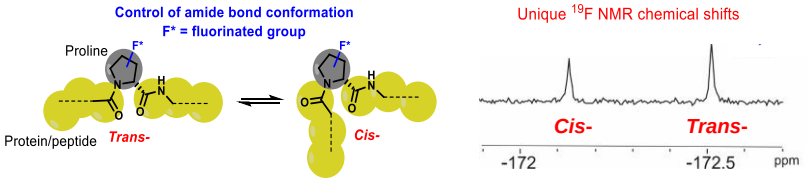
Fluorinated prolines as conformational tools and reporters for peptide and protein chemistry.

Sanne J.M. Verhoork, Patrick M. Killoran, and Christopher R. Coxon*

School of Pharmacy and Biomolecular Sciences, Byrom Street Campus, Liverpool John Moores University, Liverpool, L3 3AF, UK.

ABSTRACT: Amide bonds at the proline nitrogen are particularly susceptible to rotation, affording *cis* and *trans* isomers. Installation of a stereochemically-defined electron withdrawing fluorine atom or fluorinated groups has the power to influence the *cis-trans* conformational preferences of the amide bond in X-(F)Pro (where X = another amino acid). Advantageously, this also provides a sensitive reporter for ^{19}F NMR studies of protein conformation, interactions and dynamics. We deliberately use the term 'fluorinated prolines' as an all-encompassing term to describe proline analogues containing one or more fluorine atoms and to avoid confusion with the more well-known 4-fluoroprolines. This review presents a critical discussion of the increasing repertoire of fluorinated prolines that have been described; and importantly, provides a comparison of their uses and relative influence on amide bond conformation as well as discussing the significant potential of using ^{19}F NMR as a tool to probe for conformational change in polypeptides.



Of the twenty proteinogenic amino acids, proline is the only amino acid that has a side chain that forms a part of the protein backbone. This results in unique properties that are essential in peptide and protein three-dimensional structure.[1] Peptide backbone amide bonds are usually approximately planar and have a >1000-fold preference for a *trans* (apart in space) arrangement, yet proline allows both *trans*- and *cis* (together in space) forms to co-exist (Figure 1). Proline has two main conformational equilibria: amide bond *cis-trans* isomerism, and *endo*/*exo* pyrrolidine ring pucker. The pyrrolidine ring preferentially adopts an approximate C$^{\gamma}$-*endo* or C$^{\gamma}$-*exo* pucker (Figure 1B) and occurs as a mixture in solution (*endo*:*exo* = 66:34 for Ac-Pro-OMe at 25°C as derived from NMR Peak Intensities).[2,3]

Ring-puckering and *trans-cis* isomerisation at proline residues can influence the formation of α-helices or β-sheets as secondary protein structures through the introduction of a "kink" in the protein backbone structure. However, if proline is present inside a secondary structure it can also destabilise α-helices through the reduced hydrogen bonding capacity of the backbone nitrogen.[4,5] The presence of proline residues is often highly conserved in active-transport and ion channel proteins, where they are located in the middle of transmembrane helices, suggesting functional or structural importance.[6,7] In addition to conformational effects, prolines have also been shown to affect folding kinetics of proteins.[8] In fully folded proteins, an X-Pro (X = another amino acid) peptide bond is ~95% in the *trans* form and only ~5% in the *cis* form.[9] Proline isomerisation is often the rate-determining step in protein folding due to a ~80 kJ/mol energy barrier for interconversion.[5,10,11]

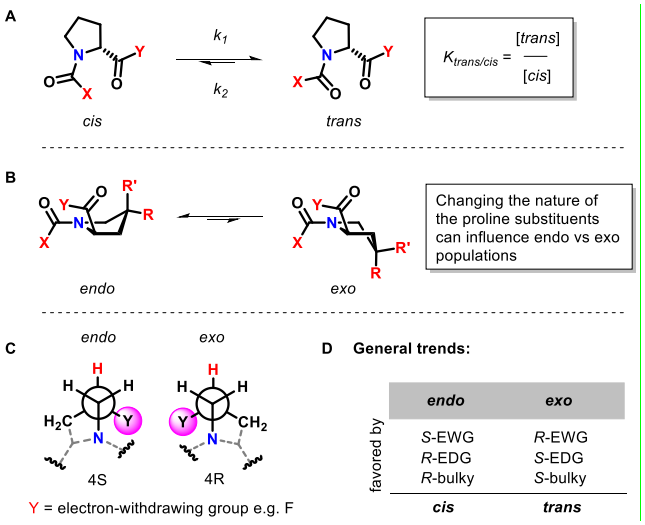


Figure 1. A) *cis*-to-*trans* conformer isomerisation within peptidyl-proline motifs. k_1 and k_2 are rate constants for *cis*-to-*trans* and *trans*-to-*cis* conformational changes, respectively. B) Interconversion between *endo* and *exo* ring-puckered forms is influenced by proline ring-substituents. C) Newman projections showing the *gauche* arrangement about an N-C-C-Z axis in a 4-substituted proline and the influence on ring pucker. D) General trends in ring pucker and X-Pro amide conformer preference (EWG = electron-withdrawing group, EDG = electron-donating group).

