Stapled Peptides Hot Paper

International Edition: DOI: 10.1002/anie.201608648
German Edition: DOI: 10.1002/ange.201608648

Stapled Peptides by Late-Stage C(sp^3)–H Activation

Anaïs F. M. Noisier, Jesús García, Ioana A. Ionuţ, and Fernando Albericio**

Abstract: *Despite the importance of stapled peptides for drug discovery, only few practical processes to prepare cross-linked peptides have been described; thus the structural diversity of available staple motifs is currently limited. At the same time, C–H activation has emerged as an efficient approach to functionalize complex molecules. Although there are many reports on the C–H functionalization of amino acids, examples of post-synthetic peptide C–H modification are rare and comprise almost only C(sp^2)–H activation. Herein, we report the development of a palladium-catalyzed late-stage C(sp^3)–H activation method for peptide stapling, affording an unprecedented hydrocarbon cross-link. This method was first employed to prepare a library of stapled peptides in solution. The compatibility with various amino acids as well as the influence of the size (i,i + 3 and i,i + 4) and length of the staple were investigated. Finally, a simple solid-phase procedure was also established.*

With the attention of the pharmaceutical industry focused on alternatives to small molecules with higher target specificity, the use of peptide therapeutics is experiencing a real breakthrough.[1] Despite the major role of conformationally restrained peptides in current drug discovery programs,[2] only a limited number of reactions have been used to prepare cross-linked peptides.[3] The ring-closing metathesis stapling method pioneered by Verdine and co-workers[4] occupies the forefront of the field. These all-hydrocarbon staples have proven exceptionally efficient in stabilizing α-helices and rendering peptides cell-penetrating. Such peptides represent potential therapeutics against intracellular targets previously thought to be unreachable.[5] The progression of a p53 stapled peptide into clinical trials (NCT02264613) has triggered a wave of interest in new technologies to access novel staples.

Simultaneously, C–H activation has emerged as an attractive method for the preparation of natural products.[6] Owing to its efficiency, it soon found applications in peptide synthesis (Figure 1).[7] Our group and others have explored

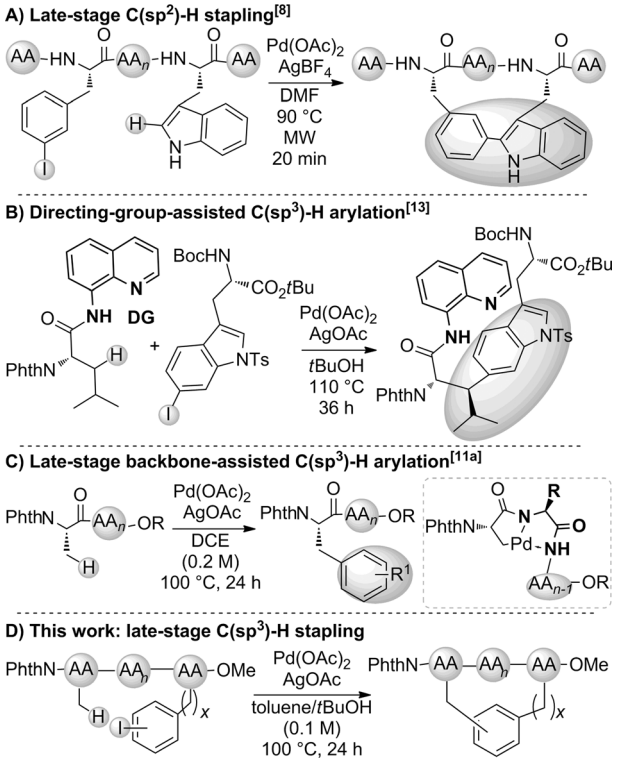

Figure 1. Strategies for C(sp^2)–H and C(sp^3)–H functionalization and peptide stapling.

