Peptide Macrocyclization

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A Thiol–Ene Coupling Approach to Native Peptide Stapling and Macrocyclization**

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Dedicated to Professor Ken-Tsung Wong

Abstract: *We report the discovery of a peptide stapling and macrocyclization method using thiol–ene reactions between two cysteine residues and an α,ω-diene in high yields. This new approach enabled us to selectively modify cysteine residues in native, unprotected peptides with a variety of stapling modifications for helix stabilization or general macrocyclization. We synthesized stapled Axin mimetic analogues and demonstrated increased alpha helicity upon peptide stapling. We then synthesized stapled p53 mimetic analogues using pure hydrocarbon linkers and demonstrated their abilities to block the p53-MDM2 interaction and selectively kill p53 wild-type colorectal carcinoma HCT-116 cells but not p53 null cells. In summary, we demonstrated a robust and versatile peptide stapling method that could be potentially applied to both synthetic and expressed peptides.*

Since the seminal work by Grubbs[1] and Verdine[2] using olefin-containing amino acids followed by ring-closing metathesis (RCM), stapled peptides have developed into promising therapeutics to block protein–protein interactions or increase protease resistance.[3] The hydrocarbon stapled peptides have been demonstrated in targeting intracellular proteins such as the BCL-2 family proteins[4] and NOTCH[5] as well as extracellular proteins such as EGFR.[6] Due to its therapeutic potential, a growing number of studies reported alternative stapling methods such as lactamization,[7] cycloaddition,[8] oxime formation,[9] thiolether,[10] and S_NAr reaction.[11] Although some of these methods still require unnatural amino acids (UAAs) in the peptide synthesis, both lactamization and cysteine modification circumvent the use of UAAs and could potentially be applied to recombinantly expressed peptides and proteins. However, the additional amide bond and perfluoroaromatic group may affect the properties of the stapled peptides and lead to unwanted interactions or immunogenic effects. Furthermore, the scope of linker length and types is limited due to the restriction on the ligation reaction.

Various chemoselective bioconjugation methods have been developed to modify native amino acid residues such as lysine, cysteine, and tyrosine.[12] The relatively low abundance of cysteine in native proteins makes it a good candidate for site-specific modifications. Nowadays, there are methods[13] available using conjugate addition, arylation, alkylation, disulfide formation, and thiol–ene coupling. Among them, the thiol–ene click reaction is attractive due to its specificity to olefins and facile transformation.[14] A wide substrate scope from sugars[14a] to fatty acids[15] in bioconjugation has been demonstrated using the thiol–ene click reaction. Furthermore, thiol–ene reaction has been used for peptide macrocyclization using cysteine residues and alkene-substituted side chains.[16] However, macrocyclization of native, unmodified peptides using thiol–ene coupling is still not available. Here we report the discovery of a two-component thiol–ene-based peptide stapling and macrocyclization method in unprotected peptides. We further show that this method can be applied in synthesizing p53 mimetics for selectively inducing cell death.

We focused on determining optimal coupling conditions using a protected cysteine **1** and 1,7-octadiene **2a** as a model system (Figure 1). Among the five different radical initiators screened, DMPA **4a** gives the best yield of the desired product **3** at 85 % under 365 nm UV irradiation.

We then moved on to peptide YCKEACAL **5** with multiple unprotected functional groups to evaluate the chemoselectivity of this stapling method as a general macrocyclization method (Figure 2). The resulting product is expected to be a cyclic peptide with five amino acids and a linker. Peptide **5** (2 mM) was incubated with diene **2a**

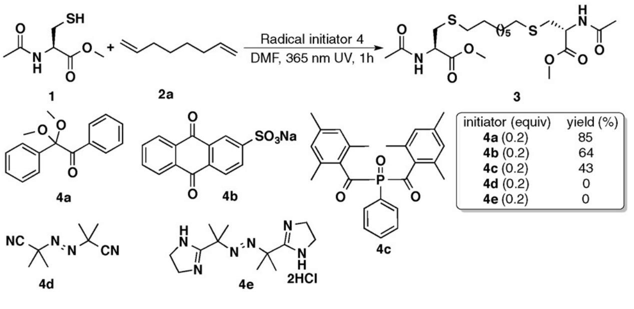

Figure 1. A model thiol–ene coupling between a protected cysteine **1** (2.5 equiv) and 1,7-octadiene **2a**.

