Fluorinated amino acids have unique properties, due to the hydrophobicity and electronegativity of fluorine and the magnetic properties of the sensitive spin-1/2 19F nucleus. The special nature of fluorine has led to broad interest in the incorporation of fluorinated amino acids in small molecules, peptides, and proteins, for applications in medicinal chemistry, in the design of stabilized proteins, and in NMR and MRI approaches to protein detection and imaging. Fluorinated amino acids allow the specific, quantitative detection of individual species in complex media, including the observation of protein synthesis and folding in living E. coli cells, due to the low background of fluorine in most environments. The sensitivity of fluorine NMR probes depends on the concentration of the molecule, the coupling patterns of the 19F nuclei to other fluorines and to hydrogens in the molecule, and the number of chemically equivalent fluorines in the molecule. For probes to be employed biologically, it is ideal to be able to work at the lowest concentration of molecule possible due to the typical nanomolar protein concentrations and affinities observed in protein–protein interactions. The most sensitive protein-based probes of biological activity employed to date have utilized aryl trifluoromethyl groups, which exhibit sharp singlets by 19F NMR of intensity 3 (one aryl-CF3 group) or 6 (two symmetrically related, chemically equivalent aryl-CF3 groups). In contrast, in the most widely used direct analogue of a canonical ω-amino acid, hexahloroelucine (6 fluorines), the two trifluoromethyl groups are diastereotopic, exhibiting separate resonances, and each is coupled to the methine hydrogen, resulting in reduced peak intensity due to each trifluoromethyl group existing as a doublet (four total peaks observed).

We previously described the synthesis of tetrapeptides containing the novel amino acids (2S,4R)- and (2S,4S)-perfluoro-tert-butyl 4-hydroxyproline (Figure 1). Peptides with these amino acids were prepared by the method of proline editing, in which a hydroxyproline amino acid within a fully synthesized peptide is site-specifically modified via reaction on solid phase to incorporate novel functionalities. The perfluoro-tert-butyl group of these amino acids, in contrast to most other fluorinated amino acids, exhibits fluorines that are singlets by 19F NMR, here with nine chemically equivalent fluorines that are uncoupled to any other nuclei. The unique magnetic nature of the perfluoro-tert-butyl group, combined with the large chemical shift dispersion of 19F nuclei and the general absence of fluorine in typical biological environments, suggests the possibility of highly sensitive detection of molecules containing this functional group in diverse media.

In addition to the magnetic properties of the perfluoro-tert-butyl group, in general, tert-butyl groups are broadly attractive in medicinal chemistry due to functional group symmetry and the consequent reduced conformational entropy penalty upon target binding. However, in amino acids and peptides, tert-butyl ethers, the most easily installed tert-butyl groups, are not stable to standard acidic cleavage/deprotection reactions. In contrast, perfluoro-tert-butyl ethers are not subject to carbocation formation and elimination reactions and thus are expected to be a chemical stable functionality that may be incorporated within small molecules, peptides, and proteins.

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The commercially available compound Boc-\((2S,4R)\)-4-hydroxyproline methyl ester (1) was used as the starting material for the synthesis of both Fmoc- and Boc-protected 4\(R\)- and 4\(S\)-perfluoro-tert-buty 4-hydroxyprolines and examine the conformational preferences of these amino acids within different secondary structure contexts where they might be functionally employed.

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hydroxyproline alcohol stereochemistry was accomplished by Mitsunobu reaction with 4-nitrobenzoic acid, followed by nitrobenzoxazolization with sodium azide, to generate the protected 4S-hydroxyproline (3), using conditions developed previously (Scheme 1).42,50−52 This alcohol was then subjected to Mitsunobu reaction with perfluoro-tert-butanol (pKₐ 5),42,44,45,53 to generate the protected 4R-perfluoro-tert-butyl hydroxyproline (4). This product was converted to the Boc-amino acid 5, the free amino acid 6, and the Fmoc-amino acid 7 using standard approaches, generating the protected derivatives for the incorporation of this amino acid in peptides or small molecules by solution or solid-phase peptide synthesis.

The 4S-substituted perfluoro-tert-butyl hydroxyproline was analogously synthesized via Mitsunobu reaction of perfluoro-tert-butanol with the same protected 4R-hydroxyproline (1) starting material used previously (Scheme 2). The product of this reaction (8) was converted to the Boc- (9), free (10), and Fmoc-amino acids (11).

