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4-Fluoroprolines: Conformational Analysis and Effects on the Stability and Folding of Peptides and Proteins

Robert W. Newberry and Ronald T. Raines

Abstract Proline is unique among proteinogenic amino acids because a pyrrolidine ring links its amino group to its side chain. This heterocycle constrains the conformations of the main chain and thus templates particular secondary structures. Proline residues undergo posttranslational modification at the 4-position to yield 4-hydroxyproline, which is especially prevalent in collagen. Interest in characterizing the effects of this modification led to the use of 4-fluoroprolines to enhance inductive properties relative to the hydroxyl group of 4-hydroxyproline and to eliminate contributions from hydrogen bonding. The strong inductive effect of the fluoro group has three main consequences: enforcing a particular pucker upon the pyrrolidine ring, biasing the conformation of the preceding peptide bond, and accelerating *cis–trans* prolyl peptide bond isomerization. These subtle yet reliable modulations make 4-fluoroproline incorporation a complement to traditional genetic approaches for exploring structure–function relationships in peptides and proteins, as well as for endowing peptides and proteins with conformational stability.

Keywords Collagen • Gauche effect • $n \rightarrow \pi^*$ interaction • Pyrrolidine • Stereoelectronic effect

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1 Introduction

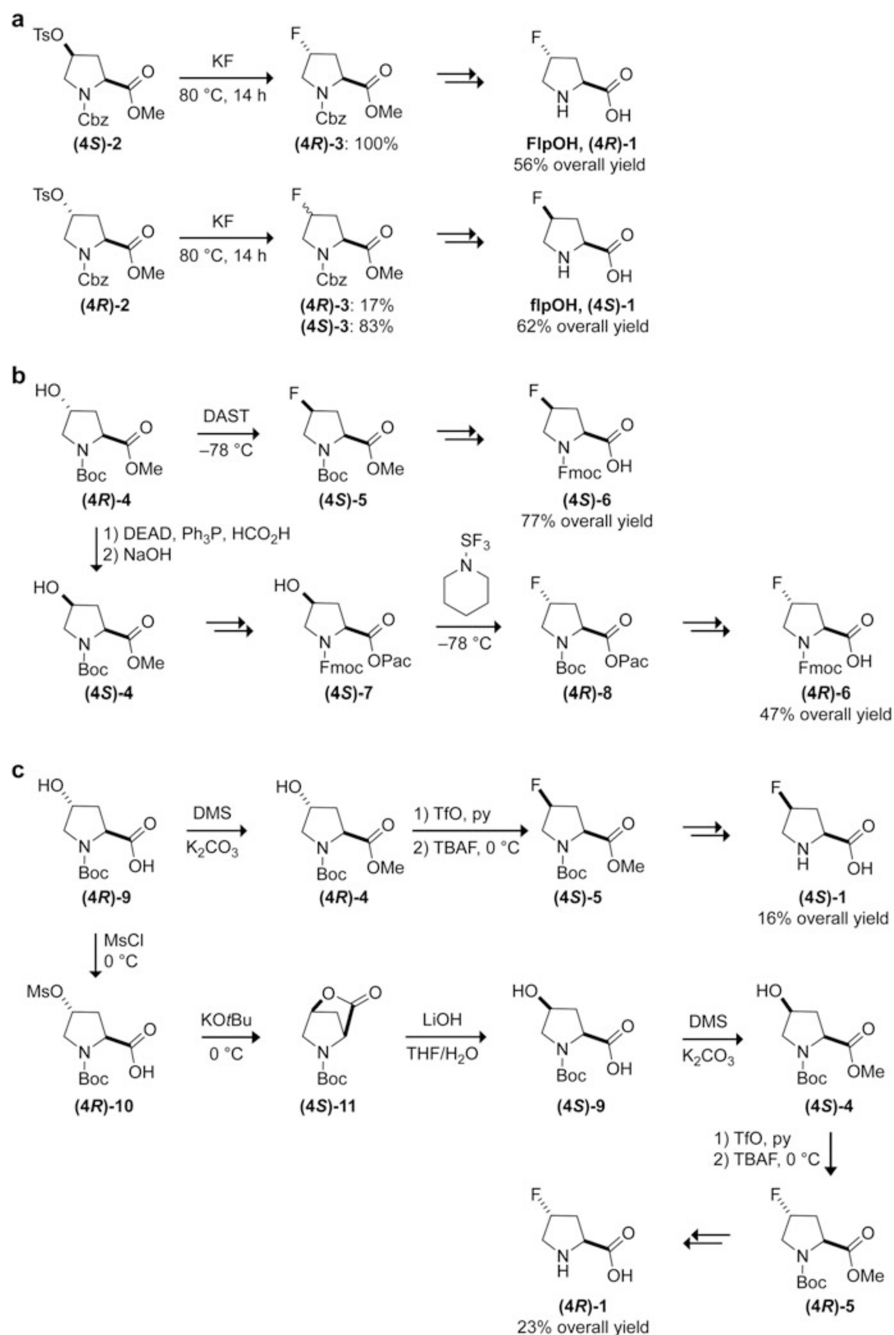
Proline is unique among proteinogenic amino acids because its α-amino group is constrained within a pyrrolidine. This heterocyclic ring restricts the ϕ (C_{i-1}'–N_i–C_i^{α}–C_i') main-chain dihedral angle, making proline an important determinant of the conformational stability of proteins [1]. Moreover, the pyrrolidine ring promotes population of both the *cis* and *trans* conformation of the preceding peptide bond, thereby enabling structural diversity. The isomerization of this "prolyl amide" bond can limit the folding rate of a protein [2].

Consistent with the conformational restriction imposed by proline relative to other amino acids, proline is prevalent in structural proteins, especially collagen [3]. In 1901, Fischer discovered proline in gelatin hydrolysates [4]. A year later, he discovered therein the product of the earliest known posttranslational modification: 4-hydroxyproline [5]. Within collagen strands, proline residues are oxidized at the 4*R* position by prolyl-4-hydroxylase (P4H [6]), an essential enzyme in animals [7–9]. Attempts to understand this modification led ultimately to the development of a wide variety of proline analogs [10]. Among these, 4-fluoroprolines have emerged as powerful tools for studying collagen, as well as for engineering conformationally biased peptides and proteins.

4-Fluoroprolines were first employed in 1965 [11], when they were used to investigate the mechanism of collagen hydroxylation and found to be incorporated successfully into collagen proteins [12–15]. Although early studies focused mainly on collagen hydroxylation [11, 16, 17], 4-fluoroprolines have now been applied to study a wide variety of systems, some of which have been reviewed previously [18–21]. In this review, the utility of these important heterocycles is presented with a thorough discussion of their synthesis and conformational properties, followed by a detailed examination of their impact on the stability and folding of peptides and proteins. We conclude with an overview of the growing interest of employing 4-fluoroprolines for medicinal applications.

2 Synthesis

4-Fluoroproline syntheses have improved significantly since the original report [11]. Nearly all of the syntheses employ displacement of the hydroxyl group of 4-hydroxyproline with a fluoride source. Activation of the hydroxyl group of Cbz-4-hydroxyproline as tosylate **2** before displacement by inorganic fluoride at



Scheme 1 Routes for the chemical synthesis of 4-fluoroprolines. Protecting group manipulations are indicated with *double arrows*. (**a**) Original synthetic route employing inorganic fluoride [11]. (**b**) Synthesis of 4-fluoroproline from a single 4-hydroxyproline stereoisomer using sulfur trifluoride reagents [22, 23]. (**c**) Synthesis of 4-fluoroproline from a single 4-hydroxyproline stereoisomer using organic fluoride [24]

elevated temperatures gave 56–62% yields of (4*S*)- and (4*R*)-*N*-Cbz-4-fluoroprolines **3** to afford, after protecting group removal, (2*S*,4*S*)-fluoroproline (flp, (4*S*)-**1**) from (2*S*,4*R*)-4-hydroxyproline (Hyp) with complete stereochemical inversion, but diastereomeric mixtures of (2*S*,4*R*)-4-fluoroproline (Flp, (4*R*)-**1**) starting from (2*S*,4*S*)-4-hydroxyproline (hyp) (Scheme 1a) [11]. Subsequent reports found difficulty in generalizing this procedure for use of alternative protecting group schemes [22]. Demand existed for syntheses that could provide *both* stereoisomers starting from naturally occurring and relatively less expensive Hyp.

Preliminary work to bypass the alcohol activation step by employing organic fluorinating agents generally resulted in inseparable mixtures of the 4*R* and 4*S* diastereomers [25–28]. Successful syntheses of both 4-fluoroproline diastereomers from a single 4-hydroxyproline diastereomer were first reported in 1998 [22]. Employing diethylaminosulfur trifluoride (DAST) as the fluorine source, both Boc- and Fmoc-flp were prepared in approximately 80% yield from Boc-Hyp-OMe (Scheme 1b). Inversion of the alcohol of (4*R*)-*N*-trityl-4-hydroxyproline methyl ester by a Mitsunobu procedure using benzoic acid as the nucleophile followed by saponification and *N*-protection gave (4*S*)-**5**, which was similarly converted to Fmoc-Flp ((4*R*)-**6**), albeit in $<25\%$ yield [22]. Substitution of formic acid for benzoic acid enabled the Mitsunobu reaction in the presence of Boc-amine protection to generate (4*S*)-**4** in 78% yield (Scheme 1b) [23]. Moreover, switching from a methyl to a phenylacetyl (Pac, $PhCOCH_2$–) ester enabled an orthogonal cleavage with Zn in AcOH in the presence of Boc and Fmoc amine-protecting groups [23]. Fluorination of Pac ester (4*S*)-**7** with morpholinosulfur trifluoride inverted the alcohol to give 4-fluoroproline (4*R*)-**8** in 79–94% yields. The configuration of Boc-flp was assigned by X-ray diffraction, and chiral HPLC established that the synthesis produced single stereoisomers. Protecting group manipulation then provided Fmoc-fluoroproline starting materials **6** for solid-phase synthesis (Scheme 1b).