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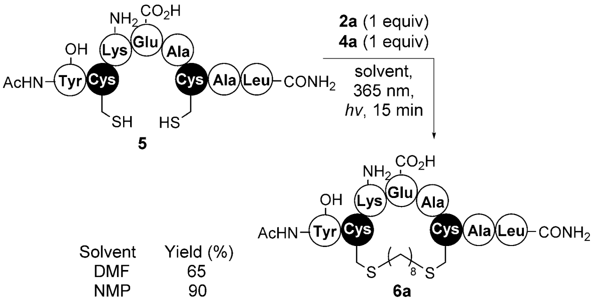
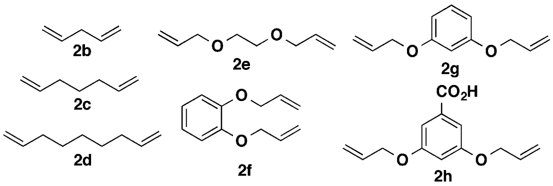


Figure 2. A model two-component thiol–ene coupling between an unprotected peptide **5** and 1,7-octadiene **2a**.

(1 equiv) in the presence of DMPA (1 equiv) in DMF and product **6a** was observed with 65% yield. By switching the solvent to NMP, we obtained **6a** in 90% yield. The yields were calculated based on the amount recovered after purification. The two-component thiol–ene coupling is chemoselective to thiol groups in the presence of functional groups such as amines, alcohols, and carboxylic acids.

With the optimized condition, we demonstrated the substrate scope using dienes with various length or heteroatom-substituted linkers (Scheme 1). All seven dienes reacted with peptide **5** in high yields (Figure 3). Next, we aimed at evaluating the possibility of using thiol–ene coupling in cyclizing longer peptides. Peptide **7** has seven amino acids between the two Cys residues and the expected cyclized peptide will have nine amino acids and a linker. As demonstrated in Figure 4, various cyclized peptides were synthesized in excellent yields using different diene linkers. Together, the two-component thiol–ene coupling represents an efficient approach in synthesizing cyclic peptides with flexible linker choices from native, unprotected peptides.



Scheme 1. Dienes used for the synthesis of macrocycles.

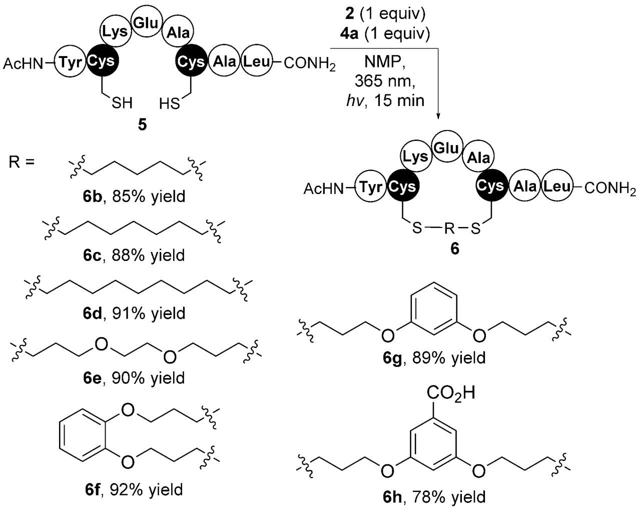


Figure 3. A two-component thiol–ene coupling between an unprotected peptide **5** and various dienes from Scheme 1. See SI for full characterization.

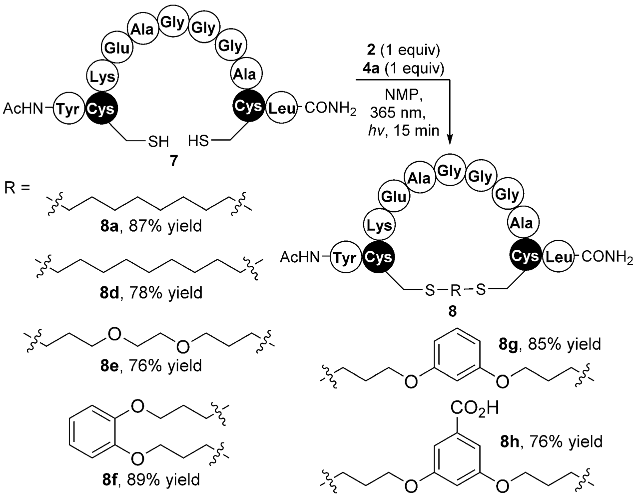


Figure 4. A two-component thiol–ene coupling between an unprotected peptide **7** and various dienes. See SI for full characterization.

