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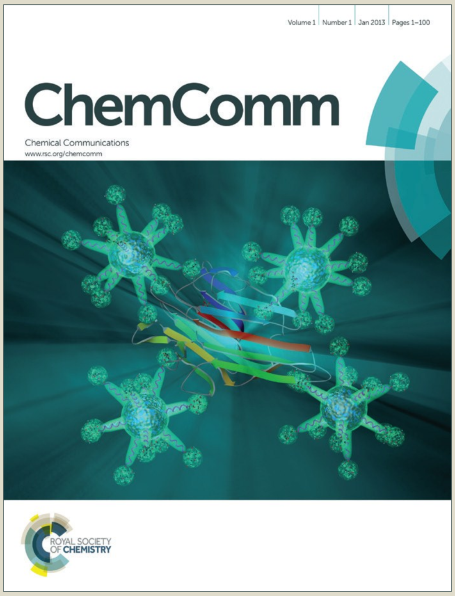

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Proline-Derived Transannular *N*-Cap for Nucleation of Short α-Helical Peptides

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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 protein secondary structure elements and are implicated in 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 sidechain cross-linking strategy, pre-organized templates for the first α-turn conformation should aid the nucleation of 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 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 as 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

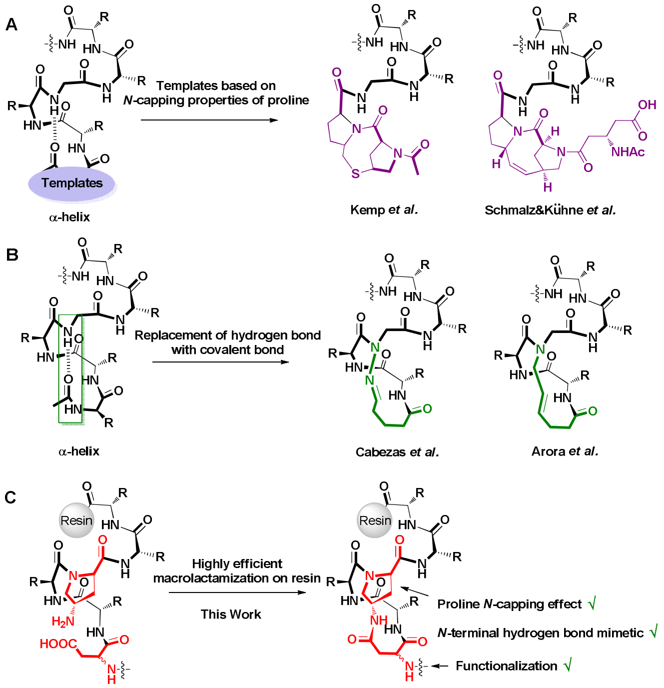

Fig. 1 A) Polycyclic Templates based on *N*-capping properties of proline; B) Hydrogen bond surrogate approach to replace one *N*-terminal hydrogen bond with a covalent bond; C) Design of proline-derived transannular *N*-cap *via* highly efficient macrolactamization on resin.

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Electronic Supplementary Information (ESI) available: Experimental procedures, supporting tables and figures. See DOI: 10.1039/x0xx00000x bond surrogates (HBS), which featured a carbon-carbon bond in replacement of a hydrogen bond at the *N*-terminus *via* microwave-assisted Ring-closing Metathesis (RCM) reaction[10]. This HBS approach has proven successful in modulating PPIs such as HIF, p53-MDM2, BCL-xl[11]. Inspired by these two strategies, we envisioned a proline-derived transannular *N*-cap for nucleation of short α-helices by exploiting both the *N*-capping effect of proline at the *N*-terminus of a peptide and also 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 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).

