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## Incorporation of CF<sub>3</sub>-pseudoprolines into polyproline type II foldamers confers promising biophysical features

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**The development and the use of fluorinated polyproline-type II (PPII) foldamers are still underexplored. Herein, trifluoromethyl pseudoprolines have been incorporated into polyproline backbones without affecting their PPII helicity. The ability of the trifluoromethyl groups to increase hydrophobicity and to act as <sup>19</sup>F NMR probes is demonstrated. Moreover, the enzymatic stability and the non-cytotoxicity of these fluorinated foldamers make them valuable templates for their use in medicinal chemistry.**

Polyproline type II (PPII) helices are one of the most abundant secondary structures found in peptides and proteins. They are left-handed helices in which the  $\varphi$ ,  $\psi$ , and  $\omega$  dihedral angles of the constituent residues are restricted (around  $-75^\circ$ ,  $145^\circ$  and  $180^\circ$  respectively). Like the better-known  $\alpha$ -helices and  $\beta$ -sheets, PPII helices are involved in numerous biological processes.<sup>1</sup> An illustrative example is collagen, the most abundant structural protein in mammals, which is characterized by its triple helix structure composed of three individual PPII strands. Additionally, the recognition of PPII sequences by SH3 protein domains is known to play a crucial role in the regulation of cellular signalling pathways.<sup>1</sup>

Foldamers are synthetic oligomers adopting a well-defined secondary structure. In parallel with the scarce but growing interest in PPII-type foldamers for biological applications,<sup>2</sup> the incorporation of fluorinated amino acids into peptides and proteins has become of major interest by modulating their physicochemical and biophysical properties (folding, stability, hydrophobicity, etc.).<sup>3</sup> Moreover, fluorine can be used as a <sup>19</sup>F NMR probe, making it a powerful tool for studying fluorinated peptide/membrane or peptide/protein interactions.<sup>4</sup> Although,

the synthesis of fluorinated foldamers is an emerging field,<sup>5</sup> fluorinated PPII-type foldamers are still underexplored and remain mainly the focus of structural studies.<sup>6</sup> Therefore, their investigation for application in a medicinal chemistry context has yet to be done.

Our group has developed the synthesis of oxazolidine-based pseudoprolines substituted by a trifluoromethyl group (CF<sub>3</sub>) at the C<sub>5</sub>-position (CF<sub>3</sub>ΨPro) and their incorporation into peptides.<sup>7</sup> Previous studies based on CF<sub>3</sub>ΨPro-containing model peptides established the stereoelectronic effects imparted by the CF<sub>3</sub> group and demonstrated that these proline surrogates are useful for tuning the *cis/trans* amide bond conformation, lowering the rotational energy barrier and freezing the puckering of the oxazolidine core.<sup>8</sup> In particular, NMR conformational studies on homo- and heterochiral Xaa-CF<sub>3</sub>ΨPro dipeptides revealed that changing the configuration of the C $\alpha$  in Xaa or in CF<sub>3</sub>ΨPro is sufficient to invert the *cis/trans* population ratio while also changing the nature of the Xaa side chain and finely tuning the conformer ratio.<sup>9</sup> Moreover, CF<sub>3</sub>ΨPro was found to meet the structural requirements for the triple helix assembly by stabilizing the PPII backbone conformation in water. They have been recently reported as valuable tools for the design of new fluorinated collagen mimetic peptides.<sup>6e</sup> Based on NMR and CD spectroscopies and X-ray crystallography, we report herein that homo-oligomers of proline can accommodate trifluoromethyl-pseudoprolines CF<sub>3</sub>ΨPro along the peptide sequence while preserving the PPII helical structure. Thanks to the 3-fold rotational symmetry of the PPII helix and a rational incorporation of the CF<sub>3</sub>ΨPro every three residues, original foldamers presenting a fluorinated face on the helix were obtained.

