Organic LETTERS

Letter

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Trp-Trp Cross-Linking: A Structure–Reactivity Relationship in the Formation and Design of Hyperstable Peptide β-Hairpin and α-Helix Scaffolds

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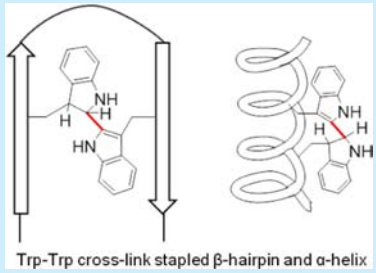
Supporting Information

ABSTRACT: Using model peptide β-hairpin scaffolds, the facile formation of a remarkably stable covalently cross-linked modification is reported in the tryptophan side chain, which confers hyperstability to the scaffold and displays a unique structure–reactivity relationship. This strategy is also validated to obtain a thermostable α-helix. Such imposition of conformational constraints can have versatile applications in peptide-based drug discovery, and this strategy may improve peptide bioavailability.



Trp-Trp cross-link stapled β-hairpin and α-helix

Conformational constraints in a polypeptide chain, imposed by natural or non-natural methods, have recently attracted attention in understanding the fundamental forces driving protein folding as well as in rational design of pharmacologically active peptides with improved stability.[1] Aromatic amino acids or engineered disulfides (natural scaffold stabilizers)[1a,c,f] and hybrid α-, β-, γ-, D-amino acids or covalent peptide modifications (non-natural)[1b,d,e,h] impart scaffold stability and specificity. Strategies employing covalent modifications have opened a new era in the generation of "stapled peptides",[2] a scenario wherein non-natural chemical linkages join two segments of a polypeptide chain. To date, several stapling strategies such as hydrogen bond surrogates,[2b] hydrocarbon cross-links,[2a] and cysteine arylation-mediated stapling[2c] have resulted in the development of potent α-helix mimetics. However, the field of stapling β-sheets and β-hairpins is still at its inception. With our increasing knowledge in the design of synthetic peptide hairpins for novel therapeutics, β-hairpin stapling can find versatile applications from peptide-based therapeutics to robust biomaterial generation.[3]

The β-hairpin is the simplest unit of a β-sheet, comprising antiparallel strands connected by a short turn. Model β-hairpin peptides have faithfully served as excellent model systems to probe a variety of chemistries.[3,4] While disulfides have been widely employed for stabilizing β-hairpin peptides and small molecule inhibitors of protein–protein interactions,[5] reported instances wherein disulfides cause structure destabilization[1f,6] accentuate the need for alternative scaffold stabilizers.

Nature has also provided us with unnatural cross-links between naturally occurring amino acids.[7] For example, biaryl cross-links, such as dityrosine and ditryptophan, can impose conformational constraints on peptide scaffolds for performing specific roles.[9] However, little is known about biaryl cross-links in scaffold stabilization, except for reports of ditryptophan cross-links to induce inverse γ-turns or bridge tripeptides,[9a,10] thus limiting its pharmaceutical application. Few studies are available on biaryl-bridged macrocyclic peptides as tools to constrain the polypeptide chain;[9c,d] however, such peptides may not adopt a regular secondary structures.

As a part of an ongoing investigation to understand the impact and geometry of aromatic interactions in β-hairpin stabilization, we identified the surprising formation of a stable Trp-Trp cross-link, which results in a hyperstable β-hairpin. We report here the synthesis, complete structural characterization, structure–reactivity relationship, and scaffold-stabilizing role of the Trp-Trp cross-link. We also validate this strategy to obtain a thermostable cross-link in an α-helix scaffold.

Table 1 lists the sequences of parent peptides and their cross-linked variants examined here. We chose model octapeptide β-hairpin scaffolds[8,11] and placed tryptophans at the non-hydrogen-bonding position to promote aryl interactions.[8,11c] Peptides ^{D}PG-WW-1 and ^{D}PG-WW-2 were commercially generated, while others were synthesized in-house (see the Supporting Information).[8,11c] The ^{L}PG-WW permutant acted as the "unfolded" control. Further, we introduced an N-terminal D-isomer in ^{D}PG-WW-1 to test its conformational effect on cross-linking.

