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An Intramolecular Tryptophan-Condensation Approach for Peptide Stapling†

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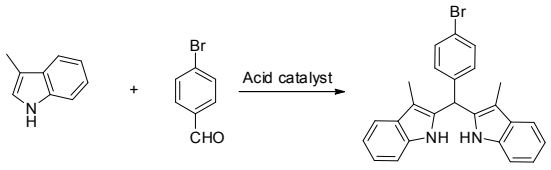
Stapled peptides are gaining tremendous interest as next-generation therapeutic agents to target protein-protein interactions. Herein, we report an intramolecular peptide stapling method which links two tryptophan residues at C2 position of the indole moieties via acid-mediated condensation with an aldehyde.

Intramolecular stapling of linear peptides is used as an approach to confer enhanced α-helicity, protease resistance and target binding affinity relative to their linear counterparts.[1-3] With the emerging trend of stapled peptides as a new class of medicine,[4] there is an urgent need to develop a feasible synthetic stapling route using natural amino acids and mild table-top chemical reagents; as an alternative to classical stapling,[5] which involves the use of expensive and synthetically challenging unnatural amino acids[5a-d] and reagents.[5e]

With the rare occurrence of tryptophan (Trp) residues in protein structures,[6] these residues are ideal for controlled single-site modification, functionalisation and derivatisation. However, it is not easy to activate the aromatic scaffold of tryptophan under mild conditions, thus posing significant challenges to site-specific functionalisation. Utilising Pd-catalysed C-H activation, Lavilla and colleagues successfully linked Trp to iodo-phenylalanine (Phe) or iodo-tyrosine (Tyr).[6] This method involves the use of transition metals which is not cost effective in large scale manufacturing and requires complex purifications to remove traces of toxic metals that may bind to the peptide. Makwana and Mahalakshmi recently reported a Trp - Trp cross linking strategy to form hyperstable peptides with β-hairpin and α-helical structures.[7] Crosslinking was achieved by incubating peptides in trifluoroacetic acid (TFA) in the absence of an inert environment, limiting this approach to peptides containing aliphatic side groups. In this report, we present a new peptide-stapling methodology that links two Trp residues via a concomitant condensation reaction with an aldehyde.

We extend the strategy used to synthesise 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene, also known as boron dipyrromethene (BODIPY),[8] to fuse indoles into a C_2-symmetric structure with an arylaldehyde. Using skatole as a model to mimic tryptophan residues, condensation reactions were carried out using skatole and 4-bromobenzaldehyde in the presence of acids. A variety of acids and methods were screened (Table 1). Desired condensation product **3** was obtained in good yield (72%) using (1*S*)-(+)-camphor-10-sulphonic acid (CSA, Table 1, entry 2). The formation of various possible side products (ESI,† Fig. S1) might have attributed to low

Table 1 Optimisation of the conditions for skatole condensation



1 2 3

Entry	Acids	Method[a]	Yield
1	CH_3SO_3H	A	0[b]
2	$C_9H_{15}C(O)SO_3H$[c]	A	72
3	$CH_3C_6H_4SO_3H$	A	62
4	CF_3COOH	A	38
5	HCl	B	4
6	H_2SO_4	C	85

[a]Method A: **1** (0.32 mmol), **2** (0.16 mmol) and acid (5 equiv) were dissolved in CH_2Cl_2 and the mixture was heated at 40 °C for 6 h in a sealed tube. Method B: **1** (0.32 mmol), **2** (0.16 mmol) were dissolved in acid and the mixture was heated at 100 °C for 3 h in a sealed tube. Method C: **1** (0.32 mmol), **2** (0.16 mmol) and acid (10 µL) were dissolved in EtOH and the mixture was stirred at room temperature for 18 hours. [b]**3** was not obtained. [c](1*S*)-(+)-Camphor-10-sulfonic acid (CSA).

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yields (Table 1). The condensation was also performed using mineral acids[9,10] (Table 1, entry 5-6), of which sulphuric acid (Table 1, entry 6) resulted in the highest yield (85%) of product **3**. After the successful linkage of skatole at the C2 position, it was envisioned that this method can be adopted to link Trp residues present in various positions of the linear peptides to produce cyclic stapled peptides as an alternative to using unnatural amino acids in conventional stapling.