The classic RCM approach represents a powerful method in synthesizing peptides stapled by a hydrocarbon linker. By using special UAAs, stapled peptides with $i, i+4$ or $i, i+7$ linkages were formed.[17] However, solid-phase peptide synthesis is needed due to the requirement of using UAAs and this limits its efficient uses in longer peptides (> 50 residues), which are generally expressed using recombinant DNA technology. Here, we aim to use the two-component thiol–ene coupling for direct peptide stapling of natural, unprotected peptides. We first synthesized peptide **9**, a stapled Axin mimetic analogue that was developed by the Verdine group to inhibit the Wnt signaling pathway[18] (Figure 5A). The incorporation of two S_5 amino acids followed by RCM gave the $i, i+4$-stapled peptide **9** (Figure 5B). We then synthesized unstapled peptide **10**, which has two Cys residues to replace the two S_5 amino acids. Peptide **10** was then reacted with dienes **2a** and **2c** to form peptides **11a** and **11c** by the two-component thiol–ene coupling (Figure 5C). Circular dichroism (CD) experiments were used to evaluate the alpha helicity of the stapled peptides. Both the unstapled peptide **10** and stapled peptide **11c** (7-carbon linker) have a low alpha-helical property. However, both literature-reported peptides **9** and stapled peptide **11a** (8-carbon linker) have strong alpha-helical characteristics as shown in Figure S1 (see the Supporting Information, SI). This suggests that the two-component thiol–ene coupling leads to the same structural features of the classic RCM method in stapling $i, i+4$ pairs.

Next, we synthesized peptide **12**, an $i, i+7$-stapled p53 mimetic developed by Walensky, Verdine, and co-workers to block the interaction between p53 and HDM2[19] (Figure 6A).

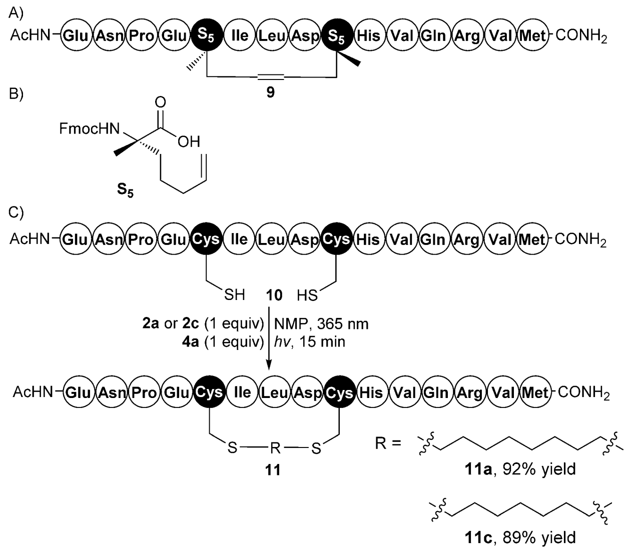


Figure 5. Peptide stapling in *I*, *i*+4 linkage. A) A stapled Axin analogue reported by the Verdine group. B) Structure of the S_5 amino acid used in the synthesis of **9**. C) Synthesis of stapled peptides from native, unprotected peptide **10** by the two-component thiol–ene coupling. See SI for full characterization.

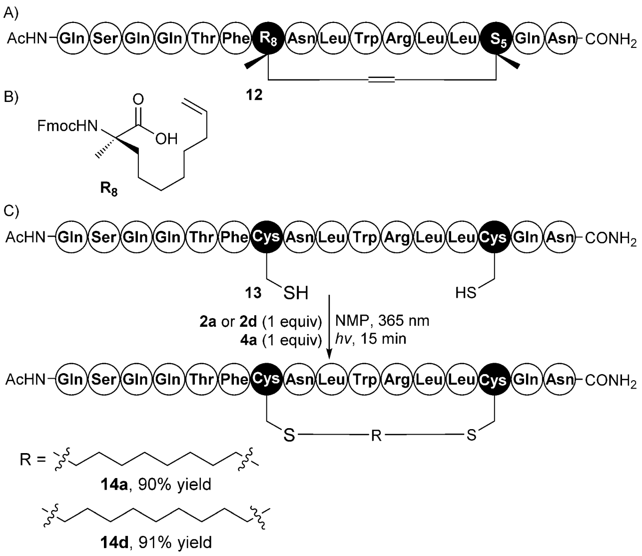


Figure 6. Peptide stapling in *i*, *i*+7 linkage. A) A stapled p53 mimetic analogue reported by the Walensky and Verdine group. B) Structure of the R_8 amino acid used in the synthesis of **12**. C) Synthesis of stapled peptides from native, unprotected peptide **13** by the two-component thiol–ene coupling. See SI for full characterization.