C(sp^2)–H arylation reactions of the tryptophan (Trp) residue to generate post-synthetically modified linear peptides and tryptophan–phenylalanine cross-linked peptides (Figure 1A).[8] However, relative to the plethora of reports on C(sp^2)–H activation,[9] the metal-catalyzed functionalization of C(sp^3)–H bonds has been rarely addressed.[10] To the best of our knowledge, only limited examples of post-synthetic C(sp^3)–H peptide functionalization have been published to date.[11] Indeed, selectively activating relatively inert C(sp^3)–H bonds represents a greater challenge. To address these regioselectivity and reactivity issues, many research groups have focused their attention on the use of chelating directing groups (DGs).[12] This approach has been employed by Feng and Chen to access the key leucine–tryptophan motif and

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complete the synthesis of celogentin C (Figure 1B).[13] Despite these advances, the introduction and removal of DG's often implies additional and non-trivial steps, thus limiting the use of C(sp^3)-H activation to the modification of building blocks prior to peptide synthesis. Recently, Yu and co-workers[11a] postulated that peptide backbones are capable of acting as DGs, forming a similar N,N-dicoordinated complex with the Pd catalyst. They implemented this strategy for late-stage site-selective C(sp^3)–H arylation at the N-terminal amino acid of short peptides (Figure 1C), thus providing a practical method for rapid peptide derivatization.

Herein, we report the synthesis of a novel class of stapled peptides based on Yu's backbone-assisted C(sp^3)–H activation method. This new process produces an original staple motif featuring an unprecedented C(sp^3)–C(sp^2) linkage between an alanine (Ala) and a phenylalanine (Phe) residue (Figure 1D). This constitutes the first example of late-stage C(sp^3)–H peptide macrocyclization.

We envisaged that the intermediate resulting from the Pd catalyzed C–H activation of the primary β-C(sp^3)–H bond of phthaloyl (Phth) protected N-terminal Ala; would react intramolecularly with an iodophenylalanine residue introduced within the same peptide sequence to provide a structurally unique staple. To test our hypothesis, we prepared the linear tetrapeptide **1a** using standard 9-fluorenylmethyloxycarbonyl solid-phase peptide synthesis (Fmoc SPPS) procedures (see the Supporting Information). We then focused on the key in-solution C–H activation step, stirring **1a** (0.05 mmol), $Pd(OAc)_2$ (10 mol%), and AgOAc (2 equiv) in 1,2-dichloroethane (DCE; 0.1 M) at 100 °C for 24 h. Pleasingly, HPLC analysis of the crude reaction mixture showed 63% conversion into the desired $S_{i,i+3}S(5)$[14] product **2a**, which was formed as a single diastereoisomer (Figure 2). The main impurities were identified as the deiodination and aryl–aryl homocoupling side products. After purification, **2a** was fully characterized by NMR analysis and high-resolution mass spectrometry. These analyses confirmed that no loss of diastereoisomeric purity had occurred and that no cyclic homodimer was formed during the C–H macrocyclization step. As previously reported by Yu and co-workers,[11a] the C–H activation was site-selective for the N-protected terminal Ala residue, and the product of C–H activation at Ala2 (Ala residue in position 2 from the N-terminus) was not observed.

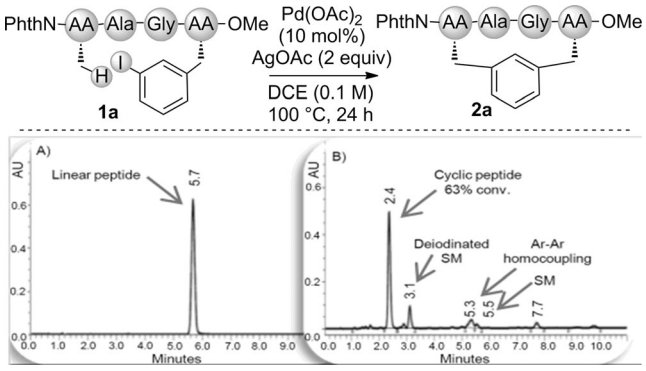


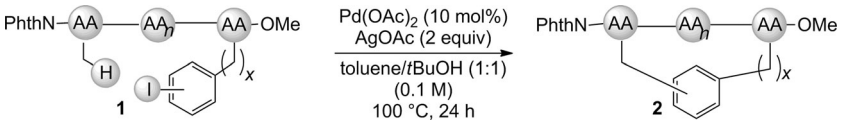
Figure 2. Initial C(sp^3)–H stapling experiment. A) HPLC chromatogram of the pure linear peptide. B) HPLC chromatogram of the crude cyclization reaction mixture.