A more scalable and cost-effective synthesis of 4-fluoroproline was later developed, which avoided potentially explosive aminosulfur trifluoride reagents [24]. To generate flp (4*S*)-**1**, Boc-Hyp-OMe (4*R*)-**4** was treated with trifluoromethanesulfonic anhydride to provide a triflate that was treated with tetrabutylammonium fluoride (TBAF). Heating of the resulting protected (4*S*)-**5** with 2N HCl at reflux removed the Boc group and methyl ester to provide flp (4*S*)-**1** in 16% overall yield from Hyp (Scheme 1c). Albeit lower-yielding than previous approaches, largely due to inefficiency in the safer fluorination step, this approach was scaled to kilogram quantities. To generate Flp (4*R*)-**1**, rather than employ the Mitsunobu approach, lactone **11** was synthesized by conversion of Boc-Hyp (4*R*)-**9** to methanesulfonate **10** and intramolecular displacement by the carboxylate on treatment with potassium *t*-butoxide. Opening of lactone **11** with lithium hydroxide provided Boc-hyp (4*S*)-**9** in 78% yield from Boc-Hyp (4*R*)-**9**. Conversion of Boc-hyp (4*S*)-**9** to Flp (4*R*)-**1** was performed using a similar protocol by way of triflate to yield (4*S*)-**5**. Alternatively, Hyp was transformed to its 2*R* diastereomer by treatment of with acetic anhydride at 90° C to form an *N*-acetyl lactone which was hydrolyzed in situ with 2N HCl at reflux.

The solid-phase synthesis of peptides possessing 4-fluoroproline residues has been achieved by conversion of Hyp residues on resin, a technique called "proline editing" [29, 30]. After coupling Fmoc-Hyp to the peptide chain, the hydroxyl group was protected as trityl ether, and the protected peptide chain was completed by standard Fmoc-based solid-phase peptide synthesis. The trityl group was to be removed selectively using 2% TFA to reveal the free hydroxyl group, which was transformed to its (4*S*)-4-hydroxyproline counterpart by Mitsunobu reaction with 4-nitrobenzoic acid and ester hydrolysis with K_2CO_3/MeOH. Transformation of the hydroxyproline containing peptides to their respective 4-fluoroproline peptides was accomplished with inversion of configuration using DAST, prior to resin cleavage and deprotection. Multiple modified proline residues bearing different 4-position substituents in the same peptide may be produced employing alternative and orthogonal protecting group strategies using *tert*-butyldimethylsilyl (TBS) ether and allyloxycarbonyl (Alloc) carbonate groups. In sum, this strategy circumvents the labor required for solution-phase purification of 4-fluoroproline building blocks.

3 Conformational Analysis

Eclipsing interactions deter population of the planar conformer of saturated five-membered rings. Instead, these rings adopt one of two predominant conformations: the envelope conformation, wherein four atoms lie in plane with the fifth distorted away, or the half-chair conformation, wherein two adjacent atoms distort on opposite sides of the plane of the other three (Fig. 1a). Early crystallographic studies [31–33], as well as data from NMR spectroscopy [34], indicated that proline generally adopts the envelope conformation. Although many envelope conformations are possible, crystallographic analysis demonstrates that two predominate: one termed C^{γ}-*endo*, in which C4 (otherwise, C^{γ}) distorts out of plane to the same side of the ring as the C1 carboxylate, and, another, C^{γ}-*exo*, in which C4 distorts out of plane to the opposite side of the ring as the C1 carboxylate (Fig. 1b) [35]. These designations are however approximate, and many molecules labeled as "C^{γ}-*exo*"

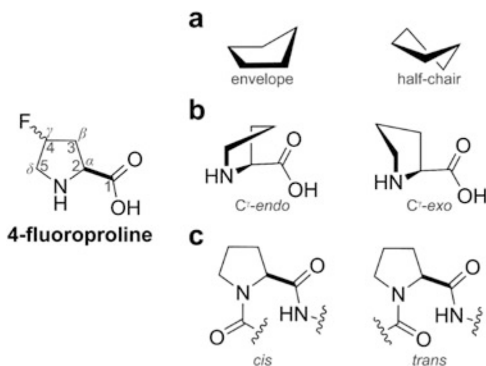


Fig. 1 Conformational attributes of proline residues. (**a**) Conformations of saturated five-membered rings. (**b**) Pyrrolidine ring puckers. (**c**) Peptide bond isomers

are better described as C^{β}-*endo*. Moreover, many proline crystal structures display some half-chair character. The C^{γ}-*endo*/*exo* terminology has become customary and is useful for discussing stereoelectronic effects on proline conformations (vide infra), because C^{γ} often experiences the largest change in orientation and because C^{γ} is typically the site of substituent modification.

The energy difference between the *endo* and *exo* puckers is small for proline (~0.5 kcal/mol), and the two forms interconvert rapidly at room temperature [36]. Using NMR spectroscopy, an approximately 2:1 *endo*/*exo* equilibrium population has been determined for Ac-Pro-OMe [36]. Fluorination of C4 serves to bias this equilibrium. Specifically, the C–F bonds in 4-fluoroprolines will orient antiperiplanar to adjacent C–H single bonds due to a gauche effect. The polarity of the C–F bond makes the substituted carbon electron deficient and therefore a potent electron acceptor. The C–F σ^* orbital is, in particular, highly electrophilic and will accept electron density from antiperiplanar C–H bonding orbitals [37]. The C–F bond in Flp is satisfied by C–H donor orbitals in an orientation that positions the fluoro group toward the opposite side of the ring as the carbonyl carbon, thereby enforcing the C^{γ}-*exo* pucker (Fig. 2a). Conversely, flp exists largely in the C^{γ}-*endo* conformation due to similar hyperconjugation (Fig. 2b). These preferences were first established using ^{1}H-NMR spectroscopy [38]. The presence of the fluoro substituent disperses the condensed proline proton signals, thereby enabling accurate calculations using the Karplus equation [39]. Refined analysis quantified the preference of flp for the *endo* conformation to be approximately 20:1; analogously, Flp prefers the *exo* conformation at a 6:1 ratio [36].

The reorientation of the pyrrolidine ring between the two puckers influences the structure and stability of proline-containing peptides (vide infra). The *exo* pucker reduces the absolute value of the proline ϕ and ψ main-chain torsion angles (Table 1) [37]. Constrained by the pyrrolidine ring, the ϕ dihedral angle varies from approximately $-60°$ in the *exo* pucker to approximately $-75°$ in the *endo* pucker [36]. Although these changes appear modest, they can have a profound effect on the structure and stability of proline-rich peptides (vide infra). Two general orientations exist for the ψ (N_i–C_i^{α}–C_i'–N_{i+1}) main-chain dihedral angle: one near 150°, corresponding to the polyproline type II (PPII) helix, and another near $-30°$, which is consistent with α-helix geometry. In isolated proline molecules and derivatives, the former ψ value is more common and calculated to be of lower energy [42]. Within the PPII conformer, 4-position fluorination of the pyrrolidine ring has been shown to affect the ψ dihedral angle by as much as 30°, from ~140° in the *exo* pucker of Flp to ~170° in the *endo* pucker of flp [36].

As a result of the influence of ring pucker on the main-chain dihedral angles, fluorination of C4 can modulate interactions between the N-terminal amide carbonyl and the proline residue carboxamide in peptides. Carbonyl groups of adjacent peptide residues have been shown to engage in so-called $n \rightarrow \pi^*$ interactions [43]. In an $n \rightarrow \pi^*$ interaction, lone-pair (n) electron density delocalizes from the oxygen of one carbonyl group into the π^* antibonding orbital of a proximal carbonyl group (Fig. 2c) [41]. Donation typically occurs from the $i-1$ residue to the i residue within a polypeptide [44]. Effective orbital mixing requires a distance

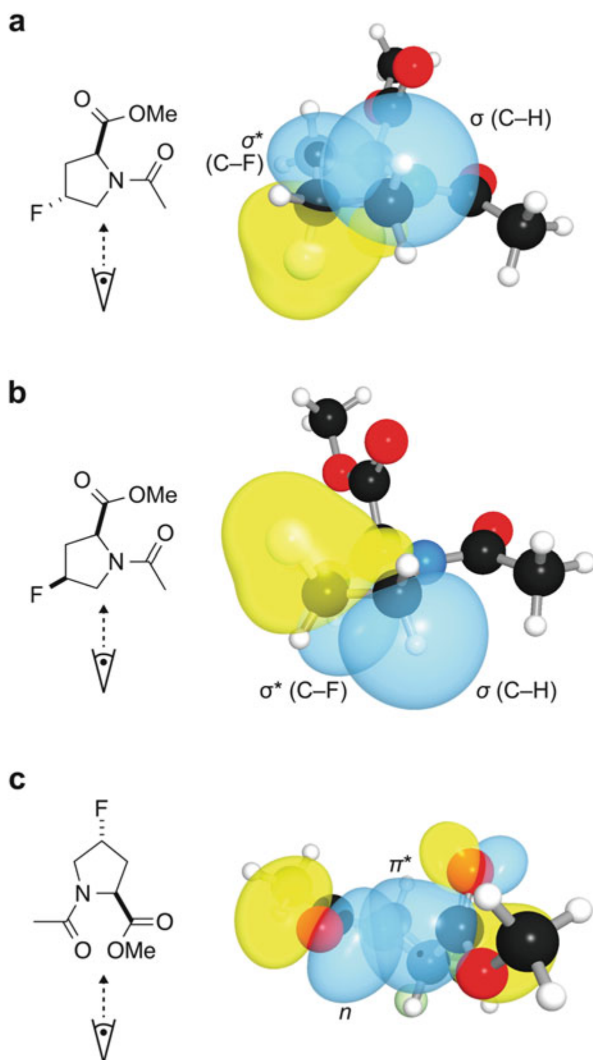


Fig. 2 Stereoelectronic effects in 4-fluoroprolines. σ(C–H) → σ*(C–F) Gauche interactions stabilizing the (**a**) C^{γ}-*exo* or (**b**) C^{γ}-*endo* conformations of Ac-Flp-OMe or Ac-flp-OMe, respectively. (**c**) The $n \rightarrow \pi^*$ interaction between adjacent carbonyl groups stabilizing the *trans* conformation of the Ac-Flp amide bond in Ac-Flp-OMe