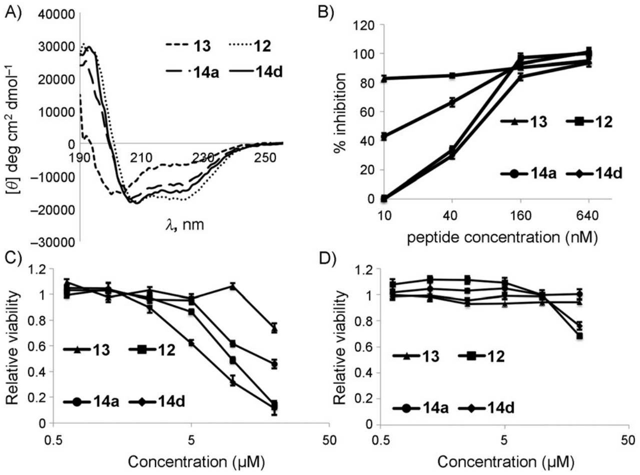


Figure 7. Biological characterization of unstapled and stapled p53 mimetics. A) CD spectra of all four peptides. B) Inhibition of p53–MDM2 interaction in an ELISA binding assay. Data represents mean values ± standard deviation (*n*=4). C,D) Cell viability assays in p53 wild-type HCT-116 cells (C) and p53 null HCT-116 cells (D). Data represents mean values ± standard deviation (*n*=4).

Due to the longer distances in the *i*, *i*+7 pair, an R_8 amino acid was used together with an S_5 amino acid for the stapling (Figure 6B). We synthesized peptide **13**, which has two Cys residues to replace the two UAAs. Peptide **13** was then reacted with dienes **2a** and **2d** to form peptides **14a** and **14d** by the two-component thiol–ene coupling (Figure 6C). As shown in the CD spectra, all the stapled peptides **12**, **14a**, and **14d** exhibit a significant increase of alpha-helicity compared to the unstapled peptide **13**. Specifically, **14d** shows similar alpha-helical characteristics with the reported stapled peptide **12** (Figure 7A). To test whether the structural feature translates to functional relevance, we performed an ELISA assay to quantify the interaction between p53 and MDM2 in the presence of the peptides (Figure 7B). Briefly, different concentrations of unstapled or stapled peptides were incubated with p53–MDM2 complexes and free MDM2 was detected by antibodies for signal readouts. For peptide **12**, we observed a similar efficacy in blocking the p53–MDM2 interaction as reported in literature. The cysteine-stapled peptide **14d** is as effective as peptide **12.** Peptide **13** is unstapled and does not have an alpha-helix structure. A similar unstapled peptide has been reported that does not block the interaction[19b] and therefore peptide **13** can be considered a negative control. Indeed, peptide **13** could not block the interaction (Figure 7B). We then performed a cell viability assay in p53 wild-type and p53-null HCT-116 colorectal carcinoma cells using peptides **12** or **14d** (Figure 7C and D). It was reported that peptide **12** selectively induces cell apoptosis in p53 wild-type cells but not p53 null cells.[19b] We were able to observe the same trend, and further showed that peptide **14d** also has this specificity. Together, we demonstrated that stapled peptides synthesized from native, unprotected peptides by the two-component thiol–ene coupling could recapitulate the structural features from the classic hydrocarbon-stapled peptides as well as the biological functions as demonstrated in the p53 mimetics.

In summary, we have demonstrated a facile and efficient synthetic platform for native peptide macrocyclization and stapling. The two-component thiol–ene coupling method operates at room temperature under 15 min UV irradiation and demonstrates excellent functional group tolerance. We first demonstrated its use as a general macrocyclization method using various diene linkers. Next, we demonstrated its use in synthesizing stapled peptides with both *i*, *i*+4 and *i*, *i*+

7 linkages. Importantly, we also demonstrated that the synthesized stapled peptides recapitulated the biological properties reported in the literature. Our method is complementary to the classic RCM method in synthesizing stapled peptides. It could be directly used on unprotected peptides without the use of UAAs and metal-based catalysts. Furthermore, similar hydrocarbon linkers were used to avoid nonspecific interactions from the bulky aromatic groups. Efforts in applying this method in stapling large peptides and proteins are currently under active investigation in our laboratory.