In an attempt to improve the outcome of the reaction, silver oxidants, acid additives,[15] and microwave irradiation were screened (see the Supporting Information) but only lower conversions were achieved. The role of the solvent appeared to be crucial as the use of *t*BuOH as the only solvent considerably suppressed side-product formation while only incomplete conversion of the starting material (SM) was observed (see the Supporting Information, Table S1, entry 9). With this in mind, we investigated the effect of 1:1 solvent mixtures. The use of toluene/*t*BuOH provided the best result (88% conversion, 29% yield; see Table S1, entry 11 and Figure S10).

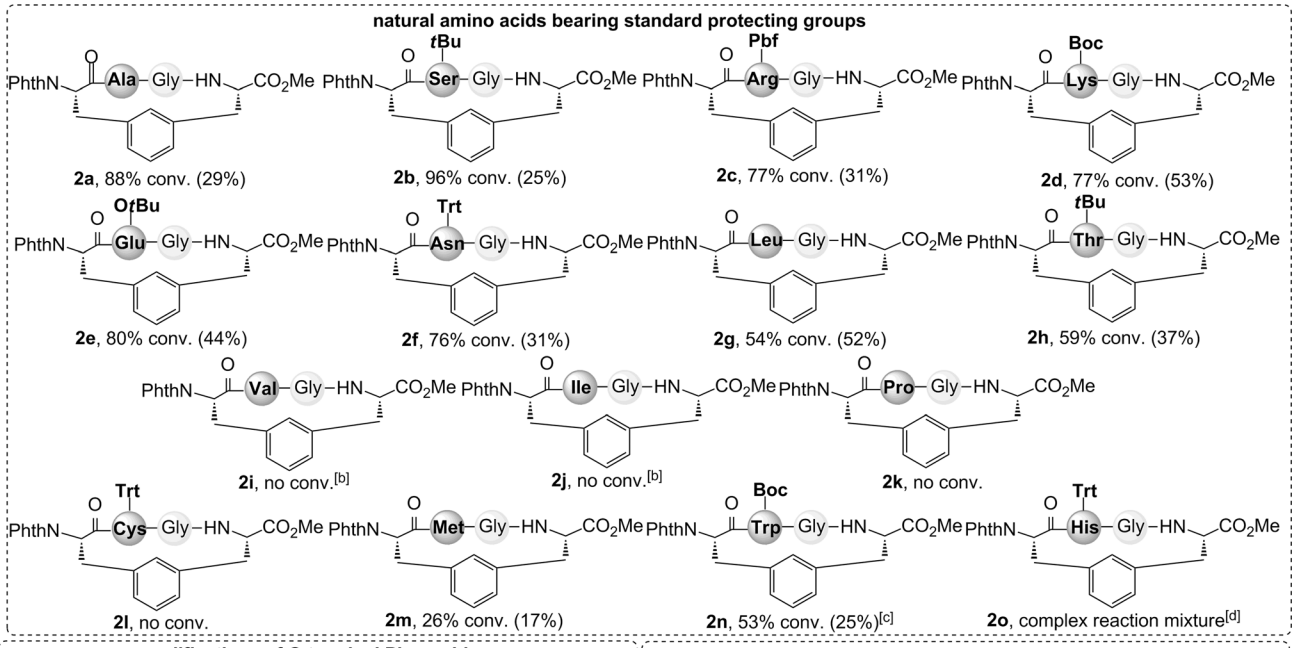
With optimized reaction conditions in hand, we next turned to the evaluation of the scope of the reaction (Table 1). We first set out to confirm the compatibility of the reaction conditions with natural amino acids bearing standard protecting groups for Fmoc SPPS, as the pioneering work by the Yu laboratory[11a] mainly focused on peptides featuring all-hydrocarbon side chains. Gratifyingly, the reaction tolerated *O-tert*-butyl-L-serine (Ser(*t*Bu)), N_{ϖ}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine (Arg(Pbf)), N_{ε}-*tert*-butyloxycarbonyl-L-lysine (Lys(Boc)), L-glutamic acid 5-*tert*-butyl ester (Glu(O*t*Bu)), and N_{γ}-trityl-L-asparagine (Asn(Trt)), affording very good to excellent conversions (**2b**–**2f**). In addition, branched amino acids such as L-leucine (Leu) and *O-tert*-butyl-L-threonine Thr(*t*Bu) in position $i+2$ did not prevent the staple formation from proceeding with good conversion (**2g** and **2h**). The effect of L-isoleucine (Ile) and L-valine (Val) on peptide stapling could not be investigated as peptides **1i** and **1j** proved to be insoluble in the solvent mixture. Unfortunately, the conformational bias resulting from the use of L-proline (Pro) in position $i+2$ (**1k**) prevented C–C bond formation. The stapling of the challenging peptides **1l**/**1m** and **1n**/**1o**, which bear Pd-deactivating sulfur atoms and reactive C(sp^2)–H bonds, respectively, was also investigated. Whereas *S*-trityl-L-cysteine (Cys(Trt)) containing **1l** underwent thiol elimination, the L-methionine (Met) containing product **2m** was obtained with 26% conversion. Surprisingly, the macrocyclization of L-Trp(Boc)-containing **1n** preferentially occurred through the C(sp^3)–H bond to afford **2n** with 53% conversion. Although C(sp^3)–H stapling of 1-trityl-L-histidine (His(Trt)) containing **1o** was observed, a complex mixture was obtained owing to partial loss of the Trt protecting group.

