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A proline-derived transannular N-cap for nucleation of short α-helical peptides†

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We report herein a proline-derived transannular N-cap as a helix nucleating template in diverse bio-related peptide sequences *via* macrolactamization on resin. This approach takes advantage of synergistic stabilization effects of both N-capping properties of proline and substitution of a main chain hydrogen bond with a covalent bond.

α-Helices constitute a large proportion of secondary structural elements of proteins and are implicated in the molecular recognition of various important protein–protein interactions (PPIs).[1] It has been extensively studied that recapitulation and stabilization of well-defined α-helices would result in peptide mimetics targeting aberrant PPIs with enhanced biophysical properties.[2] One privileged approach for helix stabilization is through sidechain–sidechain constraints between residues i, $i+3$ and/or $i+4$ and $i+7$.[3] With the exception of the sidechain cross-linking strategy, pre-organized templates for the first α-turn conformation should aid the nucleation of a helical conformation of peptides on the basis of helix–coil transition theory.[4] One advantage of this approach is that it does not block solvent-exposed molecular recognition surfaces of short helices.[4] A number of templates were reported to successfully nucleate a helix, among which, the first proline-based templates of this kind were introduced by Kemp and co-workers.[5] In 2013, inspired by Kemp, Schmalz and Kühne reported an ethylidene bridged tricyclic diproline template with the desired helix-inducing effect.[6] The design of these pre-organized templates is based on the N-capping properties of proline.[7] Proline is generally considered as a helix-breaking amino acid when internally incorporated into the peptide. However, it is frequently found at the N-terminus of a helix and is considered to stabilize and promote the capping effect of adjacent serine, aspartic acid or asparagine and may involve in additional hydrophobic interaction with valine.[7,8] In addition, the rigidity of proline is also considered to be able to constrain the orientation of the first carbonyl group of a helix, which may promote the helix nucleation process (Fig. 1A).[7] Another closely related approach was to replace one N-terminal main-chain hydrogen bond with a covalent bond (Fig. 1B).[4] Cabezas *et al.* reported the first example by replacing the N-terminal hydrogen bond with a hydrazone linkage.[9] In 2004, Arora *et al.* developed a new class of artificial α-helices called hydrogen bond surrogates (HBS), which featured a carbon–carbon bond in replacement of a

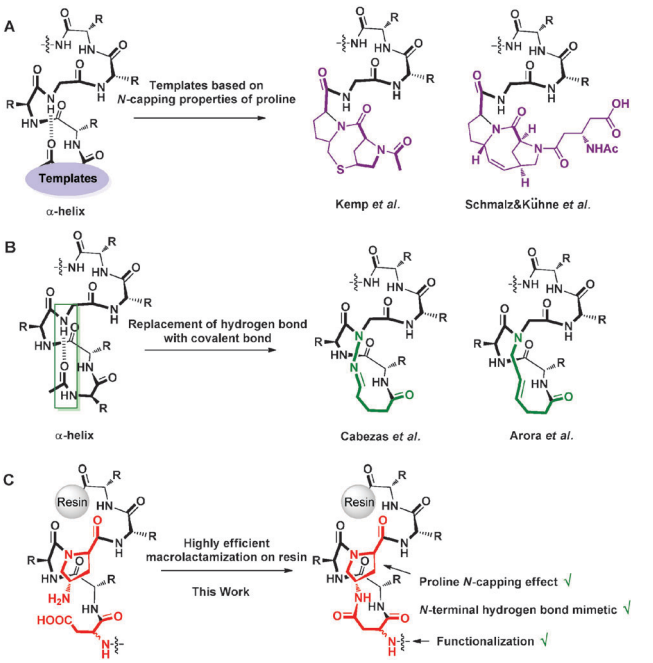

Fig. 1 (A) Polycyclic templates based on N-capping properties of proline; (B) hydrogen bond surrogate approach to replace one N-terminal hydrogen bond with a covalent bond; (C) design of a proline-derived transannular N-cap *via* highly efficient macrolactamization on resin.

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hydrogen bond at the N-terminus *via* microwave-assisted ring-closing metathesis (RCM) reaction.[10] This HBS approach has been proven to be successful in modulating PPIs such as HIF, p53-MDM2, and BCL-xl.[11] Inspired by these two strategies, we envisioned a proline-derived transannular N-cap for the nucleation of short α-helices both by exploiting the N-capping effect of proline at the N-terminus of a peptide and by replacing one N-terminal hydrogen bond with a covalent bond at the same time. This method circumvents the complex synthetic process of polycyclic proline-derived templates.[6] Additionally, the N-terminus amino group could serve as an additional attachment point for tagging the peptides through a standard solid phase peptide synthesis (SPPS) procedure and the orientation of this tag could be controlled by the stereochemistry of this amino group (Fig. 1C).