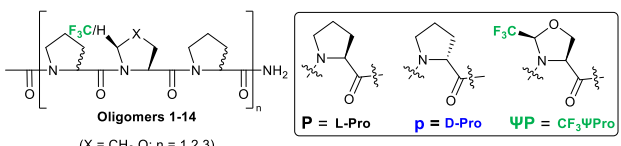
We designed the series of proline-based oligomers **1-14** (Table 1). Fluorinated foldamers **2-5**, **7-9**, and **11-14** were based on (Pro-CF<sub>3</sub>ΨPro-Pro)<sub>n</sub> scaffolds (n = 1, 2 or 3, respectively). The corresponding non-fluorinated oligomers **1**, **6** and **10** were also prepared for comparison. The first set of peptides **2-4**, corresponding to the trimers Ac-Pro-CF<sub>3</sub>ΨPro-Pro-NH<sub>2</sub>, served

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**Table 1** Synthesis of oligomers **1-14**


	Peptide sequence	N°	Yield (%)
Trimers	Ac-P-P-P-NH <sub>2</sub>	<b>1</b>	71
	Ac-P-ΨP-P-NH <sub>2</sub>	<b>2</b>	30
	Ac-p-ΨP-P-NH <sub>2</sub>	<b>3</b>	93
	Ac-P-ΨP-p-NH <sub>2</sub>	<b>4</b>	74
	Ac-p-ΨP-p-NH <sub>2</sub>	<b>5</b>	46
Hexamers	Ac-P-P-P-P-P-P-NH <sub>2</sub>	<b>6<sup>a</sup></b>	27
	Ac-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>7<sup>a</sup></b>	31
	H-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>8</b>	35
	H-P-ΨP-P-P-ΨP-P-OH	<b>9</b>	16
Nonamers	Ac-P-P-P-P-P-P-P-P-NH <sub>2</sub>	<b>10<sup>a</sup></b>	25
	Ac-P-ΨP-P-P-P-P-P-P-NH <sub>2</sub>	<b>11</b>	26
	Ac-P-P-P-P-ΨP-P-P-P-P-NH <sub>2</sub>	<b>12</b>	55
	Ac-P-P-P-P-P-P-ΨP-P-NH <sub>2</sub>	<b>13</b>	46
	Ac-P-ΨP-P-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>14<sup>a</sup></b>	45

*a.* Automated microwave-assisted synthesis.

as models to study the impact of homo- and hetero-chirality on the PPII conformation. Homochiral hexamers **7-9** constitute the second set of peptides presenting the two fluorinated groups on the same face of the helix. They differ by the nature of their termini to investigate their influence on conformational transition during solvent change, with PPII typically being preferred in an aqueous environment while the right-handed polyproline helix I (PPI) is favoured in less polar solvents such as *n*-propanol.<sup>10</sup> The final series corresponds to the homochiral nonamers **11-14** and allowed assessment of the effects of both the location and the number of the CF<sub>3</sub>ΨPro residues along the peptide chain.

### Synthesis of oligomers

The oligomers **1-14** were synthesized by solid phase peptide synthesis (SPPS) using classical coupling conditions on Rink amide resin, except for oligomer **9** for which a Wang resin was used to generate a C-terminal carboxylic acid (Table 1, ESI). Since coupling reactions at the N-terminus of the CF<sub>3</sub>ΨPro require the activation of the preceding Fmoc-amino acid as the acyl chloride, the ready-to-use for SPPS Fmoc-P-ΨP-OH and Fmoc-p-ΨP-OH dipeptides were prepared in solution.<sup>7,9</sup> After optional acetylation and cleavage from the resin, crude peptides were purified by reversed phase semi-preparative HPLC to afford pure peptides in moderate to good yields (Table 1) and high purity (>95%).

### Hetero- vs. Homo-chirality in trimers models

After proton assignment of trimers **2-5**, we were able to discriminate *trans* and *cis* amide bonds using NOESY NMR