Table 1 Conformational preferences of proline residues

Compound	Ring pucker[a]	ϕ (°)[a]	ψ (°)[a]	$K_{trans/cis}$[b]
Ac-Pro-OMe	*endo*	−79	177	4.6
Ac-Hyp-OMe	*exo*	−57	151	6.1
Ac-Flp-OMe	*exo*	−55	141	6.7
Ac-flp-OMe	*endo*	−76	172	2.5

[a]Determined with X-ray crystallography [40] or density functional theory calculations (Ac-flp-OMe) [36]
[b]Measured in D_2O at 25°C with NMR spectroscopy [41]

closer than a van der Waals contact between the donor oxygen and the acceptor carbon along the Bürgi–Dunitz trajectory for nucleophilic addition [45]. The $n \rightarrow \pi^*$ interactions have energies generally greater than 0.27 kcal/mol per interaction [46]. They have been shown to be ubiquitous in proteins [44, 47, 48], particularly about proline residues, in which the pyrrolidine ring pre-organizes the neighboring carbonyl groups to favor the $n \rightarrow \pi^*$ interaction [44]. Fluorination of C4 influences the pyrrolidine ring pucker and the main-chain dihedral angles in ways that have important consequences for the $n \rightarrow \pi^*$ interaction [49] (Fig. 3). Specifically, the distance between the oxygen–carbon donor–acceptor pair of the neighboring carbonyls is generally longer in the *endo* pucker, which leads to a

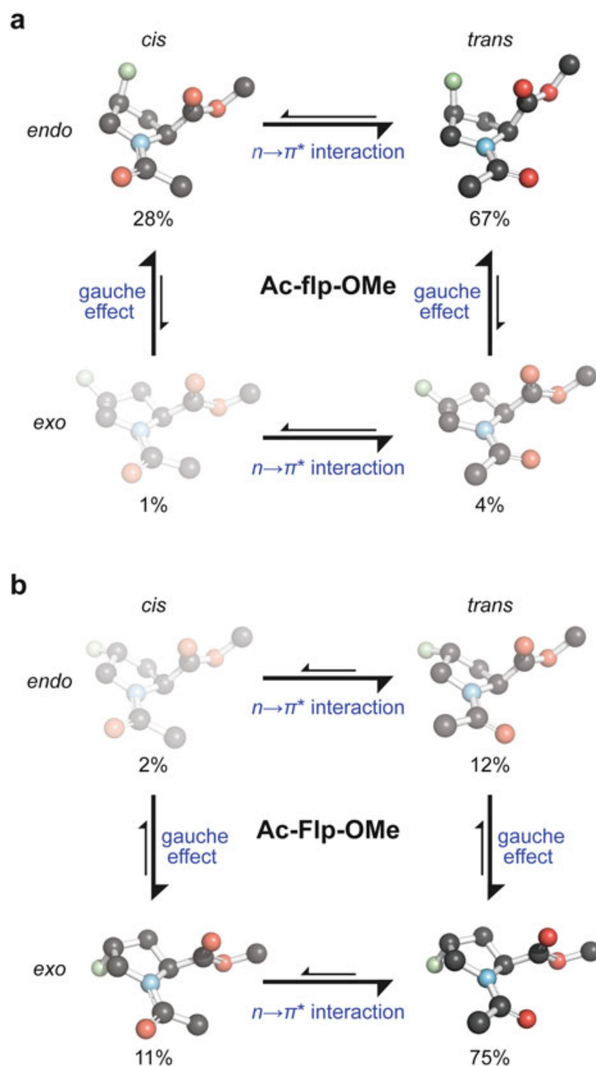


Fig. 3 (**a**) Conformational preferences of Ac-flp-OMe. (**b**) Conformational preferences of Ac-Flp-OMe. Relative populations (%) were measured in dioxane at 25°C with NMR spectroscopy [36]

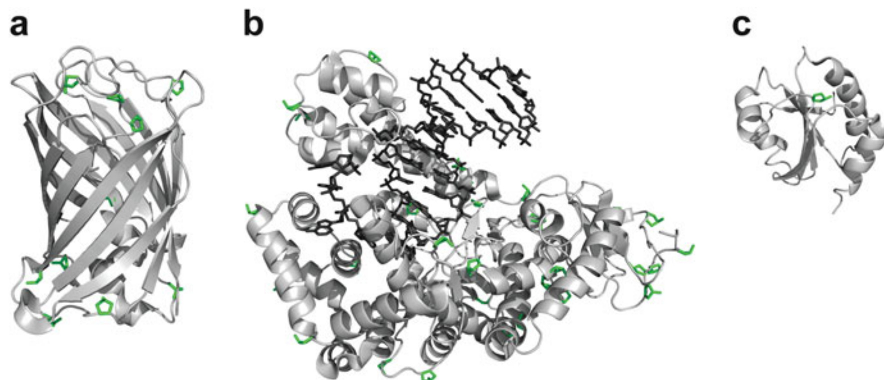


Fig. 4 Three-dimensional structures of representative proteins containing 4-fluoroproline residues (*green sticks*). (**a**) EGFP containing flp (PDB entry 2q6p) [51]. (**b**) *Taq* DNA polymerase containing Flp (4dle) [52]. (**c**) Thioredoxin containing Flp (4hua) [53]

weaker $n \to \pi^*$ interaction [50]. Conversely, the *exo* pucker is associated with shorter donor–acceptor distances and stronger $n \to \pi^*$ interactions. In turn, the $n \to \pi^*$ interaction biases the conformation of the preceding peptide bond. An attractive $n \to \pi^*$ interaction is only possible in the *trans* conformation of the peptide bond (ω: C_{i-1}^{α}–C_{i-1}'–N_i–C_i^{α}, Fig. 1c). Accordingly, a higher *trans* peptide bond population correlates with the increased preference for *exo* pucker of the pyrrolidine ring substituted with a 4*R* fluoro group (Table 1, Fig. 4). Notably, the *cis* and *trans* isomers of 4-fluoroprolines have distinct dipole moments, and the *cis–trans* ratio of their peptide bonds is sensitive to solvent polarity [54]. In toto, modification of the proline ring with a 4-fluoro substituent has structural consequences on all three backbone dihedral angles in a protein. This principle has been exploited to modulate the conformational stability of peptides and proteins (vide infra).

The inductive effect of 4-fluoro substituents has additional consequences on the peptide bond N-terminal to proline residues. Electron withdrawal by the fluoro group reduces the capacity for the proline nitrogen lone pair to contribute double-bond character to the amide N-terminal to proline [40] and thereby diminishes the rotational barrier between the *cis* and *trans* isomers in the peptide [55]. 4-Fluoroprolines have been used to study protein folding kinetics, because isomerization of peptide bonds N-terminal to proline can limit the rate of protein folding [2]. To enhance the influence of a single fluoro substituent, (2*S*)-4,4-difluoroproline (Dfp) has been developed to study protein folding (vide infra). Although detailed conformational analysis of Dfp has yet to be performed, its conformational preferences may likely be similar to those of proline.

4 Effects on Collagen Stability

4-Fluoroprolines have been particularly useful in the study of collagen stability [3, 56]. Collagen is the major structural protein in animals, forming a large portion of the dry weight of the skin. Collagen is also prevalent in the extracellular matrix, in which it performs an important structural role. Collagen consists of a distinct triple-helical structure comprised of three polypeptide strands that each have a characteristic Xaa-Yaa-Gly amino acid repeat, in which prolyl residues often occupy the Xaa and Yaa positions. This sequence enables each of the three strands to adopt PPII helices that wrap around one another with an offset of a single amino acid residue [57]. A slice through a collagen triple helix will therefore contain one Xaa, one Yaa, and one Gly residue from different strands. In the PPII secondary structure, the individual peptide chain lacks typically *intra*-strand hydrogen bonding. In a slice of the triple helix, a single interstrand hydrogen bond usually exists between the glycine NH and the carbonyl of the Xaa residue on different peptide chains. The relatively low prevalence of hydrogen bonding raised the question as to the source of the high thermal and mechanical stability of collagen.