With these preliminary results, sMTide-02, a stapled peptide that binds to MDM2 and activates p53,[11] was selected as a model system to demonstrate the applicability of our new stapling approach. The sequence was truncated to encompass key residues thus enabling us to establish a minimum-residual-spacing between two tryptophan residues that is required for cyclisation.

Although conversion using sulphuric acid is higher than when using CSA for the model cyclisation (Table 1), CSA was chosen over harsh sulphuric acid for peptide stapling considering the sensitive nature of various amino acids. Peptide **4**, 4 bromobenzaldehyde[12] and CSA in 1, 2-dichloroethane (DCE) were heated and mixed at an elevated temperature of 60 °C for three hours (Table 2, entries 1-9). Good conversions were observed for Trp residues located at *i, i+n* (n=1 to 4) positions (Table 2, entries 1-4). Moreover, this stapling strategy is used to cyclise peptides with various side chains (Table 2, entries 5-7). This methodology was also successfully used for the synthesis of cyclic peptides conta-ining Asn-Gly-Arg (-NGR-)[13] and Arg-Gly-Asp(-RGD-)[14] which are important fragments of biologically active compounds (Table 2, entries 8, and 9). When CSA-mediated tryptophan condensation was carried out in solution phase, the conversion was lower as compared to the solid phase for the formation of stapled peptide **5d.** In addition, with the usage of sul-

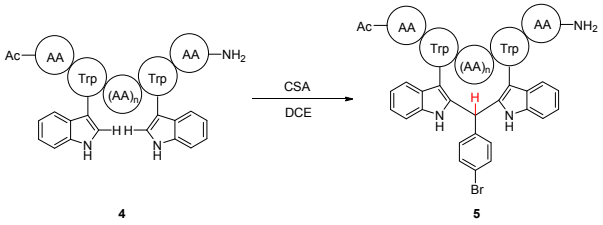
-phuric acid for condensation, crude cyclised peptide with lower purity was obtained.

The ^{1}H NMR spectrum of **5d** shows a singlet peak for the newly formed C-H at δ 6.13, which indicates the successful cyclisation of linear counterpart **4d** (ESI,† Fig. S7). The ^{1}H NMR singlet peaks for the newly formed aliphatic C-H (highlighted in red in the figure in Table 2) for stapled peptides **5a**, **5b** and **5d** (ESI,† Fig. S8) have different chemical shifts. The slight changes in chemical shifts are due to the different number of amino acid residues present between the two Trp residues that result in different ring sizes and ring strains upon peptide stapling.

One of the key benefits of stapled peptides is their enhanced proteolytic stability[2] over the linear counterparts. Hence, tryptophan-linked stapled peptide **5d** (Fig. 1A) and its linear counterpart **4d** were treated with chymotrypsin to compare their proteolytic resistance. HPLC profiles revealed that stapled peptide **5d** was resistant towards enzymatic degradation, whereas its linear precursor **4d** degraded rapidly. This suggests that peptide stapling through tryptophan condensation could bring about enhanced stability against proteolytic cleavage and may be useful for the development of systemic peptide-drugs resistant to proteolytic degradation.

Encouraged by our findings above, a further step was taken to investigate the effect of tryptophan stapling technique on peptide structure. Circular dichroism (CD) studies on peptide **5d** and its linear precursor **4d** (ESI,† Fig. S4 and S5) were carried out. It was observed that the cyclisation resulted in very marginal conformational change in the peptide (Fig. 1B). Although tryptophan condensation did not impart any α-helicity to the *i*, *i+4* peptide, we postulate that the tryptophan linkage resulted in a macrocyclic structure that is highly resistant to proteolytic degradation.