Next, we focused on modifying the C-terminal amino acid and prepared peptides **1p** and **1q** with 3-iodo-D-Phe and 4-iodo-L-Phe, respectively. While changing the stereochemistry did not affect the outcome of the C–H arylation (**2p**, 88%), the attempted cyclization of **1q,** featuring the iodo substituent in *para* position, led to a complex reaction mixture. We also examined the macrocyclization of peptides **1r** and **1s** containing N-terminal 2-aminoisobutyric acid (Aib) and Phe, respectively. Interestingly, β-C(sp^3)–H activation of the prochiral Aib residue in **1r** occurred with desymmetrization to afford product **2r** with 57% conversion and excellent diastereoselectivity (93:7 d.r.). However, the activation of the secondary β-C(sp^3)–H bond of the Phe residue failed.

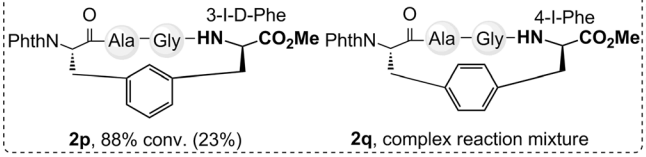
Table 1: Reaction scope.[a]



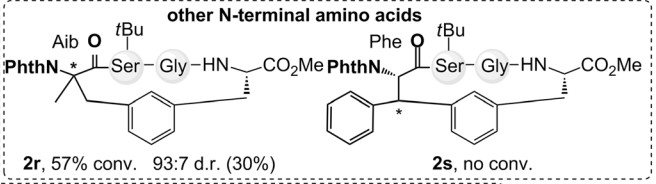
natural amino acids bearing standard protecting groups