The Fmoc-4R- and Fmoc-4S-perfluoro-tert-butyl hydroxyproline amino acids were each incorporated within peptides in two different structural contexts, a proline-rich peptide used to identify polyproline helix propensity (Ac-GPPXPGY-NH₂) and a Baldwin-type alanine-rich peptide to identify α-helix propensity (Ac-XKAAAACKAAAAKAGY-NH₂).54−56 These peptide sequences were employed to determine the conformational preferences of these amino acids within the polyproline helix and α-helix secondary structures, both of which are widely employed in molecular recognition. Matching the conformational preferences of the amino acid to the structural context in which the amino acid may be employed (protein design in “chi space”)57 can maximize the effectiveness of the amino acids toward defined applications.

Peptide synthesis with these amino acids proceeded sluggishly using HBTU as a coupling reagent. The desired peptides were obtained when amide coupling of the Fmoc-perfluoro-tert-butyl hydroxyproline was conducted using COMU as a coupling reagent and an extended reaction time.58 For the proline-rich peptides, subsequent amide coupling reactions were conducted using HATU. The relatively lower reactivity in amide bond formation associated with these amino acids was presumably due to the steric hindrance of the perfluoro-tert-butyl group.

In the Ac-GPPXPGY-NH₂ peptide series, the peptide with 4R-perfluoro-tert-butyl hydroxyproline exhibited positive (λ_max = 228 nm) and negative (λ_min = 205 nm) bands typical for polyproline helix (PPII) (Figure 2). The mean residue ellipticity at 228 nm ([θ]_228 = 3770 deg-cm²-dmol⁻¹) indicated that this amino acid had a greater propensity for the polyproline helix than proline ([θ]_228 = 2950 deg-cm²-dmol⁻¹).59 In contrast, the peptide with 4S-perfluoro-tert-butyl hydroxyproline exhibited a weaker positive band ([θ]_228 = 1700 deg-cm²-dmol⁻¹) and a red-shifted λ_max = 230 nm, both of which indicate that this amino acid has a substantially lower polyproline helix propensity than proline. These data are consistent with previous examinations on the stereoelectronic effects of 4-substituted proline residues on polyproline helix stability, where 4R-hydroxyproline and 4R-fluoroproline promote PPII relative to proline, whereas 4S-hydroxyproline and 4S-fluoroproline relatively destabilize PPII compared to proline.9,60 These data indicate that the perfluoro-tert-butyl hydroxyprolines similarly exhibit distinct conformational preferences that allow stereochemical selection to optimize their applications.

Proline is compatible with α-helices at their N-terminus, where they can function as start signals for α-helices.56,61,62

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**Scheme 2. Synthesis of (2S,4R)-Perfluoro-tert-butyl 4-Hydroxyproline (hyp(C₄F₉)) as Free, Boc-, and Fmoc-Amino Acids**

![Scheme 2](image-url)

**Figure 2.** CD spectra of peptides with (2S,4R)-hyp(C₄F₉) (red circles) and (2S,4S)-hyp(C₄F₉) (blue squares) as the guest (X) residue in Ac-GPPXPGY-NH₂ peptides. Polyproline helix (PPII) is indicated by the magnitude of the positive band at ~228 nm.59 The peptide with X = Pro exhibits an intermediate structure between the perfluoro-tert-butyl hydroxyprolines.55