One clue as to the source of collagen stability is apparent from its amino acid content. Collagen has a high prevalence of Pro and Hyp residues, which appear at approximately 28% and 38% frequencies in the Xaa and Yaa positions, respectively [58]. The importance of the Hyp hydroxyl group was shown early on by thermal denaturation studies with both synthetic peptides [59] and protocollagen [60], which is the non-hydroxylated precursor to mature collagen. A crystal structure of a collagen-mimetic peptide showed bridging water molecules between the hydroxyl substituent and main-chain carbonyl groups, and these water bridges were proposed to confer stability [61, 62]. The importance of the inductive effect

Table 2 Thermostability of triple-helical CMPs

(Xaa-Yaa-Gly)$_n$	T_m (°C)[a]	Reference
(Pro-Pro-Gly)$_7$	No helix	[63]
(Pro-Hyp-Gly)$_7$	36	[41]
(Pro-Flp-Gly)$_7$	45	[41]
(Pro-flp-Gly)$_7$	No helix	[41]
(flp-Pro-Gly)$_7$	33	[64]
(Flp-Pro-Gly)$_7$	No helix	[64]
(flp-Flp-Gly)$_7$	No helix	[63]
(Pro-Pro-Gly)$_{10}$	31–41	[65]
(Pro-Hyp-Gly)$_{10}$	61–69	[65]
(Pro-Flp-Gly)$_{10}$	91	[65]
(flp-Pro-Gly)$_{10}$	58	[66]
(Flp-Pro-Gly)$_{10}$	No helix	[66]
(flp-Flp-Gly)$_{10}$	30	[67]

[a]The value of T_m refers to the temperature at the midpoint of the thermal transition between the triple-helical and single-stranded states

of the Hyp hydroxyl group was examined by the use of 4-fluoroprolines, which cannot serve as proton donors in hydrogen bonds. In the first synthetic incorporation of 4-fluoroproline into a protein mimetic, the Hyp residues at the Yaa position of a collagen mimetic were replaced with Flp, and circular dichroism (CD) spectroscopy was used to compare the thermostability of the two triple helices (Table 2) [65, 68]. Relative to the Hyp triple helix mimetic, the Flp analog exhibited increased thermostability. Considering that fluoro groups do not form strong hydrogen bonds [69], the data from this comparison refuted the water-bridge proposal. Subsequent molecular dynamics simulations demonstrated the transient nature of bridging water molecules [70]. Moreover, experiments with (2*S*,4*R*)-4-methoxyproline showed that the water bridges actually diminished the stability of the collagen triple helix [71].

Thorough conformational analysis of 4-fluoroproline monomers provided compelling evidence that collagen is stabilized by the stereoelectronic effects of the Hyp residue that are augmented by replacement with the Flp residue [41]. For example, collagen mimetics bearing Flp at the Yaa position exhibited higher thermostability than their Pro counterparts. On the other hand, incorporation of flp at the Yaa position caused a relative decrease in thermostability (Table 2), demonstrating the importance of 4-position stereochemistry. Inspection of available crystal structures indicated a prevalence of *exo* ring puckers in the Yaa position of collagen [61]. The fluoro group in Flp enforces the *exo* ring pucker, which stabilizes collagen by organizing proper dihedral angle orientations. Moreover, Flp favors the prolyl amide *trans* isomer, organizing the proper ω dihedral angle featured in collagen (Table 1) [57]. These effects are evident with even a single Pro → Flp substitution within a collagen strand [72]. Increased and decreased stability relative to the parent peptide were respectively observed by CD and NMR spectroscopies upon substitution of Hyp by Flp and flp in a triple-helical model of the α1(IV)1263–1277 sequence of type IV (basement membrane) collagen, which is known to promote melanoma cell adhesion, spreading, and signaling [73]. Melanoma cell adhesion and spreading on the triple-helical models correlated with stability demonstrating the dramatic influences of fluoro group changes at the Hyp residue [73].

Although Flp-incorporation at the Yaa position increased thermostability in collagen relative to proline in Pro-Yaa-Gly repeats, decreased thermostability was observed with Flp relative to proline in Xaa-Hyp-Gly repeats [64]. Conversely, flp demonstrated respectively stabilizing and destabilizing effects at the Xaa and Yaa positions [66]. Inspection of available crystal structures demonstrated the Xaa residue prefers *endo* ring pucker, which is enforced by flp. The slight preference of Pro for the *endo* pucker is consistent with the stronger effect of Flp at the Yaa position relative to the milder influence of flp at the Xaa position (Table 2).

Although respective incorporation of flp and Flp at Xaa and Yaa residues in the same peptide was predicted to template the appropriate main-chain dihedral angles and confer greater stability than either substitution alone, the resulting collagen triple helix exhibited decreased thermostability relative to the peptide composed of repeats of Pro-Pro-Gly (Table 2) [63, 67]. Molecular modeling revealed a potential interstrand steric clash between the fluoro groups of 4-fluoroproline residues on

adjacent strands that prevented self-association of the peptide. Stable triple helices could be formed by mixing different ratios of (flp-Flp-Gly)$_7$ and (Pro-Pro-Gly)$_7$, [63]. The (flp-Flp-Gly)$_n$ sequence binds preferably other collagen strands but not itself. In biological collagen samples, such as in wounds, disrupted helices have provided annealing sites for (flp-Flp-Gly)$_n$ sequences coupled to fluorescent dyes to visualize damaged collagen [74]. Moreover, such annealing strands have been conjugated with moieties, such as growth factors, that modulate the physiology of the surrounding environment to aid in wound healing [75, 76].

Differential scanning calorimetry was employed to provide more insight into the thermodynamics of the influences of fluorination on collagen stability [77]. In comparisons with (Pro-Pro-Gly)$_{10}$ triple helices, the (Pro-Hyp-Gly)$_{10}$ and (Pro-Flp-Gly)$_{10}$ counterparts were shown to retard thermal denaturation by enthalpic and entropic contributions, respectively. Analysis of molecular volumes indicated that the enthalpic contribution to the stability of (Pro-Hyp-Gly)$_{10}$ was mediated significantly by enhanced water solvation. Water desolvation due to the hydrophobic effect of fluoro substitution may account for the entropic stabilization of (Pro-Flp-Gly)$_{10}$ because the fluoro group of Flp is a much weaker hydrogen bond acceptor than the hydroxyl group of Hyp [69] and cannot donate a hydrogen bond. This hypothesis was tested in a follow-up study employing Dfp, which retains the hydrophobicity of Flp, but lacks the strong preference for the *exo* ring pucker [56]. Accordingly, if the hydrophobic effect was dominant to triple-helical stability, Dfp and Flp should have similar effects; however, Dfp decreased stability relative to Flp at the Yaa position, arguing against a strong contribution from the hydrophobic effect in driving collagen-mimetic peptide assembly.

Albeit application has been limited to date [78], 4-fluoroprolines have served as NMR probes due to the utility of the ^{19}F nucleus. For example, the mechanism of strand association and dissociation was studied in a collagen triple helix model possessing a single Flp residue at the central Yaa position using ^{1}H–^{19}F and ^{19}F–^{19}F exchange experiments to measure populations of native and nonnative structures in solution [79]. As the temperature increased, denaturation of the native collagen strands was observed to give rise to multiple intermediates, contradicting the two-state model often assumed for the denaturation of collagen-mimetic peptides. Isolated monomer strands were found to possess both *cis* and *trans* peptide bonds, but collagen assembly was concluded to take place only from monomers with all-*trans* peptide bonds. Off-pathway intermediates were characterized having misaligned triple helices with more than one residue offset. Misaligned helices required dissociation to all-*trans* monomer strands to convert to the native state.

Besides imparting thermostability and binding specificity to collagen strands, 4-fluoroprolines have also been used to investigate the mechanism by which collagen strands are hydroxylated by proline 4-hydroxylase (P4H). Proline derivatives such as flp, which prefer relatively the *endo* pucker and the *cis* peptide bond conformation, proved better P4H substrates [80]. Enzymatic discrimination during substrate binding and not during turnover was evident from the hydroxylation of peptides bearing flp and the absence of binding to peptide counterparts containing Flp residues. Oxidation of flp to 4-ketoproline (Kep) by P4H was used to develop a

probe of enzymatic activity by measuring fluoride-ion release in a continuous assay [81]. Shedding light on the mechanism of collagen hydroxylation, these studies have provided information for designing inhibitors of P4H to treat fibrotic diseases associated with an overabundance of collagen.

5 Effects on Peptide Conformation

Beyond the triple-helical domain of collagen, the conformations of other peptides, especially polyproline helices, have been explored through incorporation of 4-fluoroprolines. Two major conformations of oligoproline are known: the polyproline type I (PPI) helix, which is marked by *cis* orientation of the peptide bonds, and the aforementioned PPII helix, which features prolyl amide *trans* isomers. The PPII conformation is common in both the folded [82] and unfolded states [83] of proteins and has found utility in molecular rulers [84] and scaffolds [85]. The PPII conformation is favored in relatively polar solvents, such as water. The PPI conformation is formed preferentially in less polar solvents, such as *n*-propanol [86]. The transition between these helices involves isomerization of the peptide bond. Modulating the conformation of the pyrrolidine ring was predicted to have a profound effect on oligoproline structure, because of the connection between pyrrolidine ring pucker and main-chain dihedral angles (vide supra). To evaluate this hypothesis, Pro_{10}, Flp_{10}, and flp_{10} were synthesized by solid-phase methods and subjected to analysis by CD spectroscopy [87]. In contrast to native Pro_{10}, Flp_{10} adopted a PPII conformation in both aqueous and organic solutions. Conversely, flp_{10} exhibited low PPII content in water and a high population of PPI geometry in *n*-propanol. Considering the conformational preferences of the amino acid monomers, the favored *exo* ring pucker of Flp enforced $n \rightarrow \pi^*$ interactions between adjacent carbonyl groups, leading to increased populations of prolyl amide *trans* isomers and PPII helix. Subsequently, (4*R*)- and (4*S*)-fluoroprolines were shown to respectively increase and decrease the transition-state barrier for PPII → PPI conversion [88]. This dichotomy was consistent with Flp stabilizing the prolyl amide *trans* isomer. In a follow-up study, the effects of 4-fluoroproline were shown to be more pronounced at the C versus the N terminus of the peptide [89], consistent with polyproline interconversion occurring by a mechanism that initiates at the C terminus [90].