On the contrary, poly-proline motifs (e.g., Pro-Pro-Xaa in collagen) can also promote a stable helical topology. As prolines are important for influencing overall protein or peptide stability and folding kinetics it forms an interesting strategy to control ring puckering and isomerisation and to study these events through modification or replacement of the pyrrolidine ring.

Steric control of proline conformation

Improvement of protein stability can be advantageous for reducing cold chain storage and transport problems associated with biologics, or for understanding the functional role and dynamics of a protein. A number of conformationally-biased proline analogues have been used to probe protein conformation and stability. Modifications to proline include N-alkylation or incorporation of heteroatoms into the pyrrolidine ring 4-position.[12] Sterically hindered 5-*tert*-butylproline (Tbp, **5**)[13] and 5,5,-dimethylproline (Dmp, **4**) lock L-proline in a very high proportion of the *cis*-conformation in peptides and proteins.[14] Various ring sizes have been explored through pipecolic acid (Pip, **2**) and azetidine-2-carboxylic acid (Aze, **3**). In general, constrained prolines provide a range of *trans*:*cis* preferences (Figure 2) in model peptide systems as measured using nuclear magnetic resonance (NMR) spectroscopy,[12] and are, therefore, very useful tools for understanding the stability, folding and dynamics of peptides and proteins.

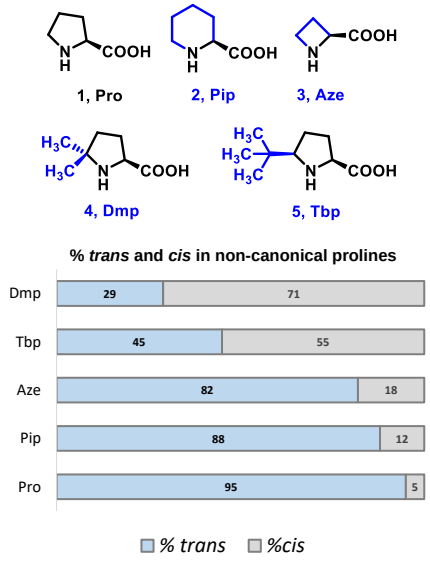


Figure 2. A) Examples of non-canonical constrained proline 'analogues' used in protein and peptide systems. B) *cis* / *trans* preference determined in model systems. Numerical data are as reported in reference 15.

Fluorinated prolines

Fluorine has enjoyed significant attention in medicinal and pharmaceutical chemistry, with an increase in occurrence from 20% to about 30% of drugs in recent years.[16,17] Introduction of fluorine has been associated with improved metabolic stability,[18] altered physicochemical properties, and increased binding affinity. Despite the prevalence of fluorine in medicinal chemistry, the main focus of research has been in small molecules, with multiple reviews describing the applicability of fluorine in this area.[16,19,20] However, the use of fluorine in peptide and protein chemistry is less explored[21,22] and has possibly been limited by the availability of commercial fluorinated Fmoc-/Boc-protected amino acid building blocks compatible with conventional solid phase peptide synthesis or methodologies to directly and specifically fluorinate peptides.

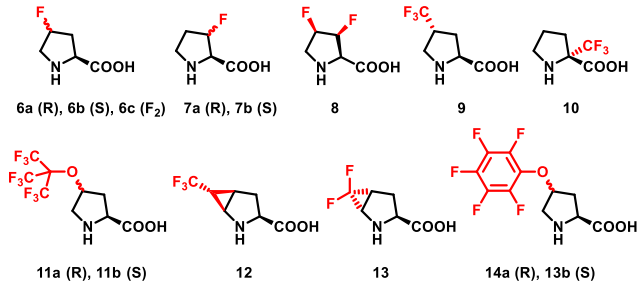


Figure 3. Various reported fluorinated-proline derivatives used in peptides and proteins.

Fluorine-labelled amino acids have been useful in studying protein-ligand interactions using ^{19}F NMR. For example, fluorinated tyrosine and tryptophans were incorporated using recombinant expression techniques into the bromodomains of Brd4, BrdT, and BPTF to measure binding of small molecule ligands.[23] Conversely, fluorine has been incorporated into a peptide ligand to elucidate ligand recognition and specificity of human protein disulfide isomerase using ^{19}F ligand-observe in conjunction with ^{15}N,^{1}H-HSQC protein-observe NMR methods.[24] For additional reading on ^{19}F NMR as a tool to study protein/peptide interactions, readers are directed to the review by Marsh and Suzuki.[25]

A number of reviews[21,26] have described the use of fluorine in peptide and protein chemistry but with little specific focus on fluorinated proline analogues, where this review will focus. Fluorinated prolines have the potential to be exceptionally useful tools in chemical biology to modulate protein or peptide stability and improve folding kinetics. Introduction of electronegative fluorine greatly influences the kind of interactions that can be formed between proline and its natural binding partners. Whilst organic fluorine (C-F) is a weaker hydrogen-bond acceptor than corresponding alcohols or carbonyls;[27,28] its introduction has been found to provide new non-native interactions (see 4-fluoroprolines).[29] Fluorine is also very electronegative and, therefore, is strongly electron-withdrawing, which can reduce the hydrogen-bond accepting ability of neighbouring groups and potentially weaken intermolecular interactions. However, the high electronegativity of fluorine makes it a good candidate for orthogonal multipolar or dipole interactions,[30] e.g., with backbone carbonyl groups of other proteins.[31]