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Ac-XKAAAKAAAAKAGY-NH$_2$ peptides, both perfluoro-tert-butyl hydroxyprolines exhibited evidence of $\alpha$-helical structure (Figure 3). However, in the compact conformation of the $\alpha$-helix, both peptides were less $\alpha$-helical than the peptide with proline at the first residue (Pro$^{56}$ $\theta_{222} = -12030$ deg cm$^2$ dmol$^{-1}$, $\theta_{122}/\theta_{208} = 0.87$, 38% $\alpha$-helix; 4R-Hyp-(C$_4$F$_9$)$_2$: $\theta_{222} = -6840$ deg cm$^2$ dmol$^{-1}$, $\theta_{122}/\theta_{208} = 0.67$, 24% $\alpha$-helix; 4S-hyp(C$_4$F$_9$)$_2$: $\theta_{222} = -4990$ deg cm$^2$ dmol$^{-1}$, $\theta_{122}/\theta_{208} = 0.64$, 19% $\alpha$-helix). The reduced $\alpha$-helicity of the 4R-perfluoro-tert-butyl hydroxyproline compared to proline was expected based on the conformational preferences of electron-withdrawing 4S-substituents on proline, which promote a more extended conformation and an endo ring pucker, which is not preferred in $\alpha$-helices.$^{63}$ The reduced $\alpha$-helicity of the 4R-perfluoro-tert-butyl hydroxyproline compared to proline was not expected based on proline conformational analysis. However, these data are consistent with trends across a range of fluorinated amino acids, where highly fluorinated amino acids strongly disfavor $\alpha$-helix independent of side chain structure.$^{49,64}$ In addition, in the 4R-perfluoro-tert-butyl hydroxyproline, the perfluoro-tert-butyl group would project toward the subsequent residues of the $\alpha$-helix, potentially providing a steric basis for reduced $\alpha$-helicity. Perfluoro-tert-butyl amino acids have substantial potential as probes in $^{19}$F-based magnetic imaging due to the sensitivity of the perfluoro-tert-butyl group (9 equivalent fluorines that do not couple to any other nuclei) and the large chemical shift dispersion inherent to $^{19}$F NMR. All peptides were examined by $^{19}$F NMR spectroscopy. In the Ac-GPPXPPGY-NH$_2$ peptides (Figure 4), both peptides exhibited one major resonance and multiple minor resonances. $X$–Pro amide bonds are inherently prone to cis–trans isomerism, with 10% of $X$–Pro–Pro amide bonds cis in the PDB. Thus, these peptides have 32 (2$^3$) potential species present in slow exchange, considering combinations of cis versus trans amide bonds at each of the five $X$–Pro amides. In the $^1$H NMR spectra of these peptides, evidence of multiple species due to cis–trans isomerism is observable, but due to the complexity of the $^1$H NMR spectra, the data only clearly indicate the presence of three or four species. In the simpler $^{19}$F NMR spectrum, where all peaks are singlets, the relative populations of the major species are confirmed.$^{30,65,66}$ Herein, however, additional minor species may be identified by $^{19}$F NMR that are not observed by $^1$H NMR. These data indicate that perfluoro-tert-butyl hydroxyprolines may function as sensitive probes of species not observable by standard $^1$H NMR approaches. $^{19}$F NMR spectra of the Ac-XKAAAKAAAAKAGY-NH$_2$ peptides (Figure 5) indicated one major species (trans Ac–Pro amide bond) and one minor species (cis Ac–Pro amide bond), with a higher population of cis amide bond for the 4S- than the 4R-perfluoro-tert-butyl hydroxyproline, as expected (Figure 1). In order to identify the sensitivity of detection of perfluoro-tert-butyl hydroxyprolines, in consideration of future applications of these amino acids in imaging, the NMR spectrum of the peptide with 4R-perfluoro-tert-butyl hydroxyproline was examined as a function of peptide concentration. The $^{19}$F NMR spectrum of this peptide was rapidly obtained (5 min, 128 scans, signal-to-noise = 7.3) at a peptide concentration of 200 nM (Figure S7, Supporting Information). The sensitivity of the perfluoro-tert-butyl group indicated by these experiments suggests broad potential applications of these amino acids in imaging. We have described the synthesis of 4R- and 4S-perfluoro-tert-butyl hydroxyproline as free amino acids and as Boc- and Fmoc-amino acids, for incorporation of the amino acids using the major approaches to peptide synthesis. These amino acids have distinct conformational preferences, suggesting alternative...
applications for each in small molecules, peptides, and proteins. These amino acids represent unique handles for molecular recognition and for use in sensitive $^{19}$F NMR and MRI imaging.

**EXPERIMENTAL SECTION**

Compounds 2 and 3 were synthesized using minor modifications to methods described. $^{51,52}$