Polyproline helices have attracted interest as motifs, because of their roles in protein–protein interactions [91]. For example, SH3 domains are a ubiquitous peptide module that bind often to proline-rich protein substrates, which adopt PPII conformations [92]. In an effort to design high-affinity ligands to SH3 domains for applications such as cancer chemotherapeutic agents, 4-fluoroprolines were introduced into various proline-rich peptides derived from the hematopoietic progenitor kinase 1 (HPK1) [93]. In affinity assays using the SH3 domain of hematopoietic-lineage cell-specific (HS1) protein, the 4-fluoroproline peptide analogs failed to exhibit enhanced binding, in spite of increasing the population of PPII

conformer, suggesting that ligand recognition involved features beyond secondary structure, albeit the fluoro groups may perturb specific binding interactions.

To study the importance of *exo* ring pucker in the proline–aromatic interaction with Trp64, Flp and flp were respectively substituted for Pro62 at the N terminus of the C-terminal helix in the subdomain of the villin headpiece (HP36), a small mostly α-helical miniprotein [91]. Although Flp and flp were respectively expected to enforce and perturb the *exo* ring pucker of Pro62 [92] with consequences of improved and depressed thermostability, thermal unfolding and urea-induced denaturation measurements indicated that the former fluoroproline significantly destabilized HP36 and the latter had little effect on its structure and protein stability, unexpected consequences suggested to be due to steric and hydrophobic effects.

To explore the importance of the *endo* pucker for Pro37 to pack into the hydrophobic core of the Pin1 WW domain, 4-fluoroprolines were incorporated into this small, three-stranded β-sheet protein of about 40 residues [94]. Consistent with the crystal structure of the parent protein [95] and the hypothesis that the native *endo* pucker is preferred for the Pin1 WW domain, Flp decreased thermostability by favoring the *exo* pucker. Although relatively hydrophilic *endo*-favoring prolines, such as hyp and (4*S*)-methoxyproline (mop), destabilized the Pin1 WW domain, flp increased stability, likely due in part to its hydrophobic nature. Moreover, flp-incorporation increased the affinity between the protein and its phosphorylated peptide ligand. The advantages of 4-fluoroprolines to perturb selectively pyrrolidine ring conformation were thus highlighted by their capacity to probe peptide structure without complications from hydrogen bonding.

4-Fluoroprolines have been used to study the significance of the *exo* ring pucker of Pro12 in the loop that joins the C-terminal polyproline helix to the N-terminal α-helix in the Trp cage miniprotein [96], a 20-residue peptide that displays tertiary structure and cooperative folding [97], physical characteristics associated with full proteins. Employing proline editing, Trp cage peptides bearing Flp and flp at position-12 were synthesized and found respectively to increase and decrease thermostability relative to the native proline peptide. The destabilizing effect of flp was greater than the stabilizing effect of Flp, effects consistent with the *exo* pucker of the native proline, and the sensitivity of small proteins that lack a hydrophobic core.

The critical role of prolyl amide *cis–trans* isomerization in the folding of the *N*-terminal domain of the cysteine-rich terminal region of minicollagen-1 was confirmed by replacement of Pro24 with Flp and flp, respectively [98]. During the folding of this important structural protein of lower animals, a fully oxidized intermediate accumulates in which the N-terminal domain possesses a prolyl amide *trans* isomer and three disulfide bonds. Conversion to the native folded peptide occurs by slow rate-determining *trans*-to-*cis* isomerization in the absence of enzyme catalysis [99]. Relative to the parent proline peptide, flp gave efficient folding with formation of the correct isomer and negligible amounts of intermediate, and Flp slowed folding by favoring the *trans* isomer in the folding intermediate. Molecular dynamics simulations and kinetic analyses demonstrated that flp

decreased the population of the *trans* isomer preventing trapping of nonnative disulfide bonds. Similarly, 4-fluoroprolines were used to investigate stability, and rates of folding and the role of disulfide bond formation in model collagen triple helices [100].

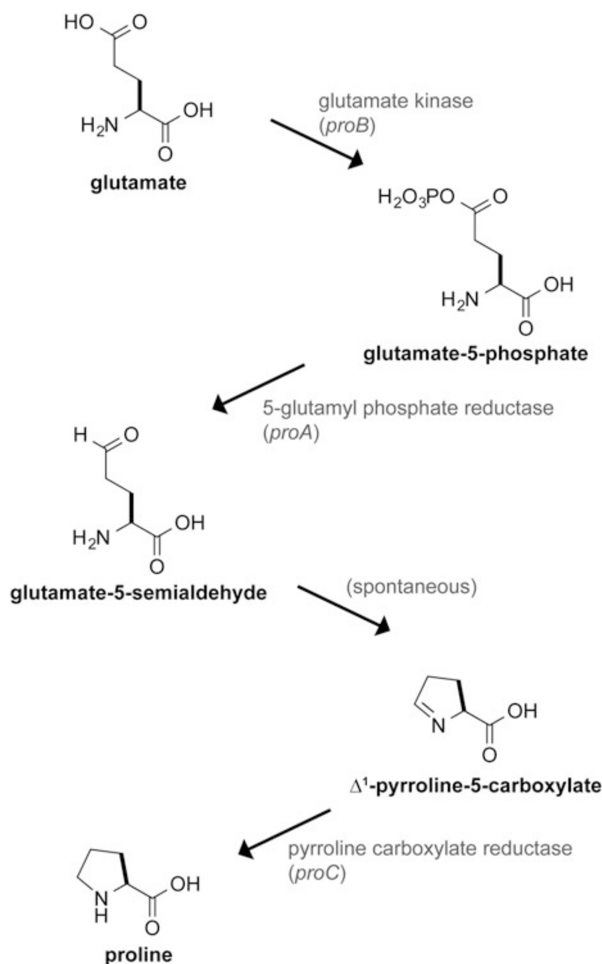
4-Fluoroprolines have been used to modulate the DNA-binding properties of a mimic of the integration host factor (IHF) [101]. Crystal structures of the complex of IHF and a 35-base pair chain of DNA have demonstrated that the so-called α-arm binds the DNA sequence in part by intercalating an *endo*-puckered proline residue between base pairs [102]. In the mimic, a lysine dendrimer was tethered to a cyclopeptide that adopted a β-sheet hairpin conformation, which presented the intercalating proline in homology to the IHF α-arm. Replacement of proline, by flp in the mimic, increased sequence-specific DNA binding.

6 Effects on Protein Folding

Broadening the applications of 4-fluoroprolines, methods have been developed to incorporate these heterocyclic amino acids into full-length proteins. For example, selective pressure incorporation (SPI) has resulted in the global substitution of 4-fluoroproline for proline by conditioning bacterial protein synthesis through genetic engineering and the control of environmental factors such as amino acid supply and fermentation parameters [103–108]. Alternatively, the site-specific incorporation of 4-fluoroproline into proteins bearing multiple proline residues has been achieved by the chemical ligation of peptides possessing 4-fluoroproline residues to protein fragments produced by recombinant DNA technology [109].

The first complete replacement of the proline residues in a protein with 4-fluoroprolines was performed on an engineered barstar variant, the cognate inhibitor of a *Bacillus* ribonuclease (barnase) [110]. Wild-type barstar has two prolines, one in each peptide bond conformation. Genetic manipulation yielded a variant containing only the *cis* proline. Efficient recognition of 4-fluoroprolines by the endogenous proline amino-acyl-tRNA synthetase was demonstrated by production of protein with approximately equal efficiency by feeding bacteria proline or either 4-fluoroproline diastereomer. In accordance with their favoring of prolyl *cis* and *trans* isomers, barstar variants containing flp and Flp exhibited respectively higher and lower thermostability than the parent protein. The fluorinated barstar variants were employed to study the mechanism of peptide bond isomerization by peptidyl prolyl *cis–trans* isomerases [111].

Bacterial strains with improved efficiency for incorporating 4-fluoroproline have been used to prepare elastin derivatives [112]. In bacterial synthesis of proline from glutamate, the *proA* gene encodes 5-glutamylphosphate reductase to generate glutamate-5-semialdehyde, which spontaneously cyclizes to a Δ^1-pyrroline intermediate. Proline is produced by reduction of the pyrroline by pyrroline carboxylate reductase, encoded by the *proC* gene (Scheme 2). Knockout of the *proC* gene, followed by supplementation of the growth medium with 4-fluoroproline, resulted



Scheme 2 Route for the biosynthesis of proline from glutamate

in efficient incorporation of 4-fluoroproline throughout the biosynthetic elastin protein, containing eighty proline residues.

Proline residues adopt the central positions of type II β-turns within the [Val-Pro-Gly-Val-Gly] repeats of elastin as the temperature approaches the phase transition of the polypeptide [113]. Elastins substituted uniformly with either Flp or flp were prepared to explore the influence of prolyl residues on the thermodynamics of the phase transition and the physiologically relevant reversible aggregation state of native elastin [114]. Differential scanning calorimetry and CD spectroscopy confirmed that Flp stabilized the structured state and lowered the critical transition temperature, likely by enforcing the *exo* conformation commonly observed in type II β-turns. Conversely, elastins incorporating flp had less stable thermal transitions at a higher temperature, indicating weaker β-turn stability. Analysis of the flp elastin using ^{19}F-NMR spectroscopy detected significant *cis* isomer population at

the valyl-prolyl peptide bond, which may exacerbate the destabilizing effect of this modification.