experiments (Table 2). In agreement with previous studies,<sup>7b,9</sup> the geometry of the Pro-CF<sub>3</sub>ΨPro amide bond can be readily tuned by using homochiral or heterochiral dipeptide moieties. The homochiral Pro-CF<sub>3</sub>ΨPro (P-ΨP) strongly favored the *trans*-amide bond (oligomers **2** and **4**). Conversely, the heterochiral (p-ΨP) showed a predominance for the *cis* amide bond (oligomers **3-5**). Interestingly, oligomer **2**, with a full homochiral sequence displayed almost exclusively the all-*trans* conformation in D<sub>2</sub>O, as required for PPII helices, while oligomer **4** with a D-Pro (p) at its C-terminus exhibited reduced content of the all-*trans* rotamer (70%). Elongated analogues of the trimer **2** (hexamer **7** and nonamer **14**) confirmed the ability of homo-chiral oligomers to promote the all-*trans* conformation. Removal of the acetyl group at the N-terminus (oligomer **8**) significantly decreased the content of the all-*trans* conformation (65%) by affecting uniquely the first Pro-CF<sub>3</sub>ΨPro amide bond. Therefore, the NMR spectra of oligomers **2**, **7** and **14** provided the first evidence of their PPII-like folding.

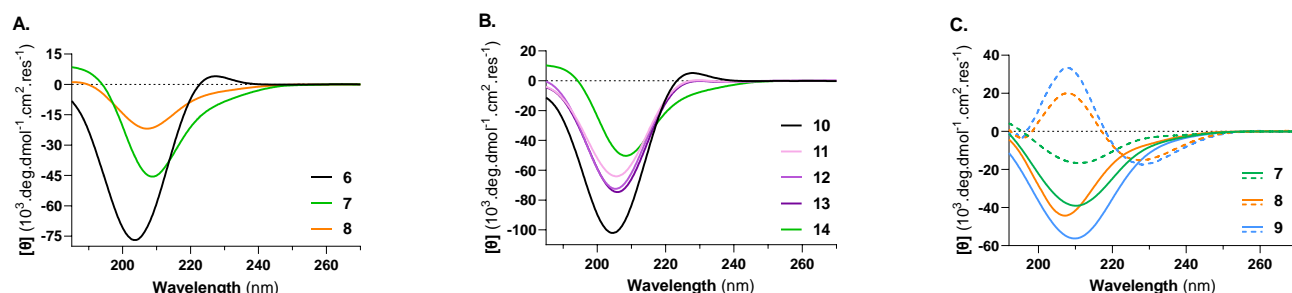
### Structural studies by CD spectroscopy

The CD spectra of hexamers **7** and **8** were almost identical, with an intense negative band around 207 nm and a positive ellipticity value below 195 nm (Fig. 1A). However, the acetylated oligomer **7** displayed a higher ellipticity than the free-amine hexamer **8** revealing greater stabilization of the secondary structure, in agreement with the NMR results (Fig. 1A). In order to assign the folding of the fluorinated hexamers, their CD profiles were compared to that of the non-fluorinated hexamer **6**, known to adopt a PPII helix. Peptide **6** revealed a typical PPII CD spectrum, with a weak positive band at 228 nm and a strong negative band at 205 nm (Fig. 1A).<sup>11</sup> The absence of the positive band in **7** and **8** described as a "hallmark" of the PPII helix may be problematic for confirming the attribution of the secondary structure of our fluorinated oligomers, the lack of this band being often linked to a random coil structure in the literature.<sup>12</sup> Nevertheless, although shifted and less intense, the negative band around 207 nm observed with the fluorinated oligomers **7** and **8** can be considered as a marker of PPII folding, as already reported in the literature.<sup>13</sup> Moreover, it is known that a modification on the proline residue can impact its features as a

**Table 2** Amide bond conformations of oligomers **2-5**, **7-8** and **14** in D<sub>2</sub>O

Compound		Rotamer*	(%)**
<i>Hetero- vs. Homo-chirality</i>			
Ac-P-ΨP-P-NH <sub>2</sub>	<b>2</b>	all- <i>t</i>	>95%
Ac-p-ΨP-P-NH <sub>2</sub>	<b>3</b>	<i>tct</i>	63%
Ac-P-ΨP-p-NH <sub>2</sub>	<b>4</b>	all- <i>t</i>	70%
Ac-p-ΨP-p-NH <sub>2</sub>	<b>5</b>	<i>tct</i>	>98%
<i>Elongation of homo-trimer 2</i>			
Ac-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>7</b>	all- <i>t</i>	>95%
H-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>8</b>	all- <i>t</i>	65%
		<i>ctttt</i>	35%
Ac-P-ΨP-P-P-ΨP-P-P-ΨP-P-NH <sub>2</sub>	<b>14</b>	all- <i>t</i>	>95%

\* *t* = *trans* amide bond and *c* = *cis* amide bond; \*\* proportion of rotamers assigned by integration of the different signals in <sup>19</sup>F NMR.