Conformational effects

Proline contains a conformationally-restricted 5 membered pyrrolidine ring, which is puckered (non-planar). Energy minima correspond to the two approximate envelope conformations with C$^{\gamma}$ *endo* and *exo* puckered[29] (Figure 1B) and the relative populations of which are to a significant extent dictated by the nature and the stereochemistry (*S*- or *R*-) of substituents at proline β (3) and γ (4) positions. 4-Hydroxyprolines and 4-fluoroprolines are by far the most common proline analogues reported. 4-Hydroxyproline occurs in nature[32] and

can be used as a precursor for the synthesis of 4-fluoroprolines.[33] Zondlo's Group performed a comprehensive study, in which the 4-position substituent on hydroxyproline (Hyp) was varied to include electron-withdrawing/ donating and different sized groups to compare the effect of steric *versus* stereoelectronic effects on proline-peptide conformation.[34] For electron-withdrawing substituents at the 4-position, *S*-stereochemistry strongly favours an *endo*-pucker; whilst *R*-stereochemistry strongly favours *exo*-pucker (Figure 1C). However, this is reversed for non-electron-withdrawing groups and/or large bulky substituents (Figure 1D). This demonstrated that proline conformation is potentially tuneable within synthetic peptides post-synthesis, opening up possibilities for stimulus-responsive conformational-switching.

The preference for a specific ring-pucker conformation following substitution at the 3- or 4- position of the pyrrolidine ring with fluorine (or another electron withdrawing group)[35–37] can be explained by the adoption of a *gauche* (60° torsion angle) conformation between the amide nitrogen and the *vicinal* fluorine atom in an N-C-C-Z (Z = N, O, F) system (Figure 1C). The ring-pucker preference is, therefore, reversed by switching the stereochemistry of the electron withdrawing group. Note: the fluorine-ammonium *gauche* effect has been thoroughly explained elsewhere.[38] In general *exo* pucker is considered to lead to higher preference for the *trans*-amide rotamer, and *endo* pucker a lower *trans* preference (higher population of *cis* amide; Figure 1D). Studies have indicated that *exo*-ring pucker in model Ac(F)ProOMe systems ((F)Pro indicates fluorinated proline – not limited to 4-fluoroprolines); stabilises the *trans* amide through an enhanced $n \rightarrow \pi^*$ interaction between the amide oxygen and the ester carbonyl carbon.[39] To examine the relationship, a series of conformationally-locked proline analogues with a fluorinated methylene bridge (5-fluoromethanoprolines) were synthesized to mimic the 'locked' *endo* and *exo* puckered prolines. This showed that, in these systems at least, substituents on proline analogues can affect the observed *trans–cis* preference in ways independent of puckering and $n \rightarrow \pi^*$ stabilising interaction.[40] Mykhailiuk probed the individual through-bond effect of the electron withdrawing group at the 4-position in 2,4-methanoproline models by measurement of the pyrrolidine nitrogen pK_a and *cis-trans* isomerisation, whilst not being confounded by ring puckering.[41] They concluded that the electron-withdrawing fluorine has a significant effect upon the kinetics but little effect upon the thermodynamics of amide bond isomerism. For example, in 4-fluoro-2,4-methanoprolines the barrier to rotation is reduced by ~3 kJ/mol leading to a rotation rate-enhancement of ~4- to 5-fold, as compared with the non-substituted analogue; which likely translates into the analogous proline system.

The well explored proline substitution with fluorine (4*R*- or 4*S*-fluoroproline) restricts the spontaneous isomerisation of the proline peptide bond, with both the rate and the equilibrium constants found to be affected.[42] For this reason, there is increasing motivation for the use of fluoroprolines to probe conformational, structural and stability properties in rational protein or peptide design. Overall, the incorporation of fluorine has been driven by two main factors a) powerful conformational effects of fluorine b) the possibility for ^{19}F to be used an NMR reporter for structural and stability studies. A number of fluorinated prolines have been reported, for which the conformational effects are typified by 4-fluoroprolines. The synthesis and main uses of 4-fluoroprolines has been thoroughly described recently in an excellent review by Raines and Newberry.[43]

4-Fluoroprolines

The use of fluoroprolines as a conformational probe in chemical biology requires the artificial introduction of these through either i) solid phase peptide synthesis or ii) recombinant protein technologies. The first report of using synthesised fluorinated prolines in peptide or protein systems was in 1965 from Bernhard Witkop's group, incorporating tritiated *R*- and *S*- 4-fluoroproline in to collagen via *E. coli* or guinea pig granulomata homogenates, and studying enzymatic hydroxylation.[44]