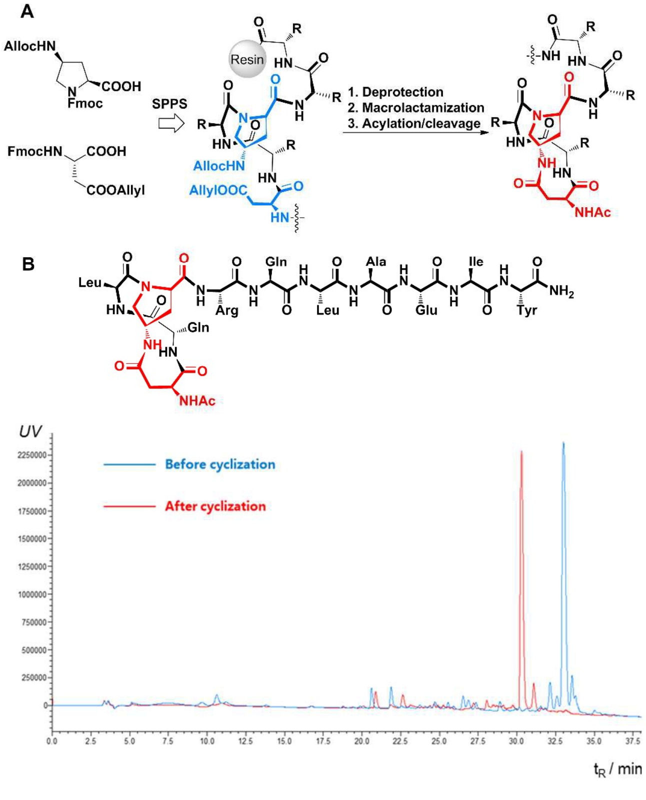

Fig. 2 Proline-derived transannular *N*-Cap peptides could be synthesized efficiently *via* macrolactamization on resin; A) Schematic presentation of peptide synthesis (Please see details in SI). B) Crude HPLC traces of macrolactamization on resin before/after cyclization. Peptide sequence: D*QL*P**RQLAEIY, where D* and *P** denote the cyclized amino acids. *P** is L-4-cis-aminoproline.

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 commercial available Boc-L-trans-4-Hydroxyproline **1** followed by protection of 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). Commercial 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). The bioactive proapoptotic protein Bak BH3 was first selected as it had been extensively studied previously [15]. 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 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, 3b]. Finally, the macrolactamization step was accomplished at room temperature for 3h on resin using PyBOP (Details for peptide preparation in SI). 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 *N*-terminus of the peptide could facilitate the amide bond formation by forcing the amino group and activated carboxylic acid into optimal reaction position.

Helix nucleation ability of this *N*-cap strategy was then investigated by Circular Dichroism spectroscopy (CD). Trifluoroethanol (TFE) titration experiments were subsequently performed. TFE is generally used as helix promoting solvents. We performed this titration to benchmark the helicity inducing effect of TFE for a certain peptide. As expected, the CD spectrum of linear Bak peptide showed a negative Cotton effect at 195nm 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

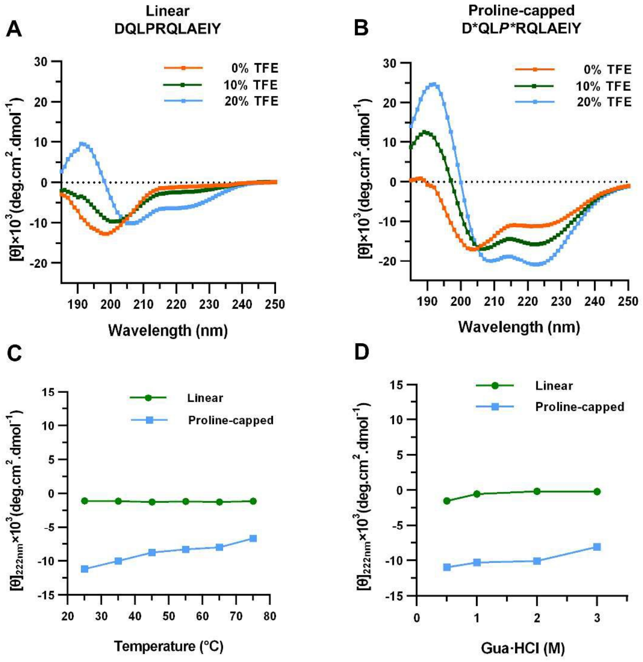

Fig. 3 Conformational analysis by Circular Dichroism spectroscopy. A) CD spectra of linear Bak peptide (DQLPRQLAEIY) with increasing percentage of TFE; B) CD spectra of proline-capped Bak peptide (D*QL*P**RQLAEIY, where D* and *P** denote the cyclized amino acids) with increasing percentage of TFE; C)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

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. 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 proline-capped Bak peptide as shown in Fig. 3C, 3D. The results suggested that proline-capped peptide remained 60% of helicity at 75 °C compared to the helicity at 25 °C. In addition, the proline-capped peptide showed remarkable conformational stability over chemical denaturation condition as the molar elipticity at 222 nm did not change much with increasing concentration of guanidine hydrochloride.

The schematic comparison of relative helical contents between HBS derived and proline-capped Bak and EK peptides were summarized in Fig. 4A respectively (See Fig. S8 for CD spectra). For Bak derived peptides, The proline-capped peptide displayed comparable helical content with the HBS-derived helices in pure ddH$_2$O as shown in Fig. 4A. In addition, both the HBS-derived peptide and proline-capped peptide showed over seven-fold helicity enhancement as compared to the linear peptide in pure ddH$_2$O. Particularly, for the helix-inducing EK peptide, we found proline-capping was more effective than HBS strategy in helix induction. Linear EK peptide which incorporated a proline at the *N*-terminus displayed some helicity in pure ddH$_2$O and the relative helicity was comparable to that of HBS-derived EK peptide. The helicity enhancement of proline-capped peptide was even more effective upon cyclization suggesting the synergistic stabilization effects of proline-capping and covalent bond substitution of *N*-terminal hydrogen bond. In addition, cellular uptake of HBS-derived or proline-capped ER targeting peptides were evaluated by confocal microscopy imaging and the data indicated a comparable cell penetration of these two strategies (Fig. 4B).