**Fig. 1** A. CD spectra of hexamers **6-8**, 100  $\mu\text{M}$  in PBS 20 mM, 4°C; B. CD spectra of nonamers **10-14**, 100  $\mu\text{M}$  in PBS 20 mM, 4°C; C. CD spectra of hexamers **7-9**, 4°C, 100  $\mu\text{M}$  in PBS 20 mM (plain lines), in *n*-propanol (dashed lines)

chromophore and thus change the profile of the PPII CD spectrum.<sup>14</sup> The CD signature of the chromophore Ac-CF<sub>3</sub>ΨPro-NHMe showed a more negative CD signal in the region around 225 nm compared to Ac-Pro-NHMe (Fig. S8). Therefore, the conformational analysis of oligomers **7** and **8** by CD spectroscopy suggests a PPII structuration, which is confirmed by the NMR results. We next studied the effect of both the location and the number of CF<sub>3</sub>ΨPro residues within the polyproline backbone (Fig. 1B). Interestingly, nonamers **11**, **12** and **13** bearing a CF<sub>3</sub>ΨPro residue at the *N*-terminal, middle and *C*-terminal positions respectively, showed similar CD spectra. Compared to the non-fluorinated nonamer **10**, the introduction of one CF<sub>3</sub>ΨPro provided only a slight effect on the profile of the CD spectra by shifting the negative band and lowering its magnitude. The introduction of the CF<sub>3</sub>ΨPro each three residues along the peptide sequence (oligomer **14**) accentuated this trend, demonstrating the additive effects of the CF<sub>3</sub>ΨPro chromophore on the CD spectra. It is well reported in the literature that polyproline peptides can adopt two different secondary structures.<sup>15</sup> The extended left-handed PPII helix is favoured in an aqueous environment and the more compact right-handed PPI helix is stabilized in an organic environment (e.g. *n*-propanol).<sup>10</sup> Reversible transition between these two forms can be easily induced by solvent change. However, the group of Wennemers showed that the PPII helix stability in organic solvents is dependent on the nature of the peptide terminal functional groups. Charged groups at both termini of polyproline dodecamers destabilized the PPII helix and favoured the PPI helix while their capped analogues displayed the opposite effect.<sup>16</sup> CD spectra of hexamers **7-9** in PBS solution (plain lines) and in *n*-propanol (dashed lines) respectively, exhibited similar trends (Fig. 1C). The acetyl and CONH<sub>2</sub> termini of hexamer **7** prevents the PPI helix formation in *n*-propanol, only inducing a significant decrease of the PPII ellipticity. However, hexamers **8** (NH<sub>2</sub><sup>+</sup>/CONH<sub>2</sub>) and **9** (NH<sub>2</sub><sup>+</sup>/COO<sup>-</sup>) presenting a charged amino group showed typical PPI helices signatures although slightly shifted (negative bands at 195 and 229 nm, positive band at 208 nm)<sup>17</sup> in *n*-propanol. Additionally, an isosbestic point is observed for hexamers **8** and **9** at 223 nm and 227 nm respectively (Fig. S9). This indicates that only two conformations are engaged in the transition process and strongly support the PPII folding of our fluorinated oligomers in aqueous media.