Peptides were synthesized using conventional solid-phase Fmoc chemistry on the Rink amide resin. After completion of the synthesis, cleavage of the peptide was achieved using a 4-h incubation at 25 °C in the cleavage cocktail comprising TFA/phenol/water/TIPS in a ratio 88:5:5:2 (details in the Supporting Information). After TFA evaporation, crude peptides were precipitated using cold diethyl ether and purified

Received: April 9, 2015
Published: May 4, 2015

DOI: 10.1021/acs.orglett.5b01017
Org. Lett. 2015, 17, 2498–2501

Table 1. Sequences of Peptides Studied

Peptide	Sequence [a]	% Folded [b]
^{D}PG-WW-1 [c]	Ac-LWV-^{D}PG-LWV-NH$_2$	59
^{D}PG-WW-1-CL	Ac-LWV-^{D}PG-LWV-NH$_2$	100
^{L}PG-WW [c]	Ac-LWV-^{L}PG-LWV-NH$_2$	08
^{D}PG-WW-2 [c]	Ac-^{D}LWV-^{D}PG-LWV-NH$_2$	76
^{D}PG-WW-2-CL	Ac-^{D}LWV-^{D}PG-LWV-NH$_2$	82
^{D}PG-WW-2a [c]	Ac-^{D}IWV-^{D}PG-LWV-NH$_2$	78
i+3-WW-CL	Ac-AAAWAAWAAAA-NH$_2$	---

[a]N- and C-termini are acetylated and amidated, respectively. Connectivity highlights side chain cross-linked indoles. [b]Normalized with ^{D}PG-WW-1-CL. [c]These peptides were named 5, 5a, 6, and 7, respectively, in a previous study.[8]

by RP-HPLC. Thus, the synthesis and purification method applied was standard and simple.

High-resolution NMR is the most powerful method to analyze the structures of molecules at the atomic level. All peptides were highly soluble in methanol. We achieved complete resonance assignment using TOCSY, HSQC-TOCSY, and ROESY spectra. The ^{1}H 1D NMR spectrum of peptide ^{D}PG-WW-1-CL was surprisingly different from ^{D}PG-WW-1, despite similar molecular masses (see the Supporting Information), with the disappearance of some aromatic protons and the appearance of new resonances. We further observed an unusual correlation pattern in the homonuclear ^{1}H−^{1}H TOCSY spectrum. This anomaly is clearly revealed upon examination of the heteronuclear ^{1}H−^{13}C HSQC-TOCSY spectrum of ^{D}PG-WW-1-CL (Figure 1). It is noteworthy that

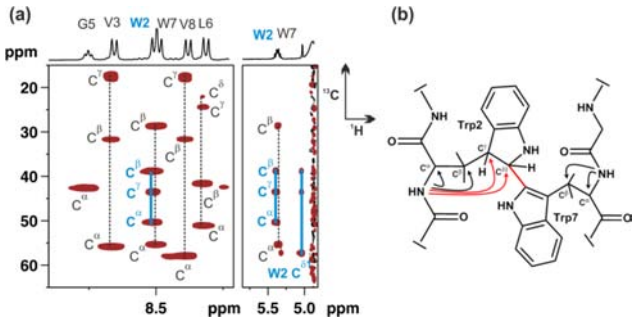


Figure 1. Partial expansions of ^{1}H−^{13}C HSQC-TOCSY spectrum in CD_3OH at 303 K, highlighting the unusual TOCSY connectivity of Trp2 in ^{D}PG-WW-1-CL (a), in blue, bold, and represented schematically in (b), indicative of chemical modification.

while ^{D}PG-WW-1 displays the anticipated TOCSY connectivity ($C^{\alpha}H \leftrightarrow C^{\beta}H \leftrightarrow C^{\gamma}H \leftrightarrow C^{\delta}H$), W2 in ^{D}PG-WW-1-CL shows the unusual $C^{\beta}H_2 \leftrightarrow \mathbf{C^{\gamma}H}$ connectivity (see the Supporting Information).