The commercial availability of L-, D- and di-substituted Fmoc-4-fluoroprolines (**6a, b, c**) allows for convenient laboratory synthesis of short peptides, e.g., proctolin (Arg-Tyr-Leu-FPro-Thr; where FPro = 4*S*- / 4*R*-fluoroproline), a neurotransmitter found in motor neurons of insects and the postural motor neurons of crayfish.[45,46] However, this is generally limited to polypeptides of <40 amino acids, without relying on chemical ligation approaches. 4-Fluoroproline analogues have also been incorporated into proteins via protein total synthesis using native chemical ligation. By incorporation of 4-fluoroprolines with different *trans* / *cis* amide preferences, Torbeev and Hilbert showed that isomeristion of Pro32 of β2-microglobulin from its native *cis* to a non-native *trans* conformation triggered misfolding and subsequent amyloid assembly.[47] 4-Fluoroprolines have been incorporated into proteins, e.g., ubiquitin[48] and collagen[37] where the 4*R*-fluoroproline was shown to increase thermal stability compared to the wild-type protein. Whilst there are several examples of introduction of 4-fluoroprolines biosynthetically into a protein, e.g., barstar C40A/C82A/P27A in *Escherichia coli*, this can present many challenges.[9,49] It is important to consider the additional effects of the introduction of 4-fluoroproline into peptides or proteins for biological studies. For example, even though the 4*S*-fluoroproline proctolin insecticide peptide had the wild-type isoform peptidyl-proline bond, only the 4*R*-fluoroproline peptide showed biological activity towards *tortrix* insects, suggesting that the ring puckering can be of greater influence on affinity than *cis-trans* isomerisation.

The *gauche* effect is eliminated when two fluorine atoms are substituted in the proline 4-position (4,4-difluoroproline, **6c**) as they counteract their electronic effects, leading to behaviour similar to native unsubstituted proline, based on its similar $K_{trans/cis}$ equilibrium ratios.[9,50] However, interestingly, it has been reported that this can be more structurally disruptive than the mono-fluorinated prolines.[15] Inserting fluorine atoms at the proline 4-position was observed to reduce the double-bond character of the prolyl amide, lowering the energy barrier for *trans-cis* isomerization in the order Pro > 4R-Flp ~ 4S-flp > F2Pro and, therefore, increasing the isomerisation rate in the model system Ac-Xaa-OMe.[47] These examples show that fluorinated prolines can stabilise or destabilise peptides or proteins compared to the wild types. Overall. when (4*R*)- or (4*S*)-fluoroproline is used the properties of the peptide or protein are altered compared to normal proline,[11] limiting the possibilities to use this as an unbiased reporter amino acid.

Polyproline peptides can adopt either type I (PPI) helices with all *cis* amide bonds or type II (PPII) helices with all *trans* am-

ide bonds. Therefore, the inclusion of conformationally-biased fluoroproline has been used to stabilise these specific conformations. For example, a C-terminal (4*R*-FPro)3 motif stabilises PPII helices, whereas, a (4*S*-FPro)3 triplet destabilises PPII helices.[51]

3-Fluoroprolines

Several groups have sought to design alternative fluorinated prolines as native proline-replacements. These developments have been largely drawn from the desire to use fluorinated probes to study protein structure and dynamics. A fluorine substituent at the proline ring 3-position (**7a, b**) is also able to adopt a *gauche* conformation with respect to the ring nitrogen, which provides control over ring-puckering akin to the 4-fluoroprolines. The *trans* and *cis* conformers of the model peptides Ac-(3-F)Pro-OMe produce distinct ^{19}F resonances separated by 0.8 ppm in (3-*S*)-fluoroproline and ~2 ppm for (3-*R*)-fluoroproline.[52] The $K_{trans/cis}$ equilibrium constants and relative populations of *cis* and *trans* amide for each and are summarised in Figure 4. The measured $K_{trans/cis}$ for (3-*S*)-fluoroproline is comparable with proline in Ac-Pro-OMe at pH 7.4, 37 °C. Interestingly, a higher entropic barrier for both *cis-trans* and *trans-cis* isomerisation was measured by Eyring analysis for (3*R*)-fluoroproline compared to the (3*S*)-, indicating that a *syn*-configured fluorine atom may sterically hinder rotation around the C-N bond. Conticello and co-workers demonstrated (using X-ray diffraction studies) that the 3-fluoroprolines series display the same C$^{\gamma}$-ring-pucker preferences as the corresponding 4-fluoroprolines, e.g., both Ac-(3*R*)-(F)Pro-OMe and Ac-(4*R*)-(F)Pro-OMe favour *exo*; both with high *trans*:*cis* amide preferences.[49] This was supported by density functional theory calculations.[53] Only a relatively small impact upon $K_{trans/cis}$ is observed in the 3-fluoroprolines as compared with 4-fluoroprolines, making them useful tools that have been biosynthetically-incorporated into an elastin peptide.[49] To date, there have been no reports of utilising 3-fluoroprolines in protein systems.

3,4-Di-fluoroprolines

One of the main challenges remaining in this field is obtaining non-invasive probes; that is non-proteinogenic fluorinated amino acids that behave like their cognate counterpart *i.e.* do not interfere with protein folding kinetics. Whilst the 4,4-difluoroproline exhibits similar energy *exo* and *endo* puckers, the geminal CF_2 with diastereotopic fluorine atoms may complicate or even distort the ^{19}F NMR spectrum. A different approach to avoid conformational-distortion by the introduction of fluorine at e.g., the 4-position, thereby maintaining 'natural' proline ring pucker and amide conformations is to introduce a second fluorine atom at the vicinal 3-position (**8**) as recently reported by Linclau *et al* (Figure 4).[54] The main advantage of this approach is the relative improvement in the NMR spectrum, which exhibits smaller $^3J_{F\text{-}F}$ coupling constants and different chemical shift as compared with geminal CF_2 (Table 1). This provides the opportunity for double-labelling of proteins and peptides e.g., polyprolines using different native-like fluorinated prolines with different characteristic ^{19}F reporter signals.