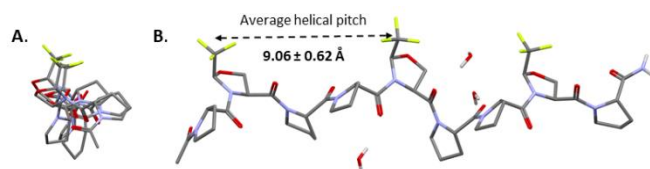
#### X-ray structure of nonamer **14**

Crystals of nonamer **14** were successfully obtained by slow evaporation of methanol giving access to the X-ray structure at the atomic resolution (Fig. 2, ESI). Three water molecules are co-crystallized and are H-bonded with the Pro<sup>4</sup>, CF<sub>3</sub>ΨPro<sup>5</sup> and Pro<sup>6</sup> carbonyls, respectively. They also participate in the interactions with neighbouring peptides and therefore in the stabilization of the PPII helical nonamer **14**. The crystal structure revealed a left-handed helix with all-*trans* amide bonds and a helical pitch of  $9.06 \pm 0.62$  Å, consistent with a PPII helix.<sup>18</sup> It is noteworthy that Pro<sup>1</sup> and Pro<sup>3</sup> residues display two potential puckering patterns in a 60/40 (*endo/exo*) mixture (Fig. S11 and S12), as observed in natural proteins. However, the 3-fold rotational symmetry is altered probably due to the fluorine incorporation close to the backbone, resulting in the distortion of dihedral angles (Table S6). Nonetheless, deviations from ideal values of PPII helix were also observed with the very few other examples of crystallized oligoproline.<sup>18</sup> Thus, the crystal structure of the nonapeptide **14** confirmed a PPII-like helical conformation.

#### Preliminary hydrophobicity, stability and toxicity data

The fluorinated oligomers showed increased retention times in reversed phase UPLC compared to their non-fluorinated counterparts, consistent with an increase of the local hydrophobicity, as observed with other fluorinated peptides (ESI).<sup>19</sup> The resistance of our fluorinated oligomers towards proteolysis was also investigated. Hexamer **7** and its non-fluorinated analogue **6** exhibit strong resistance in Pronase since no degradation was observed after two days. Interestingly, we demonstrated that the CF<sub>3</sub> groups can serve as an easy-to-handle <sup>19</sup>F NMR probe to reliably assess stability towards proteolysis (Fig. 3, ESI).<sup>4,20</sup> Finally, a cytotoxicity study on CHO-K1 cells revealed that fluorinated hexamer **7** is as non-cytotoxic as its non-fluorinated analog **6** (ESI). These results demonstrate the high stability and the non-toxicity of the fluorinated polyproline structures.

In conclusion, we reported the rational design of a series of oligomers based on (Pro-CF<sub>3</sub>ΨPro-Pro)<sub>n</sub> scaffolds (*n* = 1, 2 or 3) to access original fluorinated foldamers with a well-defined PPII-like helical structure that align the fluorinated moieties on the same side of the helix. While showing properties similar to



**Fig. 2** X-ray structure of nonamer **14** with Pro<sup>1</sup> and Pro<sup>3</sup> in *endo* puckering. **A.** Top-view of **14** from N-terminus (Pro<sup>1</sup>) to C-terminus (Pro<sup>9</sup>). **B.** Side-view of **14**

their non-fluorinated analogues such as stability towards protease activity and the absence of cellular toxicity, these foldamers present additional features. Their use as a <sup>19</sup>F NMR probe and the increase of peptide hydrophobicity make them highly valuable scaffolds for designing new original fluorinated amphipathic helices, crucial for cell-penetrating peptides (CPP) or antimicrobial peptides (AMP) applications.<sup>2e</sup>

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### Data availability

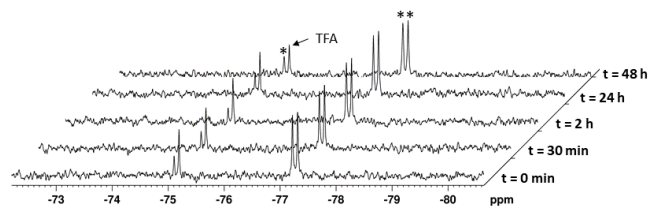
The data supporting this article have been included as part of the ESI. Crystallographic data for nonamer **14** has been deposited at the Cambridge Crystallographic Data Centre (CCDC) under deposition number 2352908 and can be obtained from [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif).

### Conflicts of interest

There are no conflicts to declare.

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**Fig. 3** Stability assay of hexamer **7** in the presence of Pronase monitored by <sup>19</sup>F NMR. \* unidentified fluorinated compounds (see ESI). \*\* two CF<sub>3</sub> signals of hexamer **7**

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