Ubiquitin has three native proline residues that were replaced with 4-fluoroproline by using the SPI method to study the influence of proline ring pucker on protein folding [115]. Ubiquitylation serves as a key posttranslational modification with a wide variety of cellular effects, notably targeting proteins for proteasomal degradation [116]. The three prolines in native ubiquitin all reside in the *trans*-*exo* conformation, according to high-resolution crystal structure data [117]. Ubiquitin uniformly incorporating Flp was efficiently expressed from a bacterial auxotroph in high yield; however, attempts failed to incorporate flp, likely due to aggregation or degradation of unfolded protein. Both wild type and Flp-ubiquitin exhibited CD spectra characteristic of native ubiquitin and similar folding pathways involving a single intermediate detected by stopped-flow fluorescence measurements. The barrier between the unfolded state and the intermediate was lower for Flp-ubiquitin, which exhibited slower unfolding from the native state to the intermediate. In thermal and guanidine · HCl-induced denaturation experiments, Flp-ubiquitin exhibited greater stability than the native protein. Furthermore, Flp-ubiquitin served as substrate for ubiquitin-processing enzymes in self-ubiquitylation. In sum, (4*R*)-fluoroproline increased thermostability, accelerated protein folding, and maintained biological function.

Fluoroprolines have also been used to improve the physical properties of antibody fragments for potential uses in therapeutics, diagnostics, and biotechnology [118]. Lack of thermostability relative to their full-length parents has prevented applications of antibody fragments, such as single-chain Fv (scFv) units prepared by linking the variable domains of IgG. Employing the SPI method, anti-c-Met scFv fragments were prepared possessing $>90\%$ substitution of 4-fluoroprolines at the five conserved proline positions as determined by mass spectrometric analysis. As observed with ubiquitin, incorporation of Flp and flp resulted respectively in the expression of properly folded soluble and misfolded, insoluble protein. Moreover, Flp-scFv demonstrated higher activity in ELISA assays at elevated temperature compared to the wild-type protein.

Applying this technology to the fluorescent protein EGFP, flp and Flp were fed to a bacterial proline auxotroph to produce respectively soluble, flp-protein, and unfolded Flp-protein that was detectable only in inclusion bodies [51]. Attempts to fold the Flp-protein failed, indicating an irrecoverable effect on the structure, which was unexpected because Flp is known to promote the prolyl amide *trans* isomer found throughout GFP. Characterization of the expressed EGFP containing flp by X-ray diffraction analysis detected no noticeable differences from native protein (Fig. 4a). Moreover, refolding experiments demonstrated that flp-incorporation accelerated the folding of EGFP relative to native protein. The only 4-fluoroproline protein reported to date that folded with flp instead of Flp residues, the flp-EGFP analog may undergo accelerated folding to the wild-type structure, due to the reduced barrier for prolyl amide isomerization of flp relative to Pro [55].

In another fluorescent protein, mRFP1, the opposite trend was observed: flp-incorporation produced only insoluble aggregates, and Flp-incorporation

resulted in a soluble mRFP1, albeit without fluorescence [119]. Molecular modeling demonstrated a steric clash between the chromophore and the fluoro group of Flp63. By genetically mutating Pro63 to alanine before incorporating Flp globally, a fluorescent mRFP1 was obtained which folded faster and exhibited increased thermostability.

Optimized conditions were devised to replace the 32 native proline residues of the 60-kDa KlenTaq DNA polymerase with Flp at 92% efficiency [120]. Attempts to incorporate flp did not result in protein production. The resulting fluorinated enzyme was less thermostable than wild type, but functioned with normal DNA replication kinetics and error rates. A refined preparation of the fluorinated protein with a substitution efficiency of 98% produced crystals of sufficient quality for diffraction to 2.4-Å resolution (Fig. 4b) [52]. The fluoro groups on the exterior of the protein were suggested to improve crystallinity by mediating contacts between individual protein molecules within the lattice. Pucker assignments were possible for 28 out of 32 proline residues in both the Flp and wild-type KlenTaq DNA polymerase crystal structures, which respectively exhibited 89% and 43% *exo* pucker, demonstrating that the strong gauche effect in Flp can alter the ring conformation in the context of structural bias from nonlocal interactions. Incorporation of Flp did not alter the two prolyl amide *cis* isomers in KlenTaq DNA polymerase, which adopted *endo* pucker, and accounted for half of the *endo*-puckered residues. Moreover, the electron density for 36% of the prolines in the wild-type polymerase was consistent with a superposition of puckers; only 7% of Flp residues in the fluorinated protein were consistent with multiple puckers in the electron-density map, indicating reduced conformational flexibility of the 4-fluoropyrrolidine ring.

Dissecting the impact of a modification at single residue is challenging using SPI, which inherently results in global substitution. To probe a single-proline position with 4-fluoroproline, a variant of *Escherichia coli* thioredoxin was conceived in which the four *trans* prolines were replaced by alanine, and the single conserved *cis* proline was retained [53]. Both Flp- and flp-proteins were expressed, folded, and shown to be stable in chemical denaturation experiments. Both modifications stabilized the reduced and destabilized the oxidized form of the protein. Both substitutions also improved the cooperativity of folding, which had been compromised by the four proline-to-alanine substitutions. Measures of catalytic activity revealed little effect from fluorination. The lack of significant differences of diastereomeric substitutions was explicable from high-resolution crystal structure analysis of all three single-proline proteins (Fig. 4c). Although Flp and flp favor usually *exo* and *endo* pyrrolidine ring puckers, respectively, both were observed in the *endo* conformation in their respective crystal structures. The Flp residue was predicted to adopt the *endo* pucker to avoid a steric clash between the 4-fluoro substituent and Cys35. The tertiary structure of thioredoxin was suggested to nullify the usual conformational preferences of 4-fluoroproline.

The first total synthesis of a 4-fluoroproline protein was achieved using β2-microglobulin (β2m), the causative agent of dialysis-related amyloidosis [121]. In

the native structure of β2m, the amide of one of the five proline residues, Pro32, adopts a *cis* isomer [122], which isomerizes to the *trans* conformation in the amyloid, as shown by solid-state NMR spectroscopy [123]. Accordingly, prolyl amide isomerization was predicted to be a key step in the mechanism of amyloid formation. Traditional mutagenic experiments yielded contradictory results: substitution of Pro32 with glycine-induced fibril formation, but substitution with valine or alanine did not [124]. 4-Fluoroprolines (Flp, flp, and Dfp) were employed to subtly and selectively evaluate the role of isomerization in the mechanism of β2m amyloid formation. To incorporate site-specifically the 4-fluoroprolines, β2m variants were produced using native chemical ligation to combine three peptide segments that were generated using solid-phase synthesis. Temporary protection of Cys25 as a thiazolidine allowed for assembly of the full-length protein, which was folded and oxidized into the native structure. Thermal denaturation of the substitution variants demonstrated that *cis*- and *trans*-favoring flp and Flp respectively increased and decreased stability of the folded state relative to wild type. Although Dfp exhibits similar conformational preferences as Pro, the Dfp variant was the least stable of the series. The Dfp variant displayed the least cooperative unfolding, the highest association with a fluorescent probe of unstructured hydrophobic patches on proteins, and the most facile amyloidogenesis. In contrast to wild-type and the monofluoroproline variants, the Dfp variant formed fibrils spontaneously after 2 weeks at neutral pH. Although the relative prolyl *cis* and *trans* isomer populations alone were demonstrated to be insufficient, the rate of amide isomerization appeared key for dictating β2m amyloid formation. Consistent with observations employing a fluorescent probe, rapid *cis*–*trans* isomerization at Pro32 may enhance flexibility of the BC loop to expose hydrophobic regions of the protein and increase the rate of amyloid formation.

7 Impact in Medicinal Chemistry

Fluorination has emerged as a powerful, general strategy for combating the oxidative metabolism of various pharmaceutical candidates [125]. In this light, 4-fluoroprolines are convenient building blocks for introducing fluorine into peptide-based pharmaceuticals [126], such as thrombin inhibitors [127]. Thrombin is an important protease for blood clotting and targeted by inhibitors in therapeutic approaches to treat clotting diseases. Substitution of the central proline residue of a tripeptide inhibitor with flp and Flp gave respectively 200-fold reduced potency and retained potency without oxidative metabolism. In crystal structures of the thrombin · inhibitor complexes, the central proline residue preferred the *exo* ring pucker [128]. These results enabled production of compounds with improved metabolic stability and selectivity toward thrombin over related proteases like trypsin.

4-Fluoroprolines have similarly been employed in the development of peptide inhibitors of Stat3 phosphorylation [129]. Phosphorylation of Stat3 occurs after

cytokines bind to their receptors and results in dimerization and translocation of Stat3 to the nucleus, where it effects changes in gene expression. High activation of Stat3 has been observed in a number of disease states, including cancers, making Stat3 an important pharmaceutical target. Mimics of the SH2 phosphopeptide hold promise as inhibitors of dimerization, because phosphorylation occurs on the SH2 domain of Stat3. Replacement of a key proline residue within a phosphopeptide prodrug with either flp or Dfp resulted in analogs exhibiting increased potency in cellular assays, in spite of decreased affinity for full-length Stat3 in vitro, suggesting that the 4-fluoroproline substitution enhanced potency by retarding deleterious metabolism.

Crystal structures of the complex between neurotensin (NT) and its G protein-coupled receptor (GPCR) NST1 have been used in structure-based drug development programs to yield new analgesics [130]. NT features a key proline residue that binds within a small pocket of NST1 as confirmed by mutagenic studies. To develop selective inhibitors of the related receptor NST2 (which also binds to neurotensin), NT8-13 analogs were produced that contain a variety of proline modifications [131]. A cellular assay was employed to determine the ability of the analogs to inhibit binding of the native ligand and demonstrated a preference of the receptor for *exo*-puckered prolines, including Flp. Further elaboration of the lead peptide with a single peptoid moiety produced a 4-fluoroproline-based ligand with nearly 10^4-fold selectivity for NST2 over NST1 and provided a probe for ^{19}F MRI imaging.