Trifluoromethylprolines

Trifluoromethylation of proline is attractive as this provides a higher signal-to-noise ratio for use in ^{19}F NMR studies. Introduction of an α-trifluoromethyl substituent at proline (**10**) can, however, adversely affect peptide backbone conformation and electronics, making them difficult substrates for standard solid phase peptide synthesis, owing to a lower nucleophilicity at the amino group,[55] and also altering the folding compared to proline.[56] Despite the difficulties, Brigaud and co-workers utilised this building block to prepare a tripeptide α-TfmPro-Leu-Gly, MIF-1 analogue for evaluation as an antinociceptive agent with enhanced analgesic activity versus the parent sequence.[57] No comment was made on the $K_{trans/cis}$ in this system and it seems that incorporation into a protein system would present significant challenges to overcome.

The direct trifluoromethyl analogue of 4-fluoroproline, 4-trifluoromethylproline (TfmPro, **9**) has been synthesised *via* several routes,[58–61] including by Ulrich and co-workers.[62] Unlike other fluorinated prolines, Ulrich also showed TfmPro in the model system Ac-TfmPro-OMe, to be significantly more 'native proline-like' with regard to i) its backbone conformational propensities, ii) $K_{trans/cis}$ equilibrium constant (TfmPro = 4.0; Pro = 4.8 at 300 K), iii) activation energy for *cis* → *trans* rotation (TfmPro = 81.8 kJ/mol; Pro = 84.5 kJ/mol) and iv) when incorporated into cyclic peptide gramicidin S, which showed virtually no structural perturbation by circular dichroism as compared with the natural product. However, this has not been incorporated into proteins at the time of submission.

Fluorinated methanoprolines

In order to address a perceived lack of conformational-rigidity of 4-trifluoromethylproline, Ulrich *et al* designed and synthesised trifluoromethyl- 3,4- and 4,5- methanoproline.[63] The use of these in peptide synthesis may be limited by a low total yield in the reported synthesis of 3,4-systems and high acid-sensitivity of some of the 4,5-epimers. However, trifluoromethyl-4,5-methanoproline **12** was incorporated specifically into the polyproline II (PPII) forming cell-penetrating "sweet arrow peptide" (VRLPPPVRLP**X**PVRLPPP; **X** = trifluoromethyl-4,5-methanoproline). The resulting peptide was found to be compatible with solid phase peptide synthesis, pH-stable and racemisation-resistant during the synthesis and was able to stabilize the PPII helical structure. To study the reasons for the excellent stabilising properties of trifluoromethyl-4,5-methanoproline (**12**), the more electron-withdrawing *trans*-4,5-difluoromethanoproline (**13**) was synthesised.[64] However, this was found to be unstable upon N-deprotection. Despite being itself unsuitable for SPPS, it was shown to be possible to