Our NMR data provides evidence for TFA-promoted dimerization of tryptophans (indole−indoline formation).[10,12] However, the noteworthy unique and important features are (i) both cross-linked and *un*cross-linked peptides have identical masses; (ii) unlike previous reports,[10] only a single stable isomer of ^{D}PG-WW-1-CL is obtained, where Trp2 is specifically modified; (iii) synthesis of the cross-linked peptide is simple and straightforward (Supporting Information), requiring TFA incubation under atmospheric pressure, 25 °C, and does not demand inert environments; (iv) ^{D}PG-WW-1-CL is a highly thermostable β-hairpin structure (discussed later); (v) previous attempts at characterization of this Trp-Trp cross-link were limited to amino acids, di- or tripeptides,[12a,b] and to our knowledge, this is the first report of the stereospecific generation of a homogeneous 100% cross-linked peptide species in well-structured β-hairpins.

Conformational analysis of ^{D}PG-WW-1-CL by ^{1}H−^{1}H ROESY revealed a well-defined ^{D}P-G-nucleated antiparallel β-hairpin, with weak self-$d_{N\alpha}$ NOEs and strong sequential $d_{\alpha N}$ NOEs characteristic of extended conformations (Figure 2).

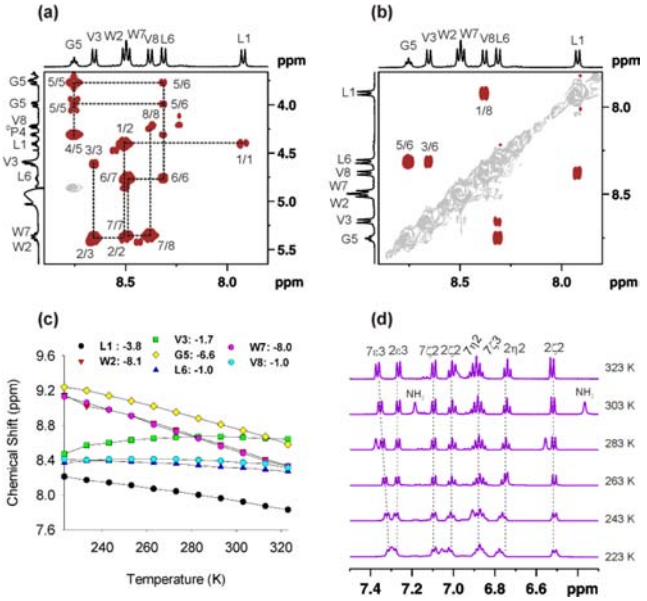


Figure 2. Partial expansion of ^{1}H−^{1}H ROESY spectrum of ^{D}PG-WW-1-CL in CD_3OH at 303 K, highlighting the characteristic β-hairpin NOEs (a and b). Note the conspicuous absence of 1NH↔2NH NOE in (b). (c) Variable-temperature NH chemical shifts to probe hydrogen-bonded amides. (d) Temperature-dependent aromatic proton chemical shifts, highlighting rigidness of the Trp-Trp cross-link.

Importantly, the 1NH↔2NH terminal NOE, indicative of strand fraying,[11b] is absent in ^{D}PG-WW-1-CL (Figure 2b). Further, amide temperature coefficients ($d\delta/dT$)[11b] are smaller for internally hydrogen-bonded amides of L1, V3, L6, and V8 (Figure 2c), confirming that the β-hairpin anatomy is preserved in ^{D}PG-WW-1-CL.

Variable-temperature experiments are also excellent tests for stability and reveal associated changes in molecular motion. While the aromatic proton of W7 $C^{\varepsilon 3}H$ in ^{D}PG-WW-1 shows an upfield shift in low temperatures (ring current effects),[8] we observe negligible temperature dependencies of the aryl protons in ^{D}PG-WW-1-CL (Figure 2d), clearly indicating rigidness and stability of the Trp-Trp cross-link to temperature. Formation and rigidity of the cross-link was further confirmed by observation of the NOEs W2 $C^{\gamma}H \leftrightarrow$ W7 $N^{\varepsilon 1}H$, W2 $C^{\delta 1}H \leftrightarrow$ W7 $C^{\beta}H$ in methanol, and W2 $C^{\delta 1}H \leftrightarrow$ W7 $N^{\varepsilon 1}H$, W2 $C^{\gamma}H \leftrightarrow$ W7 $N^{\varepsilon 1}H$, W2 $N^{\varepsilon 1}H \leftrightarrow$ W7 $N^{\varepsilon 1}H$ in DMSO (see the Supporting Information).