The effect of a fluoro substituent on ring conformation or peptide bond isomerization is usually the dominant contribution to systems incorporating *N*-acyl-fluoroprolines. Another consequence arises in *N*-alkyl-fluoroproline derivatives. In attempts to develop γ-aminobutyric acid (GABA) reuptake inhibitors related to the *N*-alkyl (*R*)-nipecotic acid drug tiagabine, 4-fluorination of proline and homoproline analogs decreased potency [132], likely due to their decreased amine basicity.

In addition, ^{18}F 4-fluoroproline analogs have begun to be developed as probes for positron emission tomography (PET), due to the relatively short half-life (110 min) of the fluorine isotope, the lack of necessary chelators, and opportunity for incorporation into nascent proteins, such as collagen. In spite of challenges to radio-synthesize, administer, and allow for trafficking and biosynthetic incorporation of the analog, ^{18}F 4-fluoroprolines have been made [133–136], and their pharmacokinetics have been studied [137, 138]. Their future application in imaging of tumors [138–141] or collagen synthesis [142, 143] remains to be advanced.

8 Outlook

Research to date has clearly demonstrated the utility of 4-fluoroprolines for the rational probing of peptide and protein stability, dynamics, and activity. Conformational analysis of 4-fluoroproline monomers has provided predictive insight into

their effects upon incorporation into biopolymers, albeit complications have been observed due to the chemical and structural constraints imposed by these complex molecules. Complementing traditional genetic approaches for exploring protein structure–function relationships, the application of 4-fluoroprolines is expected to continue to find utility for probing key proline residues in a wide variety of proteins due to their subtle yet reliable modulation of polypeptide structure. Incorporation of 4-fluoroprolines has increased conformational stability and accelerated folding of a variety of important proteins. Future application of 4-fluoroprolines is thus well merited to enhance physical, chemical, and biological attributes in the development of chemotherapeutic agents and protein-based technologies.

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References

1. MacArthur MW, Thornton JM (1991) J Mol Biol 218:397
2. Brandts JF, Halvorson HR, Brennan M (1975) Biochemistry 14:4953
3. Shoulders MD, Raines RT (2009) Annu Rev Biochem 78:929
4. Fischer E (1901) Z Physiol Chem 33:151
5. Fischer E (1902) Chem Ber 35:2660
6. Gorres KL, Raines RT (2010) Crit Rev Biochem Mol Biol 45:106
7. Winter AD, Page AP (2000) Mol Cell Biol 20:4084
8. Friedman L, Higgin JJ, Moulder G, Barstead R, Raines RT, Kimble J (2000) Proc Natl Acad Sci U S A 97:4736
9. Holster T, Pakkanen O, Soininen R, Sormunen R, Nokelainen M, Kivirikko KI, Myllyharju J (2007) J Biol Chem 282:2512
10. Mauger AB, Witkop B (1966) Chem Rev 66:47
11. Gottlieb AA, Fujita Y, Udenfriend S, Witkop B (1965) Biochemistry 4:2507
12. Bakerman S, Martin RL, Burgstahler AW, Hayden JW (1966) Nature 212:849
13. Takeuchi T, Prockop DJ (1969) Biochim Biophys Acta 175:142
14. Takeuchi T, Rosenbloom J, Prockop DJ (1969) Biochim Biophys Acta 175:156
15. Uitto J, Prockop DJ (1974) Biochim Biophys Acta 336:234
16. Hutton JJ Jr, Marglin A, Witkop B, Kurtz J, Berger A, Udenfriend S (1968) Arch Biochem Biophys 125:779
17. Dieglemann RF, Ondrejickova O, Katz E (1969) Arch Biochem Biophys 131:276
18. Yoder NC, Kumar K (2002) Chem Soc Rev 31:335
19. Salwiczek M, Nyakatura EK, Gerling UI, Ye S, Koksch B (2012) Chem Soc Rev 41:2135
20. Merkel L, Budisa N (2012) Org Biomol Chem 10:7241
21. Odar C, Winkler M, Wiltschi B (2015) Biotechnol J 10:427
22. Demange L, Ménez A, Dugave C (1998) Tetrahedron Lett 39:1169
23. Doi M, Nishi Y, Kiritoshi N, Iwata T, Nago M, Nakano H, Uchiyama S, Nakazawa T, Wakamiya T, Kobayashi Y (2002) Tetrahedron 58:8453

24. Chorghade MS, Mohapatra DK, Sahoo G, Gurjar MK, Mandlecha MV, Bhoite N, Moghe S, Raines RT (2008) J Fluor Chem 129:781
25. Kronenthal DR, Mueller RH, Kuester PL, Kissick TP, Johnson EJ (1990) Tetrahedron Lett 31:1241
26. Hudlický M, Merola JS (1990) Tetrahedron Lett 31:7403
27. Avent AG, Bowler AN, Doyle PM, Marchand CM, Young DW (1992) Tetrahedron Lett 33:1509
28. Hudlický M (1993) J Fluor Chem 60:193
29. Thomas KM, Naduthambi D, Tririya G, Zondlo NJ (2005) Org Lett 7:2397
30. Pandey AK, Naduthambi D, Thomas KM, Zondlo NJ (2013) J Am Chem Soc 135:4333
31. Donohue J, Trueblood KN (1952) Acta Crystallogr 5:419
32. Matheison AM, Welsh HK (1952) Acta Crystallogr 5:599
33. Mitsui Y, Tsuboi M, Iitaka Y (1969) Acta Crystallogr B25:2182
34. Higashijima T, Tasumi M, Miyazawa T (1977) Biopolymers 16:1259
35. Allen FH (2002) Acta Crystallogr B58:380
36. DeRider ML, Wilkens SJ, Waddell MJ, Bretscher LE, Weinhold F, Raines RT, Markley JL (2002) J Am Chem Soc 124:2497
37. Improta R, Benzi C, Barone V (2001) J Am Chem Soc 123:12568
38. Grieg JT, McLeod RS (1973) J Am Chem Soc 95:5725
39. Karplus M (1959) J Chem Phys 30:11
40. Panasik N Jr, Eberhardt ES, Edison AS, Powell DR, Raines RT (1994) Int J Pept Prot Res 44:262
41. Bretscher LE, Jenkins CL, Taylor KM, DeRider ML, Raines RT (2001) J Am Chem Soc 123:777
42. Jakobsche CE, Choudhary A, Miller SJ, Raines RT (2010) J Am Chem Soc 132:6651
43. Hinderaker MP, Raines RT (2003) Protein Sci 12:1188
44. Bartlett GJ, Choudhary A, Raines RT, Woolfson DN (2010) Nat Chem Biol 6:615
45. Bürgi HD, Dunitz JD, Shefter E (1974) Acta Crystallogr B30:1517
46. Newberry RW, VanVeller B, Guzei IA, Raines RT (2013) J Am Chem Soc 135:7843
47. Bartlett GJ, Newberry RW, Vanveller B, Raines RT, Woolfson DN (2013) J Am Chem Soc 135:18682
48. Newberry RW, Bartlett GJ, Vanveller B, Woolfson DN, Raines RT (2014) Protein Sci 23:284
49. Jenkins CL, Lin G, Duo J, Rapolu D, Guzei IA, Raines RT, Krow GR (2004) J Org Chem 69:8565
50. Choudhary A, Gandla D, Krow GR, Raines RT (2009) J Am Chem Soc 131:7244
51. Steiner T, Hess P, Bae JH, Wiltschi B, Moroder L, Budisa N (2008) PLoS One 3:e1680
52. Holzberger B, Obeid S, Welte W, Diederichs K, Marx A (2012) Chem Sci 3:2924
53. Rubini M, Schärer MA, Capitani G, Glockshuber R (2013) ChemBioChem 14:1053
54. Siebler C, Maryasin B, Kuemin M, Erdmann RS, Rigling C, Grünenfelder C, Ochsenfeld C, Wennemers H (2015) Chem Sci 6:6725
55. Eberhardt ES, Panasik N Jr, Raines RT (1996) J Am Chem Soc 118:12261
56. Shoulders MD, Kamer KJ, Raines RT (2009) Bioorg Med Chem Lett 19:3859
57. Rich A, Crick FHC (1961) J Mol Biol 3:483
58. Ramshaw JAM, Shah NK, Brodsky B (1998) J Struct Biol 122:86
59. Sakakibara S, Inouye K, Shudo K, Kishida T, Kobayashi Y, Prockop DJ (1973) Biochim Biophys Acta 303:198
60. Berg RA, Prockop DJ (1973) Biochem Biophys Res Commun 52:115
61. Bella J, Eaton M, Brodsky B, Berman HM (1994) Science 266:75
62. Bella J, Brodsky B, Berman HM (1995) Structure 3:893
63. Hodges JA, Raines RT (2005) J Am Chem Soc 127:15923
64. Hodges JA, Raines RT (2003) J Am Chem Soc 125:9262
65. Holmgren SK, Bretscher LE, Taylor KM, Raines RT (1999) Chem Biol 6:63