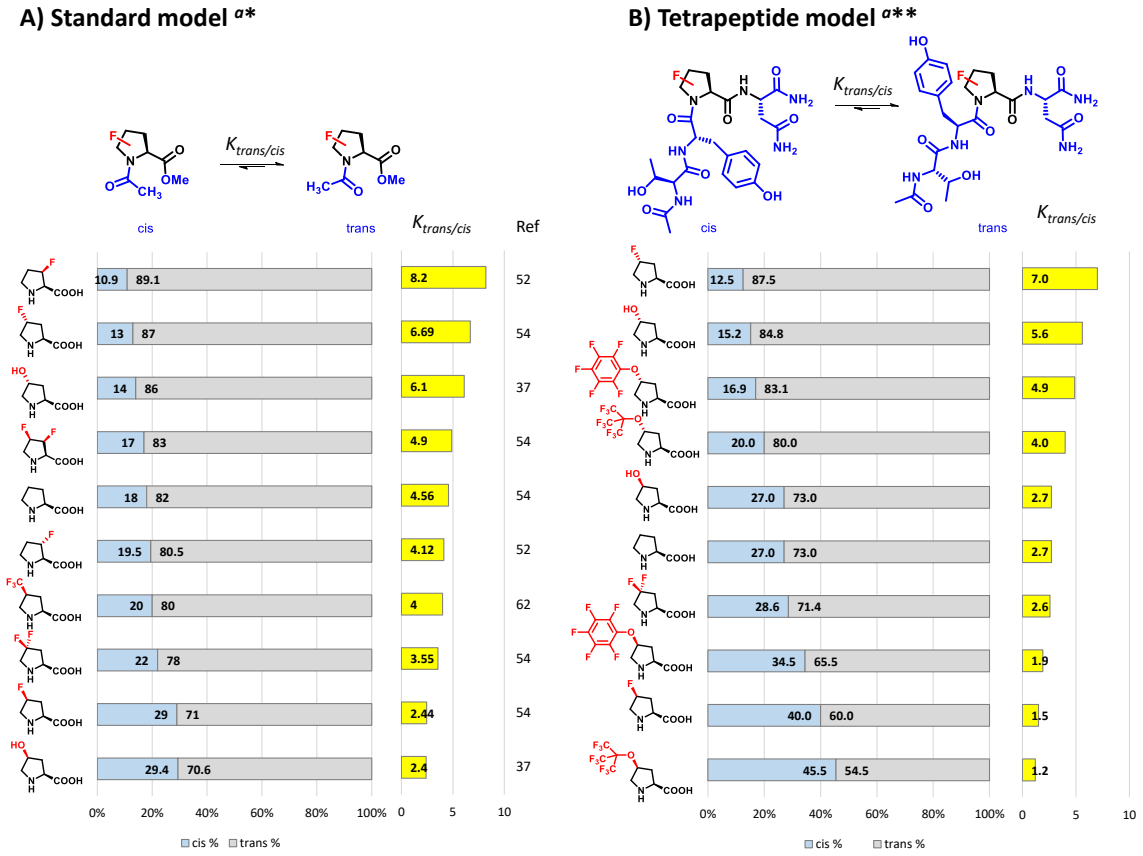


Figure 4. Graphical ranking of differential %*cis* and *trans* conformers (blue/grey) depending on nature of the fluorinated proline in A) the standard AcProOMe* and B) a tetrapeptide model showing the similar trends but quantitative differences between a simple model and a short peptide sequence. The corresponding $K_{trans/cis}$ (ratio of peptide with *trans* amide bond compared to peptide with *cis* amide bond as determined by NMR) is also presented graphically (yellow). Notes: [a] Where more than one value is reported in different publications, the values are generally in good agreement with values shown. * Data obtained Ref 52 (D_2O at 37 °C), Ref 54 (D_2O at 25 °C), Ref 37 (D_2O at 25 °C) and Ref 62 (D_2O at 25 °C). ** Data obtained in 25 mM NaCl, 5 mM phosphate [pH 4], 9:1 H2O/D2O at 298K.[34]**

incorporate this into peptides using a rather long semi-solution phase route, as a stable label for ^{19}F NMR structure analysis.[65] Unfortunately, for each of the methanoproline systems, no data has yet been reported to indicate *trans*:*cis* preferences or equilibrium constants.

Perfluoro-tert-butyl-hydroxyproline

Perfluoro-*tert*-butyl-hydroxyproline **(11a, b)** was designed to further enhance the signal-to-noise ratio in ^{19}F NMR studies.[66] Initially, the perfluoro-*tert*-butyl group was directly added to hydroxyproline as part of a peptide, *via* a so-called 'proline-editing' approach.[34] In the model peptide system, Ac-TYXN-NH_2 (X = either 4*R*-perfluoro-*tert*-butyl hydroxyproline or 4*S*-perfluoro-*tert*-butyl hydroxyproline) 4*S*-perfluoro-*tert*-butyl hydroxyproline exhibited an impressive *trans*:*cis* conformational preference ($K_{trans/cis}$ = 1.2) compared to the corresponding proline peptide ($K_{trans/cis}$ = 2.7). Subsequently, the Boc- and Fmoc-(2S, 4R)- and (2S, 4S)- perfluoro-*tert*-butyl hydroxyprolines were synthesised and utilised successfully in SPPS in the preparation of an alanine-rich α-helical peptide and a proline-rich sequence. The coupling reaction of these fluorinated prolines was slow as compared with standard amino acids and was attributed to steric hindrance associated with the perfluoro-*tert*-butyl group. The 4*R*-OC$(CF_3)_3$ derivative was found to promote α-helicity more than proline itself, whereas, 4*S*-OC$(CF_3)_3$ discouraged helix formation. Both were found to lower the propensity to form a polyproline helix. However, increased steric demand, significant modulation of pyrrolidine ring electronics and high lipophilicity may significantly affect its utility as a probe for protein structure and dynamics.

Pentafluorophenyl-hydroxyproline

Pentafluorophenyl-hydroxyproline[34] **(14a, b)** contains 5 fluorine atoms. However, this produces a more complex ^{19}F NMR spectrum than more symmetrical fluorinated groups. While these strongly-favour a *trans*-conformation, so far such groups have not yet been exploited to study protein systems. However, the corresponding perfluorinated reagents have the distinct advantage that they are particularly suited to direct chemoselective 'tagging' of synthetic and possibly native peptide/protein side chains rather than using SPPS, with similar

functionalities having been incorporated by the Cobb and Pentelute groups into peptides at sidechains other than hydroxyproline, e.g., cysteine and lysine.[67–71] Therefore, they may be excellent candidates for proline editing on a protein.