**Boc-(2S,4S)-p-nitrobenzoate-4-hydroxyproline Methyl Ester (2).** Compound $2 (1.02 g, 2.90 mmol)$ was dissolved in acetone (50 mL). Sodium azide (0.49 g, 7.55 mmol) was added, and the solution was heated at reflux for 14 h. The solution was allowed to cool to room temperature, and the solvent was removed under reduced pressure. The crude product was redissolved in ethyl acetate (50 mL) and washed with distilled water (2 × 50 mL). The solution was then stirred on ice for another 5 min. The solution was removed from the ice bath, warmed to 45 °C in an oil bath, and stirred for 24 h. The solvent was removed under reduced pressure, and the crude product was dissolved in CH$_2$Cl$_2$ (50 mL). The crude product was purified via column chromatography (0–7% ethyl acetate in hexanes v/v) to obtain compound 8 as a white solid (0.83 g, 1.45 mmol) in 50% yield. $^1$H NMR (600 MHz, CDCl$_3$) δ 7.77–7.76 (dd, $J = 7.5$ Hz, 1H, trans), 7.74–7.73 (dd, $J = 7.6$ Hz, $0.7H$, cis) 7.55–7.52 (dd, $J = 12.4$, 7.6 Hz, 2H), 7.42–7.38 (m, 2H), 7.32–7.29 (dd, $J = 7.4$, 7.3 Hz, 2H), 4.92 (s, 0.7H), trans), 4.89 (s, 0.3H, cis), 4.59–4.57 (dd, $J = 7.8$, 7.4 Hz, 0.7H, trans), 4.52–4.43 (m, 2H), 4.38–4.37 (dd, $J = 7.4$, 7.4 Hz, 0.3H, cis), 4.30–4.27 (dd, $J = 7.0$, 6.6 Hz, 0.7H, trans), 4.17–4.16 (dd, $J = 6.6$, 6.6 Hz, 0.3H, cis), 3.83–3.73 (m, 1H), 3.71 (s, 1H), 2.49–2.45 (m, 1.7H, mixture of cis and trans), 2.32–2.29 (m, 0.3H, cis). $^{13}$C NMR (150.8 MHz, CDCl$_3$) δ 173.1, 156.1, 143.5, 143.4, 141.4, 127.9, 127.2, 124.9, 124.8, 120.12, 120.09, 77.9, 68.6, 67.6, 57.6, 56.7, 53.0, 52.7, 47.2, 47.0, 37.6, 35.8. $^{19}$F NMR (367.3 MHz, CDCl$_3$) δ −70.47 (trans conformation), −70.53 (cis conformation). HRMS (EI-TOF) m/z: [M$^+$ H$^+$]$^+$ calculated for C$_2$H$_4$F$_2$NO$_3$, 350.0439, found 350.0420.

**Fmoc-(2S,4S)-tert-butyloxycarbonyl-4-hydroxyproline Methyl Ester (7).** Crude compound 6 (1.01 g, 2.90 mmol) was dissolved in a solution of 1,4-dioxane (15 mL) and 4 M HCl (15 mL). The solution was allowed to stir at reflux for 6 h. The solvent was removed under reduced pressure. Compound 6 (0.78 g, 2.2 mmol) was used as a crude reagent in the next step without purification. Alternatively, compound 5 could be subjected to identical conditions to yield compound 6. $^1$H NMR (600 MHz, MeOD-d$_4$) δ 5.22 (br s, 1H), 3.69–3.68 (1H, minor), 3.72 (s, 3H), 3.68 (s, 3H). $^{13}$C NMR (150.8 MHz, CDCl$_3$) δ 171.9, 171.7, 153.1, 152.4, 119.1 (q, $J = 293$ Hz), 79.8, 77.1, 56.5, 56.1, 51.5, 51.2, 36.6, 35.6, 27.2. $^{19}$F NMR (367.3 MHz, CDCl$_3$) δ −70.47 (cis conformation). HRMS (EI-TOF) m/z: [M$^+$ Cl$^-$]$^-$ calculated for C$_7$H$_5$F$_3$NO$_3$, 571.0141, found 571.0127.