66. Doi M, Nishi Y, Uchiyama S, Nishiuchi Y, Nakazawa T, Ohkubo T, Kobayashi Y (2003) J Am Chem Soc 125:9922
67. Doi M, Nishi Y, Uchiyama S, Nishiuchi Y, Nishio H, Nakazawa T, Ohkubo T, Kobayashi Y (2005) J Pept Sci 11:609
68. Holmgren SK, Taylor KM, Bretscher LE, Raines RT (1998) Nature 392:666
69. Dunitz JD, Taylor R (1997) Chem Eur J 3:89
70. Mooney SD, Kollman PA, Klein TE (2002) Biopolymers 64:63
71. Kotch FW, Guzei IA, Raines RT (2008) J Am Chem Soc 130:2952
72. Persikov AV, Ramshaw JAM, Kirkpatrick A, Brodsky B (2003) J Am Chem Soc 125:11500
73. Malkar NB, Lauer-Fields JL, Borgia JA, Fields GB (2002) Biochemistry 41:6054
74. Chattopadhyay S, Murphy CJ, McAnulty JF, Raines RT (2012) Org Biomol Chem 10:5892
75. Chattopadhyay S, Raines RT (2014) Biopolymers 101:821
76. Chattopadhyay S, Guthrie KM, Teixeira L, Murphy CJ, Dubielzig RR, McAnulty JF, Raines RT (2015) J Tissue Eng Regen Med. doi:10.1002/term.1886
77. Nishi Y, Uchiyama S, Doi M, Nishiuchi Y, Nakazawa T, Ohkubo T, Kobayashi Y (2005) Biochemistry 44:6034
78. Marsh EN, Suzuki Y (2014) ACS Chem Biol 9:1242
79. Kawahara K, Nemoto N, Motooka D, Nishi Y, Doi M, Uchiyama S, Nakazawa T, Nishiuchi Y, Yoshida T, Ohkubo T, Kobayashi Y (2012) J Phys Chem B 116:6908
80. Gorres KL, Edupuganti R, Krow GR, Raines RT (2008) Biochemistry 47:9447
81. Gorres KL, Raines RT (2009) Anal Biochem 386:181
82. Adzhubei AA, Sternberg MJE (1993) J Mol Biol 229:472
83. Shi Z, Chen K, Liu Z, Kallenbach NR (2006) Chem Rev 106:1877
84. Wilhelm P, Lawandowski B, Trapp N, Wennemers H (2014) J Am Chem Soc 136:15829
85. Siebler C, Erdmann RS, Wennemers H (2013) Chimia 67:891
86. Kuemin M, Engel J, Wennemers H (2010) J Pept Sci 16:596
87. Horng JC, Raines RT (2006) Protein Sci 15:74
88. Chiang YC, Lin YJ, Horng JC (2009) Protein Sci 18:1967
89. Lin YJ, Horng JC (2014) Amino Acids 46:2317
90. Lin L-N, Brandts JF (1980) Biochemistry 19:3055
91. Berisio R, Vitagliano L (2012) Curr Protein Pept Sci 13:855
92. Feng S, Chen JK, Yu H, Simon JA, Schreiber SL (1994) Science 266:1241
93. Ruzza P, Siligardi G, Donella-Deana A, Calderan A, Hussain R, Rubini C, Cesaro L, Osler A, Guiotto A, Pinna LA, Borin G (2006) J Pept Sci 12:462
94. Tang HC, Lin YJ, Horng JC (2014) Proteins 82:67
95. Wintjens R, Wieruszeski JM, Drobecq H, Rousselot-Pailley P, Buée L, Lippens G, Landrieu I (2001) J Biol Chem 276:25150
96. Naduthambi D, Zondlo NJ (2006) J Am Chem Soc 128:12430
97. Neidigh JW, Fesinmeyer RM, Andersen NH (2002) Nat Struct Biol 9:425
98. Boulègue C, Milbradt AG, Renner C, Moroder L (2006) J Mol Biol 358:846
99. Pokidysheva E, Milbradt AG, Meier S, Renner C, Häussinger D, Bächinger HP, Moroder L, Grzesiek S, Holstein TW, Özbek S, Engel J (2004) J Biol Chem 279:30395
100. Barth D, Musiol HJ, Schütt M, Fiori S, Milbradt AG, Renner C, Moroder L (2003) Chem Biol 9:3692
101. Scholz S, Liebler EK, Eickmann B, Fritz HJ, Diederichsen U (2012) Amino Acids 43:289
102. Rice PA, Yang S-W, Mizuuchi K, Nash HA (1996) Cell 87:1295
103. Merkel L, Schauer M, Antranikian G, Budisa N (2010) ChemBioChem 11:1505
104. Lepthien S, Merkel L, Budisa N (2010) Angew Chem Int Ed 49:5446
105. Hoesl MG, Acevedo-Rocha CG, Nehring S, Royter M, Wolschner C, Wiltschi B, Budisa N, Antranikian G (2011) ChemCatChem 3:213
106. Hoesl MG, Budisa N (2011) ChemBioChem 12:552
107. Oldach F, Al Toma R, Kuthning A, Caetano T, Mendo S, Budisa N, Sussmuth RD (2012) Angew Chem Int Ed 51:415

108. Larregola M, Moore S, Budisa N (2012) Biochem Biophys Res Commun 421:646
109. Nilsson BL, Soellner MB, Raines RT (2005) Annu Rev Biophys Biomol Struct 34:91
110. Renner C, Alefelder S, Bae JH, Budisa N, Huber R, Moroder L (2001) Angew Chem Int Ed 40:923
111. Golbik R, Yu C, Weyher-Stingl E, Huber R, Moroder L, Budisa N, Schiene-Fischer C (2005) Biochemistry 44:16026
112. Kim W, George A, Evans M, Conticello VP (2004) ChemBioChem 5:928
113. Debelle L, Tamburro AM (1999) Int J Biochem Cell Biol 31:261
114. Kim W, McMillian RA, Snyder JP, Conticello VP (2005) J Am Chem Soc 127:18121
115. Crespo MD, Rubini M (2011) PLoS One 6:e19425
116. Komander D, Rape M (2012) Annu Rev Biochem 81:203
117. Vijay-Kumar S, Bugg CE, Cook WJ (1987) J Mol Biol 194:531
118. Edwardraja S, Sriram S, Govindan R, Budisa N, Lee SG (2011) Mol Biosyst 7:258
119. Deepankumar K, Nadarajan SP, Ayyadurai N, Yun H (2013) Biochem Biophys Res Commun 440:509
120. Holzberger B, Marx A (2010) J Am Chem Soc 132:15708
121. Torbeev VY, Hilvert D (2013) Proc Natl Acad Sci U S A 110:20051
122. Trinh CH, Smith DP, Kalverda AP, Phillips SE, Radford SE (2002) Proc Natl Acad Sci U S A 99:9771
123. Jahn TR, Parker MJ, Homans SW, Radford SE (2006) Nat Struct Mol Biol 13:195
124. Eichner T, Radford SE (2011) FEBS J 278:3868
125. Park BK, Kitteringham NR, O'Neill PM (2001) Annu Rev Pharmacol Toxicol 41:443
126. Tran TT, Patino N, Condom R, Frogier T, Guedj R (1997) J Fluor Chem 82:125
127. Staas DD, Savage KL, Sherman VL, Shimp HL, Lyle TA, Tran LO, Wiscount CM, McMasters DR, Sanderson PE, Williams PD, Lucas BJ Jr, Krueger JA, Lewis SD, White RB, Yu S, Wong BK, Kochansky CJ, Anari MR, Yan Y, Vacca JP (2006) Bioorg Med Chem 14:6900
128. Tucker TJ, Brady SF, Lumma WC, Lewis SD, Gardell SJ, Naylor-Olsen AM, Yan Y, Sisto JT, Stauffer KJ, Lucas BJ, Lynch JJ, Cook JJ, Stranieri MT, Holahan MA, Lyle EA, Baskin EP, Chen I-W, Dancheck KB, Krueger JA, Cooper CM, Vacca JP (1998) J Med Chem 41:3210
129. Mandal PK, Ren Z, Chen X, Kalurachchi K, Liao WS-L, McMurray JS (2013) Int J Pept Prot Res 19:3
130. White JF, Noinaj N, Shibata Y, Love J, Kloss B, Xu F, Gvozdenovic-Jeremic J, Shah P, Shiloach J, Tate CG, Grisshammer R (2012) Nature 490:508
131. Held C, Hübner H, Kling R, Nagel YA, Wennemers H, Gmeiner P (2013) ChemMedChem 8:772
132. Zhuang W, Zhao X, Zhao G, Guo L, Lian Y, Zhou J, Fang D (2009) Bioorg Med Chem 17:6540
133. van der Ley M (1983) J Label Compd Radiopharm 20:453
134. Hamacher K (1999) J Label Compd Radiopharm 42:1135
135. Mazza SM (2000) J Label Compd Radiopharm 43:1047
136. Azad BB, Ashique R, Labiris NR, Chirakal R (2012) J Labelled Cpd Radiopharm 55:23
137. Wester H-J, Herz M, Senekowitsch-Schmidtke R, Schwaiger M, Stöcklin G, Hamacher K (1999) Nucl Med Biol 26:259
138. Börner AR, Langen K-J, Herzog H, Hamacher K, Müller-Mattheis V, Schmitz T, Ackermann R, Coenen HH (2001) Nucl Med Biol 28:287
139. Langen K-J, Mühlensiepen H, Schmieder S, Hamacher K, Bröer S, Börner AR, Schneeweiss FHA, Coenen HH (2002) Nucl Med Biol 29:685
140. Langen K-J, Jarosch M, Hamacher K, Mühlensiepen H, Weber F, Floeth F, Pauleit D, Herzog H, Coenen HH (2004) Nucl Med Biol 31:67
141. Langen KJ, Hamacher K, Bauer D, Bröer S, Pauleit D, Herzog H, Floeth F, Zilles K, Coenen HH (2005) J Cereb Blood Flow Metab 25:607

142. Zimny M, Klosterhalfen B, Conze J, Hamacher K, Fehler S, Schumpelick V, Coenen HH, Buell U (2002) Nucl Med Commun 23:695
143. Skovgaard D, Kjaer A, Heinemeier KM, Brandt-Larsen M, Madsen J, Kjaer M (2011) PLoS One 6:e16678