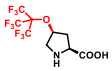
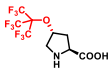
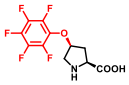
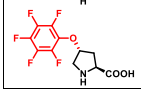
A comparison of conformational effects

It is clear that different fluorinated prolines offer differing degrees of *cis*-conformer stabilisation. Using the reported $K_{trans/cis}$ values, it was possible for us to calculate the expected % *trans* and % *cis* to provide a useful comparative reference. This provides a useful tool-kit for probing conformational and stability properties of a protein or peptide. There has previously been no collective assessment of the many published effects of wider proline fluorination on the relative *trans*:*cis* ratio for the fluorinated prolines discussed. These data have been collated from a number of references that agree (in some cases cite each other) in Figure 4. Helpfully, most groups have chosen to study the model Ac-(F)ProOMe ((F)Pro = fluorinated proline). However, some of the data shown is derived from an alternative model peptide Ac-TYPN-NH_2. Where comparison is possible, there is general agreement between the two systems, with *S*-stereochemistry fluorinated groups favouring a higher proportion of *cis* conformation than their enantiomeric partner in both model systems. However, an interesting observation is the different extents to which the same fluorinated proline promotes the *cis*-conformer in the different systems. For example, 4S-fluoroproline in Ac(F)ProOMe exists in around 29% *cis*, whereas, in Ac-TY(F)PN-NH2 it promotes a significantly larger population of 40% *cis*. What is clear from the comparison of reported data in the Ac-TYPN-NH_2 system is that the fraction of *cis*- and *trans*- isomers is affected by the preceding amino acid, with aromatic residues favouring the *cis* isomer slightly in most cases.[72] Other notable examples within Ac-TYPN-NH_2 include, 4-perfluoro-*tert*-butyl- and 4-pentafluorophenyl- hydroxyprolines that display significant $K_{trans/cis}$ preferences for *cis* amides in the *S*-configuration, whilst the *R*-enantiomers seem to behave more like the parent hydroxyproline. The equilibrium constants derived for each fluorinated proline may not be directly comparable as they are measured in different systems and conditions, however, this provides a rough guide to the relative ranking of each.

Potential applications of fluorinated prolines and ^{19}F NMR to study dynamic biological processes

Peptides and proteins are large biomolecules making it difficult to study them by the conventional chemical methods of ^{1}H and ^{13}C NMR. For example, protein dynamics (e.g., isomerisation, folding) can be difficult to study as substrate and product have the same chemical constitution and exist in dynamic equilibrium. However, the ratio between *cis* and *trans* proline species was originally demonstrated using a 4-position ^{13}C-labelled proline in collagen. The significant drawback of this approach is the requirement for an expensive NMR cryoprobe to enhance the low sensitivity of ^{13}C nuclei.[73]

Table 1. Reported characteristic ^{19}F NMR chemical shifts for *trans* and *cis* conformers in model systems as indicated.

Fluorinated proline	Ac-FProOMe *trans* (ppm)	Ac-FProOMe *cis* (ppm)	Ac-TY-FPro-N-NH_2 *trans* (ppm)	Ac-TY-FPro-N-NH_2 *cis* (ppm)
	-177.85 [54, A]	-177.79	-177.1 [34,D]	-177.9
	-173.26 [54, A]	-173.25	-173.2 [34,D]	-173.5
	-98.4 (pro-*S*) [54, A] -102.2 (pro-*R*)	-95.5 (pro-*S*) -104.7 (pro-*R*)	-100.6 [34,D]	-103.2, -103.9, -94.8, -95.4
	-203.3 (F^4)[54, A] -210.4 (F^3)	-200.3 (F^4) -208.5 (F^3)	-	-
	-108.5[52, B]	-106.5	-	-
	-97.5 [52, B]	-98.6	-	-
	-71.0 [62, C]	-71.3	-	-
	-	-	-70.0 [67,D]	-70.7
	-	-	-70.7 [67,D]	-70.8
	-	-	-163.4 [34,D] -162.4 -156.3	-163.6 -162.9 -156.3
	-	-	-163.2 [34,D] -161.9 -156.5	-163.6 -162.1 -156.1

Notes: NMR conditions A) D_2O, 25˚C; B) 9:1 H_2O/D_2O, 35˚C (note: no exact values provided in ref 52 – chemical shift values in above table are estimated from raw NMR spectra); C) D_2O, 27˚C; D) 5 mM phosphate buffer pH 4 with 25 mM NaCl in 9:1 H_2O/D_2O at 23 °C.

Several examples mentioned previously, have used ^{19}F NMR for easier and more accurate determination of the populations of *cis* and *trans* conformations in fluorinated prolines and this should be applicable as a tool to study dynamic biological processes. However, so far there are rather few examples of this. ^{19}F NMR has the significant advantage that it has a very wide chemical shift range and characteristic signals for *cis*/*trans* isomers. Whilst there are some small discrepancies between reported ^{19}F NMR chemical shift values, we have provided referenced examples in Table 1. Advantageously, the low number of atoms present (e.g., one ^{19}F NMR signal for proline compared with seven ^{1}H) means the ^{19}F spectrum of a protein containing multiple fluorinated amino acids can be well-resolved.[74] Natural occurrence of fluorine in biomolecules and in nature is very low,[75] thus making fluorine a perfect reporter atom for ^{19}F NMR. Fluorine NMR studies of proteins with their ligand provides valuable insight into ligand-target interactions and dynamics.[76–79] Fluorinated amino acids, and by proxy peptides and proteins, allow sensitive detection in complex media, biological samples and even microorganisms. However, the sensitivity of the detection can be limited by the signal intensity of the F-containing analyte. Moreover, in a biological system, only very low concentrations may be possible. As such, the use of proline derivatives containing several symmetrical fluorine nuclei e.g., trifluoromethyl- prolines (**9**, **10**), methanoprolines (**12**) (three ^{19}F nuclei) or perfluoro-*tert*-butyl hydroxyprolines (**11**) (nine ^{19}F nuclei), are exciting as they offer enhanced sensitivity. Zondlo showed that peptides containing