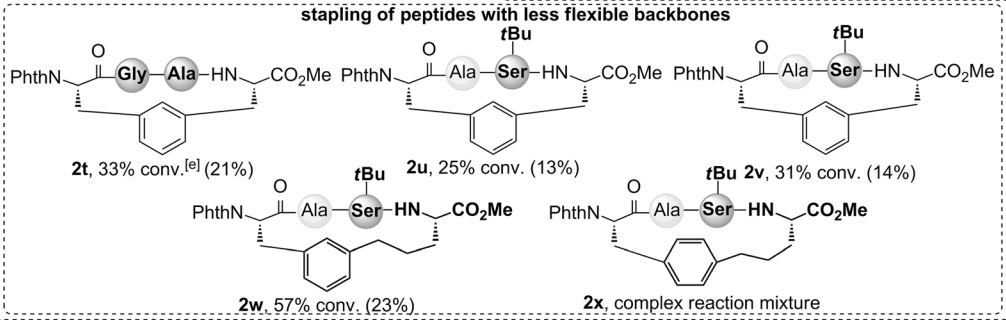
modifications of C-terminal Phe residue



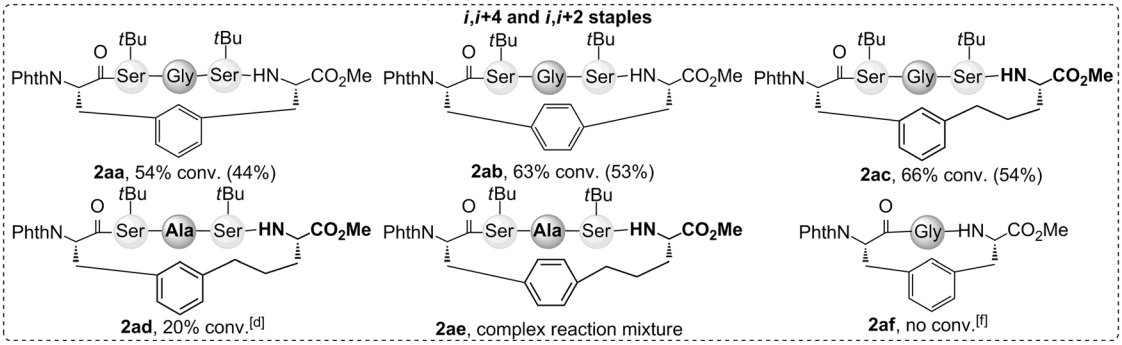
other N-terminal amino acids



stapling of peptides with less flexible backbones



***i,i*+4 and *i,i*+2 staples**



[a] Conversion into the desired product determined by HPLC analysis.[16] Yields of isolated products given in parentheses. [b] Starting material not soluble. [c] Yield of isolated Boc-deprotected product **2n′**. [d] Not isolated. [e] In DCE. [f] The cyclic dimer **2ag** was formed instead of **2af**.

Intrigued by the role of the flexible glycine (Gly) residue in the formation of the relatively short staple, we designed peptide **1t** where the positions of Ala2 and Gly3 were exchanged. Surprisingly, **1t** showed poor solubility in the solvent mixture, and after 24 h at 100 °C, only the starting material was observed. However, carrying out the reaction in DCE afforded **2t** with 33 % conversion. To overcome the solubility issue, peptides **1u** (Ala2–Ser3) and **1v** (Ala2–D-Ser3) were synthesized. Independent of the side-chain orientation, both **1u** and **1v** reacted with moderate conversions (25 % and 31 %, respectively), thus attesting to the role of the Gly3 residue as an efficient staple-enabling amino acid.

Next, we investigated the influence of the length of the cross-link on the $i,i+3$ stapling of Gly-free peptides. First, unnatural amino acids with longer side chains; *meta*- (**3a**) and *para*-iodophenylpropylglycine (**3b**); were prepared using a diastereoselective Mitsunobu-type alkylation of a Ni complex of Gly Schiff base (see the Supporting Information).[17] These novel building blocks were introduced into **1w** and **1x**, which, after C–H activation, should give rise to an $S_{i,i+3}S(7)$ and an $S_{i,i+3}S(8)$ peptide, respectively. While the reaction reached completion with **1w** (57 % conv. into the desired product), which features the iodo group in the *meta* position of the aromatic ring, the macrocyclization of peptide **1x** afforded a complex mixture of products.

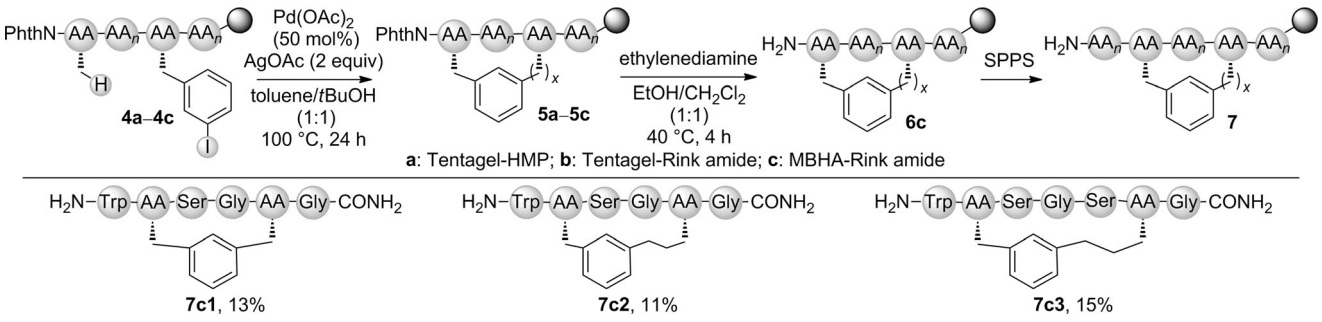
With a general method for $i,i+3$ stapling in hand, we turned towards $i,i+4$ cross-linked systems. We prepared a series of Gly3-containing $S_{i,i+4}S(5)$, $S_{i,i+4}S(6)$, and $S_{i,i+4}S(7)$ peptides **2aa**–**2ac** (54–66 % conv.) using *meta*- and *para*-iodo-L-Phe as well as **3a**, respectively. Unfortunately, our attempt to prepare Gly-free $S_{i,i+4}S(7)$ peptide **2ad** led to an incomplete reaction (20 % conv.) after 24 h, thus underlining the importance of the length of the staple for the efficient stapling of peptides with less flexible backbones. As previously observed for peptides **2q** and **2x** with the *para*-iodo-Phe residue, stapling of peptide **1ae** led to a complex reaction mixture. On the other hand, attempts at $i,i+2$ stapling did not lead to monomeric **2af** but to the dimeric cross-linked peptide **2ag** (see the Supporting Information).