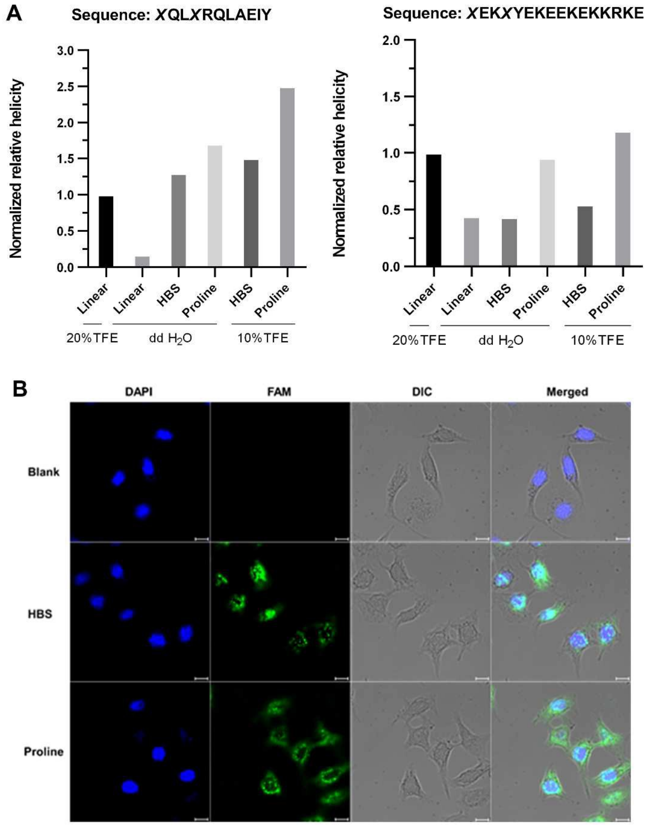

Fig. 4 Proline-capped helices showed comparable biophysical properties with HBS-derived helices. A) Relative helicity in different solvent system. Bak peptide: *X*QL*X*RQLAEIY; EK peptide: *X*EK*X*YEKEEKEKKRKE; *X* denote cyclized amino acids in different method. B) Confocal microscopy images of Hela cells treated with 10 μM FAM-labelled ER peptides (Sequence: *X*RK*X*ILRRLLQGW, where *X* denote cyclized amino acids in different method. FAM was site-specifically incorporated into the ε-NH$_2$ group of lysine residue.). Scale bar, 10 μm.

To further support the helical structure of the proline capped peptide. A detailed 1D and 2D ^{1}H NMR experiment was performed in 20% TFE-*d3* in PBS (pH 5.0) at 25 °C as previously reported[14]. Linear Bak analog was used as reference. As expected, the Rotating-frame Overhauser Effect (ROE) signals of proline capped Bak peptide displayed more signals than the linear counterpart, which indicated an increase in helical content at the same condition (Fig. 5). The ROESY spectrum of 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) ROEs, which indicated the helical propensity. Sequential low temperature dependence for amide NH chemical shifts ($\Delta\delta/T \leq 4.5$ ppb/K) was an evidence for intramolecular hydrogen bonding.(Fig. S9) The CD spectra and NMR characterization unambiguously demonstrated that the nucleation ability of proline-derived transannular *N*-cap was effective in our model peptides.

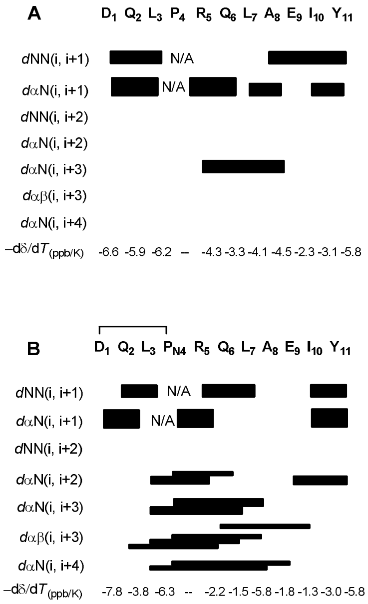

Fig. 5 ROE summary diagram of linear Bak peptide (A) and proline-capped Bak peptide (B). Bar thickness reveals the intensity of the ROE signals.

As a conclusion, the proline-derived transannular *N*-cap methodology takes advantages 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. In 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 standard SPPS procedure.

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