Table 2 Synthesis of various stapled peptides using CSA



4 5

Entry (%)[a]	*i, i+n*	Linear Peptide **(4)**	Cyclic Peptide **(5)**	Conv.
1	*i, i+*1	Ac-WWALL-NH_2 **(4a)**	**5a**	100
2	*i, i+*2	Ac-WAWLL-NH_2 **(4b)**	**5b**	98
3	*i, i+*3	Ac-WALWL-NH_2 **(4c)**	**5c**	93
4	*i, i+*4	Ac-WALLW-NH_2 **(4d)**	**5d**	93
5	*i, i+*4	Ac-EYWALLW-NH_2 **(4e)**	**5e**	95
6	*i, i+*4	Ac-YWALLWS-NH_2 **(4f)**	**5f**	95
7	*i, i+*4	Ac-ALWALLW-NH_2 **(4g)**	**5g**	100
8	*i, i+*4	Ac-WNGRW-NH_2 **(4h)**	**5h**	81
9	*i, i+*4	Ac-WRGDW-NH_2 **(4i)**	**5i**	94

Coupling conditions: Peptide **4** (1 equiv), CSA (6 equiv) and 4-bromobenzaldehyde (10 equiv) in 1, 2-dichloroethane (DCE) were heated at 60 °C for 3 h in a sealed tube. The resin was agitated with a stream of nitrogen gas. Isolated yields of 1% are obtained for **5a**, **5b** and **5d** after solid phase peptide synthesis and CSA mediated condensation. [a]Percent conversion was determined by estimating the consumption of linear peptide **4** using HPLC.[15] AA– amino acid, HPLC- high-performance liquid chromatography, conv.- conversion.

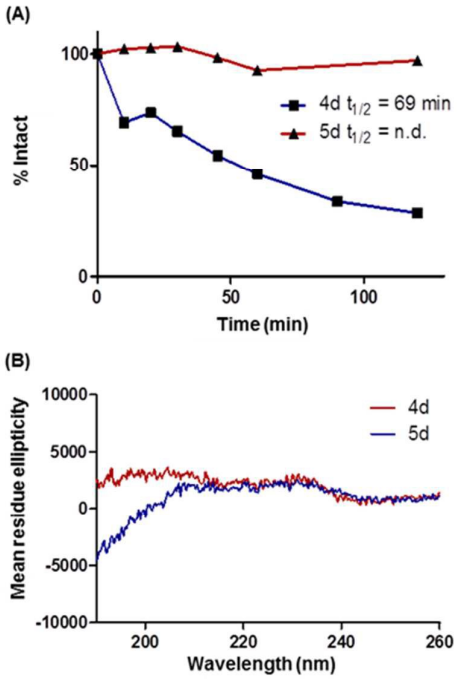


Fig. 1 (A) Chymotrypsin proteolysis and B) circular dichroism study of linear peptide **4d** (dark red) and corresponding cyclic peptide **5d** (blue).

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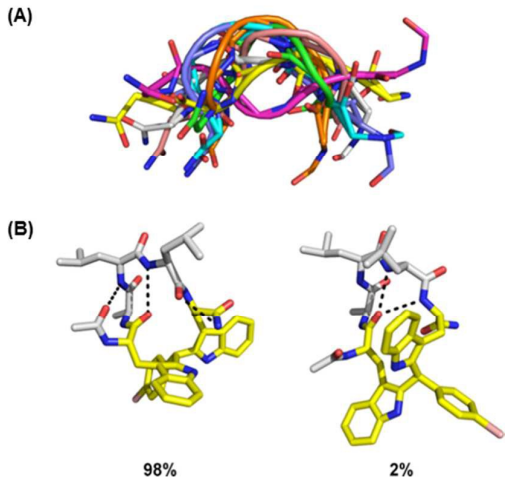

Fig. 2 Conformations of **4d** and **5d** observed in MD simulations. (A) Superimposition of representative conformations of **4d**. Side chain atoms are not shown. (B) Representative conformations of **5d**. Percentage populations of the respective conformations are indicated. The tryptophan linker is in yellow while the rest of the peptide is in grey.