The magnitude of $C^{\alpha}H$ chemical shift deviation (CSD) correlates with the extent of secondary structure formation.[8,11c] Assuming a similar influence of ring current effects, greater positive values for strand segments in ^{D}PG-WW-1-CL compared to *un*cross-linked controls (Figure 3) is suggestive of the cross-linking induced increase in β-hairpin population. As

DOI: 10.1021/acs.orglett.5b01017
Org. Lett. 2015, 17, 2498−2501

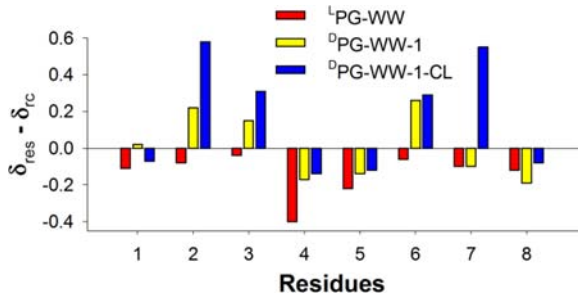


Figure 3. Variation of $C^{\alpha}H$ chemical shift with respect to random coil values. The cross-linked peptides show significantly more β-hairpin structure compared to the *un*cross-linked analogues. Note that ^{L}PG-WW is unstructured. See the Supporting Information for a description of ring current effects.

proof of principle, we also extended the TFA-promoted cross-linking of Trp in ^{D}PG-WW-2 to obtain ^{D}PG-WW-2-CL and carried out the complete structural characterization using NMR (see the Supporting Information). Put together, our results suggest that cross-linking can play an important structure-stabilizing role in β-hairpins or in α-helical scaffolds (described later).

Circular dichroism (CD) is a rapid method for secondary structure estimation and thermal stability measurement of biomolecules and offers many advantages over NMR, including low sample concentration requirement and wider choices of solvent systems. Interacting Trp rings in β-hairpins give rise to a signature exciton couplet in the far-UV CD region,[13] with characteristic negative and positive CD bands at ~214 nm and ~228 nm, respectively, as observed for ^{D}PG-WW-1[8] (Figure 4a). Hence, we investigated the nature of the exciton couplet and the associated thermal stability of the *un*cross-linked and cross-linked peptides, using CD. Cross-linked ^{D}PG-WW-1-CL displays one unusual negative band at ~228 nm (Figure 4b), which may highlight that the Trp-Trp cross-link is not coplanar.[14] While ^{D}PG-WW-1 shows loss of the exciton couplet with thermal denaturation, ^{D}PG-WW-1-CL is highly thermostable, with no significant change in the CD band intensity (Figure 4a,b).

Previous studies have reported that the peptide sequence plays an important role in TFA-mediated Trp dimerization.[12d] We were therefore curious to examine whether a predefined scaffold is necessary for efficient cross-linking by measuring the rate of structural transition upon Trp cross-linking using CD and HPLC. Note that ^{L}PG-WW is largely unstructured due to poor turn nucleation at ^{L}P-G.[8] Moreover, the cross-linked and *un*cross-linked peptides show different HPLC retention times (see the Supporting Information). Our far-UV CD (Figure 4c,d) and HPLC (Figure 4e) measurements clearly indicate that a 10-fold enhancement in the cross-linking rate is achieved in the presence of a predefined structure (Figure 4f), with the reaction progressing over days, in the absence of defined structural fold. We thereby establish the existence of a structure−reactivity relationship in the cross-linking process.

Our results with "stapled" stable β-hairpin formation with TFA encouraged us to test whether this can be extended to helical scaffolds to obtain Trp-Trp cross-linked α-helical stapled peptides. We designed and characterized a poly-Ala helix, wherein we centered tryptophans at the *i* and *i*+3 positions, to promote Trp-Trp interaction (Table 1). Complete characterization of the product *i*+3-WW-CL (Figure 5; also see the Supporting Information), obtained from TFA incubation,