11 could be used to visualise several different conformations and was detectable at a low concentration of 200 nM, demonstrating the utility of this as a biological probe.[66]

Whilst, it should be considered that introduction of a fluorine can interfere with the natural characteristics of a peptide/protein (through e.g., stabilised conformation, altered electronegativity or increased hydrophobicity);[42] fluorinated prolines favouring one isoform over the other are useful tools for measuring kinetics of isomerisation, and in turn by slowing down isomerisation, can be a useful probe for enzyme mechanism and function. As such, the combination of conformational/kinetic-control and provision of a sensitive NMR reporter group, gives fluorinated proline derivatives exceptional potential as tools to measure peptide and protein conformation.

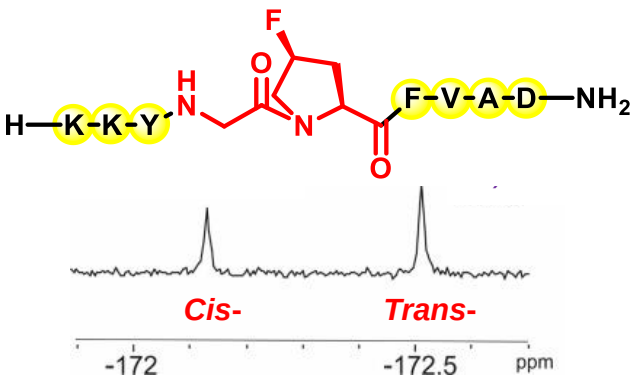


Figure 5. ^{19}F NMR is a powerful approach to study peptide and protein dynamics, such as isomerisation.

Conclusions and future directions

An increasing number of fluorinated proline analogues have been reported, principally for the purpose of controlling conformation *via* changing the ring-pucker of the proline and/or the amide bond conformation. Some even behave as more proline-like by not interfering with natural *cis-trans* isomerism. Given that these possess a range of effects upon *trans:cis* amide bond conformation, they provide a useful tool-kit for exploring protein folding, dynamics, stability and function. Whilst the *trans:cis* ratios provided from model systems are informative from the perspective of being able to rank the effects of various proline-modifications, the effects are likely to be more subtle in a protein where the *gauche* effect is overpowered by the many hundreds or thousands of interactions that promote the folding of a protein into a tertiary structure. Furthermore, much of this work using simple model systems has neglected to fully rationalise the effect of neighbouring amino acids upon the observed *trans:cis* ratio. Therefore, a more thorough investigation of how the amino acid 'X' in an 'X-(F)Pro' system affects the observed conformational properties is required as a small step towards understanding the longer distance effects in proteins. Other future developments will likely use novel fluorinated prolines with distinct conformational properties to understand new protein systems or for the *de novo* design of proteins with new functions. This may be achievable through increasingly sophisticated recombinant technologies or through protein total chemical synthesis. The value of being able to use ^{19}F NMR to measure dynamics and protein properties is yet to be fully-realised and undoubtedly holds much promise. This is due to the natural scarcity of organic fluorine as well as its wide chemical shift range and high-sensitivity to local environments, making analysis relatively simple and achievable in real-time. Moreover, newer NMR technologies will continue to enable more precise measurements and require lower fluorinated-protein concentrations increasing the future utility of these tools in protein and peptide chemical biology.

AUTHOR INFORMATION

Corresponding Author

* Email c.r.coxon@ljmu.ac.uk

ORCID

Christopher R Coxon: 0000-0002-3375-3901

Author Contributions

The manuscript was written through contributions of all authors.

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KEY WORDS

Conformation/conformer: A form of stereoisomerism, in which the isomers can be interconverted by rotations about single bonds. Such isomers are generally referred to as conformers or rotamers.
Cis: A molecular structure in which two particular atoms or groups lie on the same side of a given plane in the molecule.
Trans: A molecular structure in which two particular atoms or groups lie on the opposite side of a given plane in the molecule.
***Gauche*-effect:** The tendency to adopt a conformation that places vicinal electron-withdrawing groups (e.g., fluorine in 1,2-difluoroethane) at a torsion angle of approximately 60° with respect to one another. This is generally due to the avoidance of destabilising electronic effects.
Ring pucker: The non-flat 3D shape adopted by a cyclic molecule that minimise ring-strain and/or eclipsing interactions.
Peptide: A polymer consisting of a chain of amino acids (usually less than 50) adjoined by amide bonds. The amide bonds usually have a 'trans'-conformation.
NMR: Nuclear Magnetic Resonance spectroscopy - an analytical technique used for identifying and quantifying the components in a mixture of chemicals e.g., conformers.
Chemical shift: In NMR spectroscopy (see above), the chemical shift is the characteristic resonant frequency of a specific nucleus relative to a standard in a magnetic field. Chemical shift is a useful way to identify specific components in a mixture and is represented as δ 'delta' in ppm.

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