Hydroxyproline represents approximately 13% of amino acids of collagen and is formed by posttranslational hydroxylation of proline in the collagen chain.[12] In 2014, Wennemers *et al.* reported a pH responsive conformation change of collagen using *cis*-4-aminoproline.[13] The synthesis of *cis*-4-aminoproline started from commercially available Boc-L-*trans*-4-hydroxyproline **1** followed by protection of the carboxyl group to give Boc-L-*trans*-4-hydroxyproline *tert*-butyl ester **2**. Then intermediate **2** was converted into Boc-L-*cis*-4-azidoproline *tert*-butyl ester **3** *via* Mitsunobu reaction. Reduction of **3** afforded Boc-L-*cis*-4-aminoproline *tert*-butyl ester **4**, followed by protecting group manipulation to give the final non-natural amino acid Fmoc-L-*cis*-4-((allyloxy)carbonyl)aminoproline **5** (Scheme S1, ESI†). Commercially available Fmoc-L-Asp(OAll)-OH was also used in this study. With these two amino acids in hand, a series of bio-related model peptides were selected to evaluate the nucleation effect of this N-cap (Table S1, ESI†). The bioactive proapoptotic protein Bak BH3 was first selected as it had been extensively studied previously.[14] A proline-capped peptide was synthesized *via* SPPS and the protected *cis*-4-aminoproline and L-aspartic acid were easily incorporated into the peptide sequence using a standard activation reagent (Fig. 2A). After peptide assembly, allyl and alloc protecting groups were removed as previously reported in the synthesis of lactam cross-linked peptides.[3a,b] Finally, the macrolactamization step was accomplished at room temperature for 3 h on resin using PyBOP (see the details of peptide preparation in the ESI†). The cyclized peptides were isolated easily with high conversion and purity as shown in Fig. 2B. To our delight, we did not observe any dimer or oligomer products which were generally detected in peptide macrolactamization.[3b] And this highly efficient intramolecular lactam formation on resin indicated that proline at the N-terminus of the peptide could facilitate the amide bond formation by forcing the amino group and activated carboxylic acid into the optimal reaction position.

The helix nucleation ability of this N-cap strategy was then investigated by circular dichroism (CD) spectroscopy. Trifluoroethanol (TFE) titration experiments were subsequently performed.

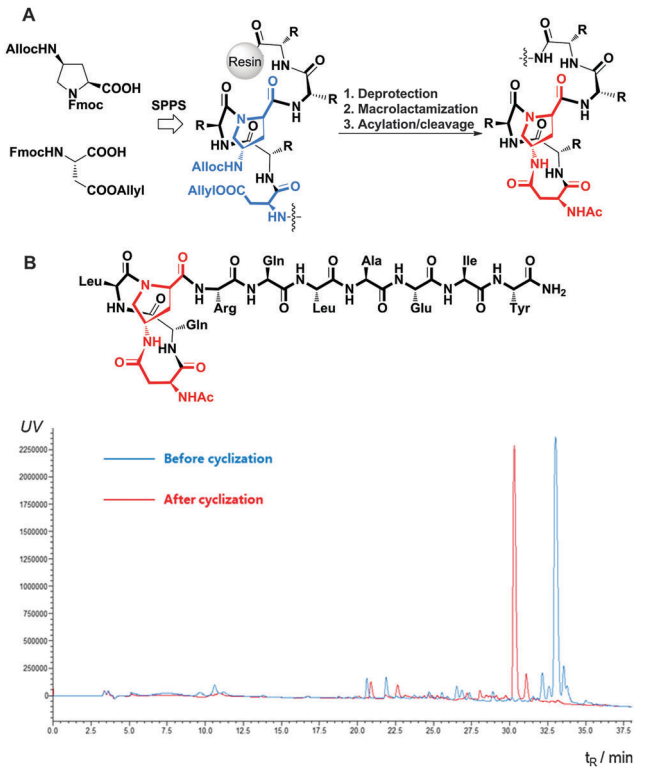

Fig. 2 Proline-derived transannular N-cap peptides synthesized efficiently *via* macrolactamization on resin. (A) Schematic presentation of peptide synthesis (please see details in the ESI†). (B) Crude HPLC traces of macrolactamization on resin before/after cyclization. Monitored at 220 nm. Peptide sequence: D*QLP*RQLAEIY, where D* and P* denote the cyclized amino acids. P* is L-4-*cis*-aminoproline.