Finally, we endeavored to transpose the method developed in solution onto solid phase (Scheme 1). Initial on-resin experiments using ChemMatrix 4-[(*R*,*S*)-(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide) or ChemMatrix derivatized with 4-hydroxymethylphenoxyacetic acid (HMP) linker failed. Pleasingly, when Tentagel-HMP and Tentagel-Rink amide resin linkers were employed, the resin-bound stapled peptides **5a** and **5b** were obtained with 73 % and 57 % conversion, respectively (see the Supporting Information). Interestingly, despite the use of *t*BuOH in the solvent mixture, significant amounts of the deiodinated side products were formed, with higher amounts observed for resin **5b** featuring the Rink amide linker. Unfortunately, the HMP linker was not stable under the reaction conditions required for phthalimide deprotection. An improved stapling conversion of 62 % was achieved using the methylbenzhydryl amine (MBHA) Rink amide resin linker **5c**. The N-terminal phthalimide protecting group was removed by treatment with 20 % ethylenediamine in $EtOH/CH_2Cl_2$ (1:1) at 40 °C, affording resin-bound peptide **6c**. Next, the peptide could be further elongated by standard SPPS. Peptides **7c1**–**7c3**, containing $S_{i,i+3}S(5)$, $S_{i,i+3}S(7)$, and $S_{i,i+4}S(7)$ staples, were prepared in 11 to 15 % yield following this method (see the Supporting Information). The effect of these novel cross-links on the secondary structure of the peptides was determined by circular dichroism (see the Supporting Information). Whereas peptides **7c1** and **7c2** showed a positive maximum at 195 nm, thus indicating some structural organization, peptide **7c3** and linear peptides exhibited flattened profiles typical of unfolded structures.

In conclusion, we have developed a new method for the late-stage stapling of peptides by a $C(sp^3)$–H activation approach that does not rely on the use of external directing groups. A library of peptides featuring a structurally unique staple was prepared in solution, demonstrating the compatibility of the reaction with a wide range of side-chain functional groups. Aside from $i,i+3$ stapling, $i,i+4$ cross-linking was also achieved. Furthermore, a practical solid-phase procedure was developed, affording rapid access to new peptide motifs. This technology is a powerful addition to the repertoire of currently available stapling techniques and should make a significant contribution to the design of future peptide therapeutics. The application of this new method to biologically active peptides is in progress in our laboratory.

Acknowledgements

This project has received funding from the European Union's Seventh Framework Programme for research, technological



Scheme 1. On-resin synthesis of stapled peptides.

development and demonstration under grant agreement no 623737.

Conflict of interest

The authors declare no conflict of interest.

Keywords: C–H activation · macrocycles · palladium · peptides · stapled peptides

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Manuscript received: September 4, 2016
Final Article published: ■■ ■■, ■■■■

Communications

Stapled Peptides

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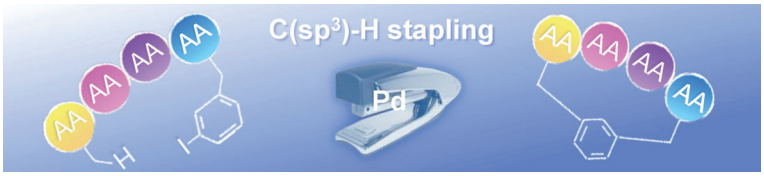
Stapled Peptides by Late-Stage C(sp^3)–H Activation



Staple 'em all: A new peptide stapling method based on palladium-catalyzed late-stage C(sp^3)–H activation is described, which provides peptides featuring a structurally novel all-hydrocarbon crosslink between an Ala and a Phe residue. A library of (i,i+3)- and (i,i+4)-stapled peptides was prepared using both in-solution and on-resin procedures.