Keywords: bioconjugation · peptide macrocyclization · peptide stapling · thiol–ene coupling

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[1] H. E. Blackwell, R. H. Grubbs, *Angew. Chem. Int. Ed.* **1998**, *37*, 3281–3284; *Angew. Chem.* **1998**, *110*, 3469–3472.
[2] C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891–5892.
[3] a) L. D. Walensky, G. H. Bird, *J. Med. Chem.* **2014**, *57*, 6275–6288; b) P. M. Cromm, J. Spiegel, T. N. Grossmann, *ACS Chem. Biol.* **2015**, *10*, 1362–1375.
[4] L. D. Walensky, A. L. Kung, I. Escher, T. J. Malia, S. Barbuto, R. D. Wright, G. Wagner, G. L. Verdine, S. J. Korsmeyer, *Science* **2004**, *305*, 1466–1470.
[5] R. E. Moellering, M. Cornejo, T. N. Davis, C. Del Bianco, J. C. Aster, S. C. Blacklow, A. L. Kung, D. G. Gilliland, G. L. Verdine, J. E. Bradner, *Nature* **2009**, *462*, 182–188.
[6] J. K. Sinclair, E. V. Denton, A. Schepartz, *J. Am. Chem. Soc.* **2014**, *136*, 11232–11235.
[7] A. D. de Araujo, H. N. Hoang, W. M. Kok, F. Diness, P. Gupta, T. A. Hill, R. W. Driver, D. A. Price, S. Liras, D. P. Fairlie, *Angew. Chem. Int. Ed.* **2014**, *53*, 6965–6969; *Angew. Chem.* **2014**, *126*, 7085–7089.
[8] Y. H. Lau, P. de Andrade, S.-T. Quah, M. Rossmann, L. Laraia, N. Sköld, T. J. Sum, P. J. Rowling, T. L. Joseph, C. Verma, *Chem. Sci.* **2014**, *5*, 1804–1809.
[9] C. M. Haney, M. T. Loch, W. S. Horne, *Chem. Commun.* **2011**, *47*, 10915–10917.
[10] a) H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. DeGrado, D. C. Greenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 17704–17713; b) P. Timmerman, J. Beld, W. C. Puijk, R. H. Meloen, *ChemBioChem* **2005**, *6*, 821–824; c) P. Timmerman, W. C. Puijk, R. H. Meloen, *J. Mol. Recognit.* **2007**, *20*, 283–299.
[11] a) A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y. S. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946–5949; b) S. P. Brown, A. B. Smith 3rd, *J. Am. Chem. Soc.* **2015**, *137*, 4034–4037.
[12] E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; *Angew. Chem.* **2009**, *121*, 7108–7133.
[13] P. M. Cal, G. J. Bernardes, P. M. Gois, *Angew. Chem. Int. Ed.* **2014**, *53*, 10585–10587; *Angew. Chem.* **2014**, *126*, 10758–10760.
[14] a) A. Dondoni, A. Massi, P. Nanni, A. Roda, *Chem. Eur. J.* **2009**, *15*, 11444–11449; b) F. Li, A. Allahverdi, R. Yang, G. B. Lua, X. Zhang, Y. Cao, N. Korolev, L. Nordenskiold, C. F. Liu, *Angew. Chem. Int. Ed.* **2011**, *50*, 9611–9614; *Angew. Chem.* **2011**, *123*, 9785–9788; c) Y. Li, M. Yang, Y. Huang, X. Song, L. Liu, P. R. Chen, *Chem. Sci.* **2012**, *3*, 2766–2770.
[15] T. H. Wright, A. E. Brooks, A. J. Didsbury, J. D. MacIntosh, G. M. Williams, P. W. Harris, P. R. Dunbar, M. A. Brimble, *Angew. Chem. Int. Ed.* **2013**, *52*, 10616–10619; *Angew. Chem.* **2013**, *125*, 10810–10813.
[16] a) A. A. Aimetti, R. K. Shoemaker, C. C. Lin, K. S. Anseth, *Chem. Commun.* **2010**, *46*, 4061–4063; b) C. Hoppmann, R. Kuhne, M. Beyermann, *Beilstein J. Org. Chem.* **2012**, *8*, 884–889.
[17] Y. W. Kim, T. N. Grossmann, G. L. Verdine, *Nat. Protoc.* **2011**, *6*, 761–771.
[18] T. N. Grossmann, J. T. Yeh, B. R. Bowman, Q. Chu, R. E. Moellering, G. L. Verdine, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 17942–17947.
[19] a) F. Bernal, A. F. Tyler, S. J. Korsmeyer, L. D. Walensky, G. L. Verdine, *J. Am. Chem. Soc.* **2007**, *129*, 2456–2457; b) F. Bernal, M. Wade, M. Godes, T. N. Davis, D. G. Whitehead, A. L. Kung, G. M. Wahl, L. D. Walensky, *Cancer cell* **2010**, *18*, 411–422.

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