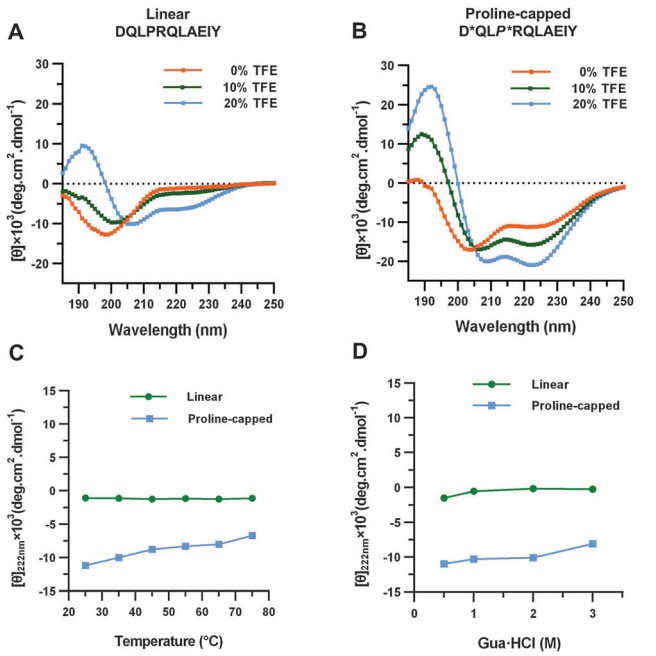

Fig. 3 Conformational analysis by circular dichroism spectroscopy. (A) CD spectra of the linear Bak peptide (DQLPRQLAEIY) with increasing percentage of TFE; (B) CD spectra of the proline-capped Bak peptide (D*QLP*RQLAEIY, where D* and P* denote the cyclized amino acids) with increasing percentage of TFE; (C) thermal denaturation experiment; (D) guanidine hydrochloride denaturation experiment.

TFE is generally used as helix promoting solvent. We performed this titration to benchmark the helicity inducing effect of TFE for a certain peptide. As expected, the CD spectrum of the linear Bak peptide showed a negative Cotton effect at 195 nm even in the presence of 10% TFE, which indicated a major random coil conformation as shown in Fig. 3A. The proline-capped peptide displayed double negative minima at 208 nm and 222 nm in pure ddH_2O and the helical content increased with the addition of TFE (Fig. 3B). The CD spectra of different bio-related sequences were also tested including peptides derived from hormone urocortin (EK),[6] estrogen receptor-coactivator interaction (ER)[3c] and HIF-1α (HIF)[11a] as shown in Fig. S1–S7 (ESI†). All the peptides tested showed an increased helicity *via* N-terminal proline-capping. We then performed thermo and guanidine hydrochloride denaturation experiments to evaluate the conformational stability of the proline-capped Bak peptide as shown in Fig. 3C and D. The results suggested that the proline-capped peptide retained 60% of helicity at 75 °C compared to the helicity at 25 °C. In addition, the proline-capped peptide showed remarkable conformational stability under chemical denaturation conditions as the molar ellipticity at 222 nm did not change much with increasing concentration of guanidine hydrochloride.