To understand the conformation of linear and cyclic peptides, *insilico* studies on **4d** and **5d** were performed. Molecular dynamics (MD) simulations of the peptides in water followed by root-mean-square-deviation-based clustering of the sampled structures revealed that **4d** was highly flexible and adopted a variety of extended and folded conformations (See Supplementary Information for computational details). In contrast, the stapled peptide **5d** assumed two main conformations (Fig. 2). The dominant conformation of **5d** (98%) was non-α-helical and characterised by the absence of an *i*, *i* + 4 backbone hydrogen bond between the stapled residues. There is a small cluster of α-helical conformations (2%). The paucity of helical conformations obtained from the simulations explained the non-helical conformation of **5d** in solution, as indicated by its circular dichroism (CD) spectrum.

We observed that the two indole rings of **5d** are nearly perpendicular to each other in the MD simulations (Fig. 2B). Hence, emission spectra of the tryptophan on the linear **4d** and cyclized **5d** were obtained (Fig. 3). The high intensity of emission for **4d** in solution probably results from π-π stacking[16] between the two aromatic indole rings in the tryptophan residues. Fluorescence intensity reduced drastically upon cyclisation indicating that the indole rings are structurally restricted[17,18] and are unable to align properly to form π-π stacking interactions. This photophysical observation is in agreement with the MD simulations (Fig. 2).

Conclusions

In summary, a new intramolecular tryptophan stapling strategy using an aldehyde under mild acidic condition for peptides is reported. Several stapled peptides were synthesized, of which two, peptides **4d** vs. **5d** were further investigated for helicity, proteolytic stability, fluorescence measurements and their conformational landscape explored using MD simulations. The use of natural amino acid i.e. tryptophan as a cyclization fragment and the resulting enhanced proteolytic stability upon condensation are key new developments we report. Further investigations could result in improved yields, making this method more synthetically viable. This study provides a strong complement to existing methods for the synthesis of stapled peptides using natural amino acids.

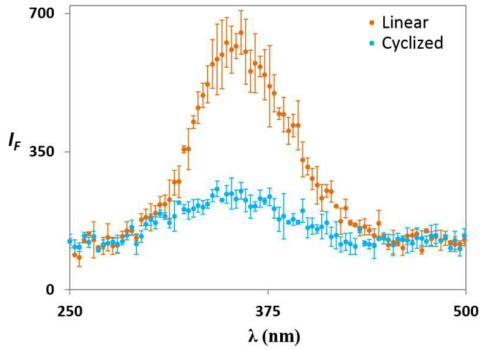

Fig. 3 Emission spectra of the 10 μM solution of the linear peptide **4d** (orange) and cyclized peptide **5d** (blue) in methanol. Each spectrum represents average of three different measurements with standard deviations. λexc. = 230 nm.

Conflicts of interest

There is no conflict of interest.

Acknowledgement

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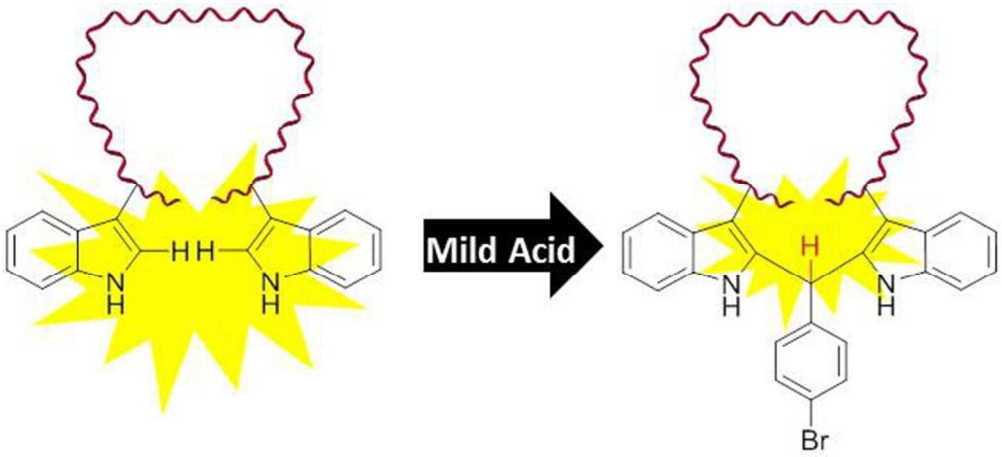

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