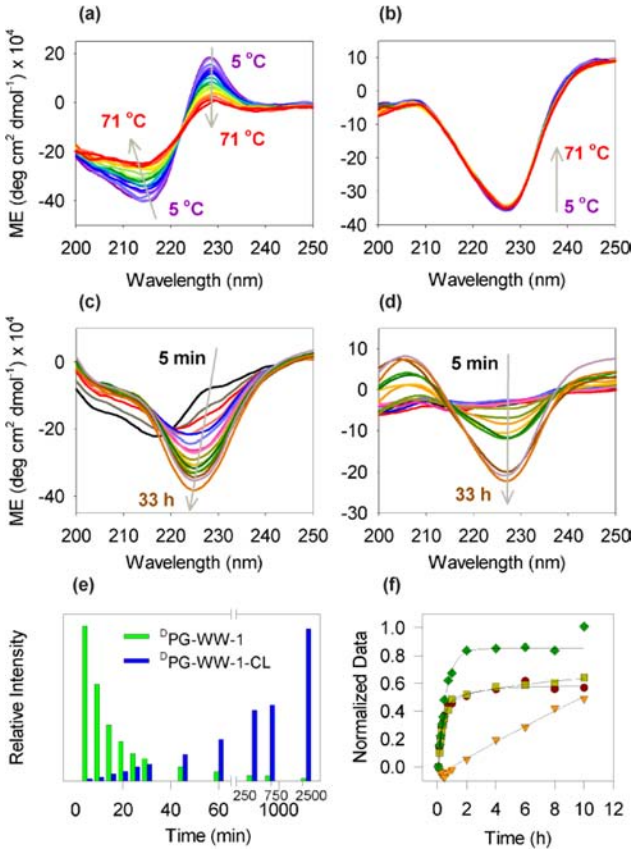


Figure 4. Thermal stability and structure−reactivity relationship investigated by CD and HPLC. CD wavelength scans in methanol for ^{D}PG-WW-1[8] (a) and ^{D}PG-WW-1-CL (b). Structural and chemical transition−induced spectroscopic variation upon cross-linking, captured with time using CD (c and d) for ^{D}PG-WW-1 (c) and ^{L}PG-WW (d), and HPLC (e). Postbreak values in *x*-axis in (e) are in logarithmic scale. (f) Progress of cross-linking monitored using CD for ^{D}PG-WW-1 (●), ^{L}PG-WW (▼), ^{D}PG-WW-2 (■), and ^{D}PG-WW-2a (◆). Note that the reaction rates (shown as fits) are rapid for all of the structured peptides (see Table 1).

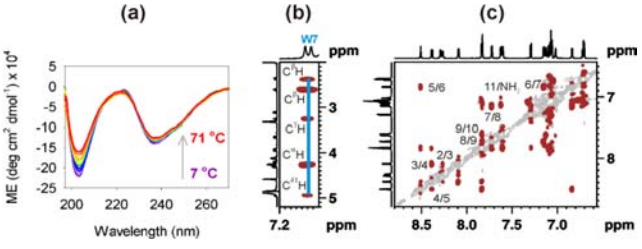


Figure 5. i+3-WW-CL. (a) Variable-temperature CD measurements in methanol. (b) ^{1}H−^{1}H TOCSY spectrum highlighting unusual connectivity in W7. (c) Partial expansion of ROESY spectrum showing sequential d_{NN} NOEs, characteristic of a helical scaffold.

confirms intrahelical Trp dimerization and endorses that TFA-mediated cross-linking for β-hairpins can be extended to obtain thermostable α-helical peptides. Remarkably, while stereospecific 100% conversion is achieved in i+3-WW-CL, the cross-linking in α-helical scaffolds involves Trp7 modification, and not Trp4 (in β-hairpins, Trp2, and not Trp7, is modified). Further, the indoline displays a *cis* and *trans* isomer[12a] in the hairpin and helix, respectively (Supporting

DOI: 10.1021/acs.orglett.5b01017
Org. Lett. 2015, 17, 2498−2501

Information) and may account for differences in the observed CD spectra.

Our findings for simplified generation of "stapled" Trp-Trp highly stable β-hairpin or α-helical peptides, in biomolecules with few reactive side chains, can have enormous applications in the design of potent peptide-based therapeutics and in the production of robust biomaterials.

■ ASSOCIATED CONTENT

Supporting Information

Peptide synthesis, purification, HPLC analysis, mass spectrometry, NMR, and CD measurements. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01017.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

K.M.M. acknowledges a senior research fellowship from the University Grants Commission. R.M. is a Wellcome Trust/DBT India Alliance Intermediate Fellow. This work was supported by intramural funds.

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DOI: 10.1021/acs.orglett.5b01017
Org. Lett. 2015, 17, 2498−2501