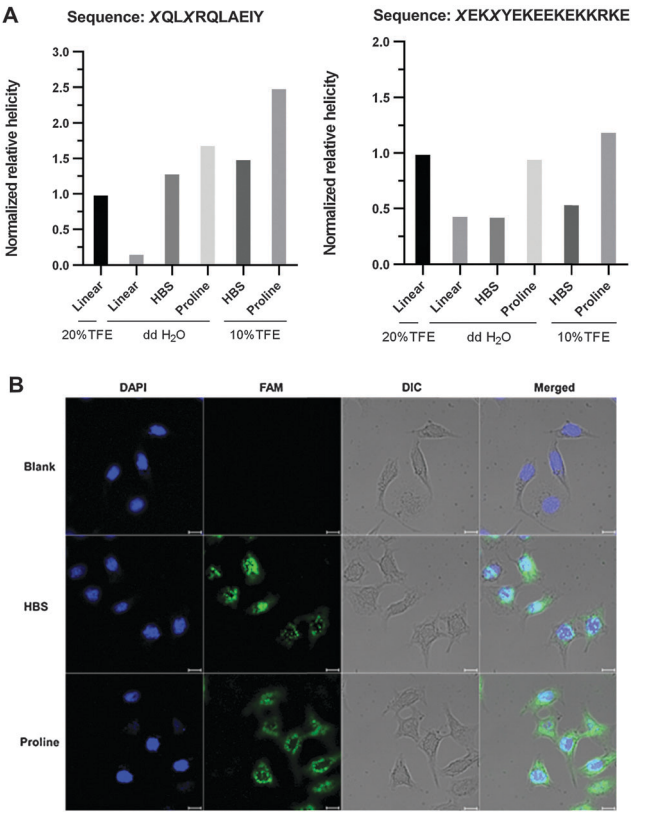

Fig. 4 Proline-capped helices showing comparable biophysical properties with HBS-derived helices. (A) Relative helicity in different solvent systems. Bak peptide: *X*QL*X*RQLAEIY; EK peptide: *X*EK*X*YEKEEKEKKRKE; *X* denotes cyclized amino acids in different methods. (B) Confocal microscopy images of HeLa cells treated with 10 μM FAM-labelled ER peptides (sequence: *X*RK*X*ILRRLLQGW, where *X* denotes cyclized amino acids in different methods; FAM was site-specifically incorporated into the ε-NH_2 group of the lysine residue). Scale bar, 10 μm.

The schematic comparison of relative helical contents between HBS derived and proline-capped Bak and EK peptides is summarized in Fig. 4A respectively (see Fig. S8 for CD spectra, ESI†). For Bak derived peptides, the proline-capped peptide had a comparable helical content to the HBS-derived helices in pure ddH_2O as shown in Fig. 4A. In addition, both the HBS-derived peptide and the proline-capped peptide showed over seven-fold helicity enhancement as compared to the linear peptide in pure ddH_2O. In particular, for the helix-inducing EK peptide, we found that proline-capping was more effective than the HBS strategy in helix induction. The linear EK peptide which incorporated a proline at the N-terminus displayed some helicity in pure ddH_2O and the relative helicity was comparable to that of the HBS-derived EK peptide. The helicity enhancement of the proline-capped peptide was even more effective upon cyclization suggesting the synergistic stabilization effects of proline-capping and covalent bond substitution of the N-terminal hydrogen bond. In addition, cellular uptake of HBS-derived or proline-capped ER targeting peptides was evaluated by confocal microscopy imaging and the data indicated comparable cell penetration of these two strategies (Fig. 4B).

To further support the helical structure of the proline capped peptide, a detailed 1D and 2D ^1H NMR experiment was performed in 20% TFE-d_3 in PBS (pH 5.0) at 25 °C as previously reported.[14] A linear Bak analog was used as reference. As expected, the Rotating-frame Overhauser Effect (ROE) signals of the proline capped Bak peptide displayed more signals than the linear counterpart, which indicated an increase in helical content under the same conditions (Fig. 5). The ROESY spectrum of the proline-capped Bak peptide also displayed several $d_{\alpha N}(i, i+3)$, $d_{\alpha N}(i, i+4)$ and $d_{\alpha\beta}(i, i+3)$ ROE signals, which indicated the

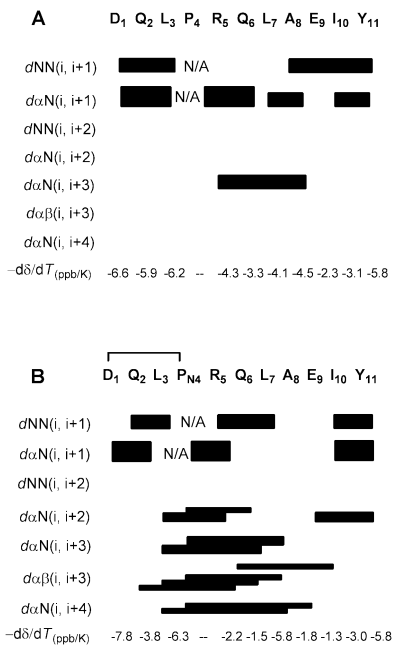

Fig. 5 ROE summary diagram of the linear Bak peptide (A) and the proline-capped Bak peptide (B). Bar thickness reveals the intensity of the ROE signals.

helical propensity. Sequential low temperature dependence of amide NH chemical shifts ($\Delta\delta/T \leq 4.5$ ppb K^{-1}) was evidence for intramolecular hydrogen bonding (Fig. S9, ESI†). The CD spectra and NMR characterization unambiguously demonstrated that the nucleation ability of the proline-derived transannular N-cap was effective in our model peptides.

In conclusion, the proline-derived transannular N-cap methodology takes advantage of both the N-capping properties of proline and the substitution of a main chain hydrogen bond with a covalent bond as two distinct helix-stabilizing elements to nucleate a helical structure. From a synthetic point of view, this methodology circumvents the construction of complex polycyclic proline-derived templates and could facilitate a fully automated solid-phase peptide synthesis of proline-capped helices. In addition, the preserved amino group provides a facile way to further functionalize the constrained peptides through a standard SPPS procedure.

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