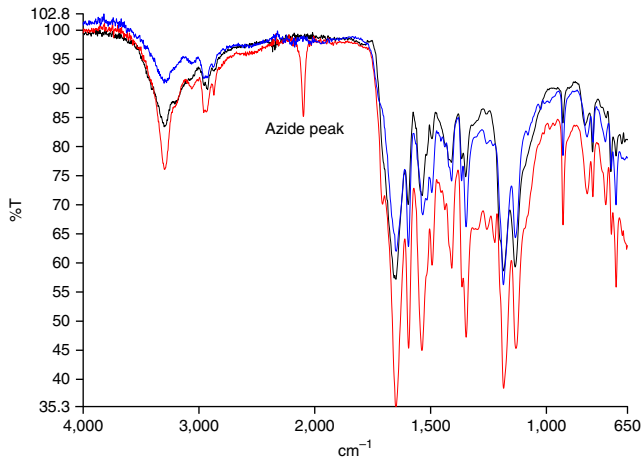


Figure 9 | IR spectra of stapled peptide **2** (black), unstapled peptide **2** (red) and wild-type $p53_{17-29}$ (blue). Only the spectrum of the unstapled peptide contains a peak at ~2,100 cm^{-1} for azides.

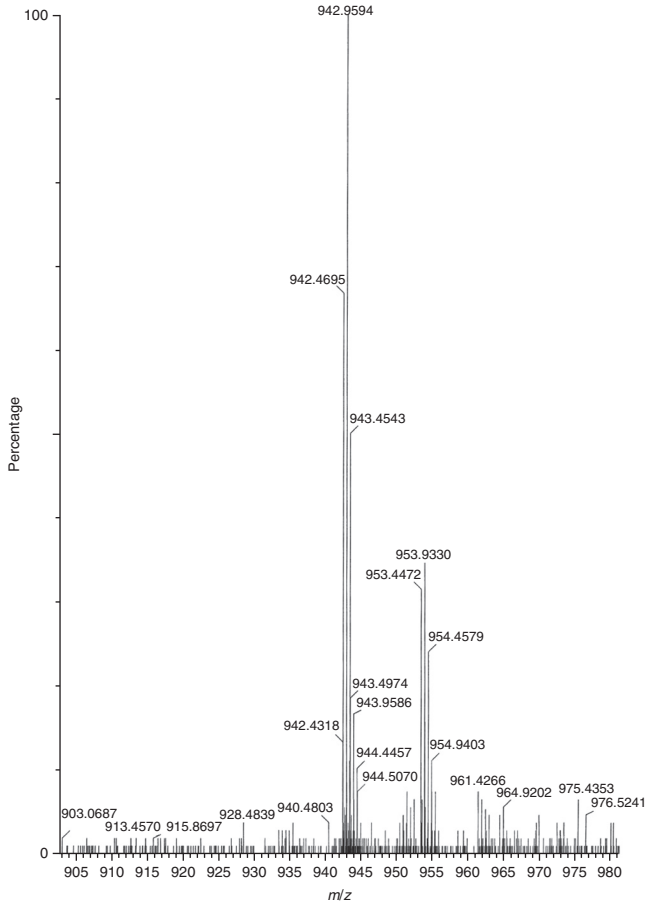


Figure 10 | Mass chromatograph of stapled peptide 1 showing the isotope peaks for $[M+2H]^{2+}$ separated by ~0.5 AMU, corresponding to peptides containing different numbers of the ^{13}C isotope.

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