**Boc-(2S,4S)-p-nitrobenzoate-4-hydroxyproline Methyl Ester (2).** Compound 2 (1.02 g, 2.90 mmol) and Ph$_3$P (2.66 g, 10.1 mmol) were dissolved in toulene (91 mL) under a nitrogen atmosphere. The solution was cooled to 0 °C and stirred on ice for 10 min. DIAD (2.40 g, 2.15 mL, 10.9 mmol) was added dropwise to the solution over 15 min. Perfluoro-tert-butanol (4.30 g, 2.54 mL, 18.2 mmol) and DIPEA (2.35 g, 3.16 mL, 18.2 mmol) were added to the solution, which was then stirred on ice for another 5 min. The solution was removed from the ice bath, warmed to 45 °C in an oil bath, and stirred for 24 h. The solvent was removed under reduced pressure, and the crude product was dissolved in ethyl acetate (50 mL). The crude product was washed with brine (2 × 50 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, and the crude product was dissolved in CH$_2$Cl$_2$ (50 mL). The crude product was purified via column chromatography (0–7% ethyl acetate in hexanes v/v) to obtain compound 5 (0.790 g, 2.28 mmol) as a colorless oil in 25% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 4.91 (s, 1H), 4.49–4.47 (dd, $J = 8.6$, 6.4 Hz, 0.4H, cis), 4.40–4.36 (dd, $J = 7.8$, 7.8 Hz, 0.6H, trans), 3.84–3.75 (m, 1H), 3.75 (s, 3H), 3.69–3.66 (dd, $J = 12.5$ Hz, 0.6H, trans), 3.60–3.58 (dd, $J = 12.3$, 0.4H, cis), 2.47–2.44 (m, 1H), 2.28–2.23 (m, 1H), 1.46 (s, 4H, cis), 1.42 (s, 4H, trans). $^{13}$C NMR (100.8 MHz, CDCl$_3$) δ 171.9, 171.7, 153.1, 152.4, 119.1 (q, $J = 293$ Hz), 79.8, 77.1, 56.5, 56.1, 51.5, 51.2, 36.6, 35.6, 27.2. $^{19}$F NMR (367.3 MHz, CDCl$_3$) δ −70.47 (cis conformation), −70.53 (trans conformation). HRMS (EI-TOF) m/z: [M$^+$ H$^+$]$^+$ calculated for C$_8$H$_5$F$_3$NO$_3$, 463.1041, found 463.1051.
**Boc-(25,4S)-perfluoro-tart-butyl-4-hydroxyproline (9).** Compound 8 (1.75 g, 3.78 mmol) and LiOH (0.1086 g, 4.53 mmol) were dissolved in a solution of water (20 mL) and 1,4-dioxane (20 mL). The solution was stirred at room temperature for 14 h. The reaction mixture was acidified to pH 2 with dilute HCl and extracted with ethyl acetate (2 × 75 mL). The solvent was removed under reduced pressure, and the crude product was redissolved in CH2Cl2. The crude product was purified via column chromatography (0–2% methanol in CH2Cl2/v/v) to obtain compound 9 (1.05 g, 2.34 mmol) as an off-white solid in 62% yield.1H (600 MHz, CDCl3) δ = 7.90 (d, J = 6.7 Hz, 2H), 7.67 (d, J = 6.7 Hz, 2H), 7.42–6.78 (m, 4H), 4.92 (s, 0.5H), 4.88 (s, 0.5H), 4.61–4.56 (m, 1H), 4.53–4.49 (m, 0.5H), 4.43–4.31 (m, 2H), 4.16–4.13 (d, J = 6.3, 6.1 Hz, 0.5H), 3.90–3.86 (m, 1H), 3.76–3.63 (m, 1H), 3.74 (s, 1H), 2.57–2.51 (m, 2H).13C NMR (150.8 MHz, CDCl3) δ = 177.4, 175.5, 154.7, 153.5, 120.1 (q, J = 293 Hz), 80.9, 57.5, 57.1, 52.9, 52.7, 52.1, 37.7, 37.6, 36.1, 36.1, 28.1.19F NMR (31.0 MHz, CDCl3) δ = −70.44 (major), −70.44 (major). HRMS (LIFDI-TOF) m/z: [M]+ calcd for C24H18F9NO5 571.1048, found 571.1047.

**Fmoc-(25,4S)-perfluoro-tart-butyl-4-hydroxyproline (11).** Crude compound 10 (0.50 g, 1.4 mmol) was dissolved in 1,4-dioxane (7 mL). Fmoc-OSu (0.50 g, 1.4 mmol) and K2CO3 (0.39 g, 2.9 mmol) were added, and the resultant solution was stirred for 14 h at room temperature. The 1,4-dioxane was removed under reduced pressure, and the crude product was acetylated with acetic anhydride in pyridine, 3 mL, 3 equiv at room temperature. The 1,4-dioxane was removed under reduced pressure, and the resultant solution was stirred for 14 h at room temperature. The 1,4-dioxane was removed under reduced pressure, and the 1H NMR spectrum of the crude product was acquired in CDCl3.

**NMR Spectroscopy of Peptides.** Experiments were conducted in 90% H2O/10% D2O containing 5 mM phosphate buffer pH 7.4, 25 mM NaCl, and 100 μM TSP as a reference for 1H NMR spectra. Residual trifluoroacetate was used as an internal reference for 19F NMR spectra.13F NMR experiments were conducted without decoupling using a 1.5 kHz probe. NMR experiments in Figure 3 were conducted at 0.5 °C.55 The temperatures for each experiment were chosen to allow direct comparison to the analogous peptides with proline.

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**ASSOCIATED CONTENT**

*Supporting Information* 1H, 13C, and 19F NMR spectra for all new compounds; CD spectra for compounds 4, 5, 7, 8, 9, and 11; and full 1H NMR spectra, 19F NMR spectra, and TOCSY spectra of peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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