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Synthesis of Perfluoro-*tert*-butyl Tyrosine, for Application in ^{19}F NMR, via a Diazonium-Coupling Reaction

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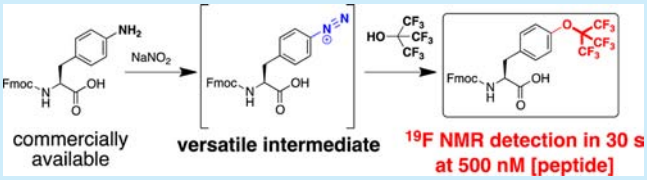
Supporting Information

ABSTRACT: A practical synthesis of the novel highly fluorinated amino acid Fmoc-perfluoro-*tert*-butyl tyrosine was developed. The sequence proceeds in two steps from commercially available Fmoc-4-NH_2-phenylalanine via diazotization followed by diazonium coupling reaction with perfluoro-*tert*-butanol. In peptides, perfluoro-*tert*-butyl tyrosine was detected in 30 s by NMR spectroscopy at 500 nM peptide concentration due to nine chemically equivalent fluorines that are a sharp singlet by ^{19}F NMR. Perfluoro-*tert*-butyl ether has an estimated σ_p Hammett substituent constant of +0.30.



Sensitivity in NMR spectroscopy is limited by the concentration of the analyte (typically requiring mid-micromolar concentration for rapid analysis) and by challenges in resolution due to spectral overlap (due to competing signals obscuring the signals of interest). These problems are particularly acute for the study of proteins and for investigations in complex solutions. The problem of spectral overlap in proteins can be partially overcome via site-specific isotopic labeling, such as by using ^{15}N-, ^{13}C-, and/or ^{2}H-labeled amino acids and selective pulse sequences to spectroscopically isolate these amino acids. These approaches have allowed the investigation of protein function in media as complex as living *E. coli* and human cells and *Xenopus* oocytes, albeit at a substantial cost in sample preparation, experimental complexity, and instrument time.[1]

Alternatively, the introduction of NMR-active nuclei that are not typically present in biological systems provides an approach that results in NMR signals that are only associated with the molecules of interest, eliminating the challenges associated with background signals and spectral assignment. ^{19}F has been the most widely used non-native NMR-active nucleus applied for interpretation of biological structure and function.[2] Fluorinated molecules have been used for analysis of events as diverse as protein folding in cells, screening of pharmaceutical inhibitors of protein function, and MRI.[3] ^{19}F has 100% natural abundance and sensitivity similar to that of ^{1}H. Moreover, due to the absence of background signals, including from water, experiments can, compared to ^{1}H NMR, be typically conducted with increased gain and simpler pulse sequences, further increasing sensitivity. However, despite these substantial advantages, the inherent sensitivity limits of NMR spectroscopy result in an inability to probe many processes at physiologically relevant concentrations.

Increasing the concentration of *equivalent* signals in a molecule can substantially increase sensitivity and decrease the concentrations accessible for analysis. Thus, highly fluorinated molecules employing multiple chemically equivalent fluorines have found applications of small molecules, polymers, and proteins in NMR spectroscopy and in MRI.[3d,4]

We recently described the synthesis of two conformationally biased amino acids containing perfluoro-*tert*-butyl groups, 4*R*- and 4*S*-perfluoro-*tert*-butyl hydroxyproline.[3c,5] These amino acids were synthesized either in the solution phase or within peptides on the solid phase via Mitsunobu reaction of perfluoro-*tert*-butyl alcohol with the diastereomeric hydroxyprolines. Peptides containing perfluoro-*tert*-butyl hydroxyprolines have distinct conformational preferences and, owing to the presence of nine chemically equivalent fluorines, can be rapidly detected at high nanomolar concentrations (5 min at 200 nM peptide concentration).

Given this high sensitivity achievable by ^{19}F NMR, we sought to extend the applications of perfluoro-*tert*-butyl groups to applications with aromatic amino acids. In particular, we were inspired by recent work showing that *tert*-butyl tyrosine (Tby) (Figure 1) can be incorporated in expressed proteins via amber codon suppression using an evolved aminoacyl tRNA synthetase or pyrrolysyl tRNA synthetase.[4g,6] Tby was observed by ^{1}H NMR in expressed proteins as a sharp singlet due to nine equivalent hydrogens.[7] Moreover, this sharp singlet is present even when this amino acid is in proteins as large as 320 kDa: multiple bond rotations of the side chain result in isotropic

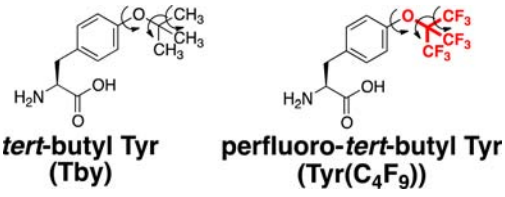


Figure 1. *tert*-Butyl tyrosine (Tby) and its fluorinated analogue. Arrows indicate bond rotations that result in isotropic averaging of the NMR-active ^{1}H nuclei in Tby, even in large proteins.

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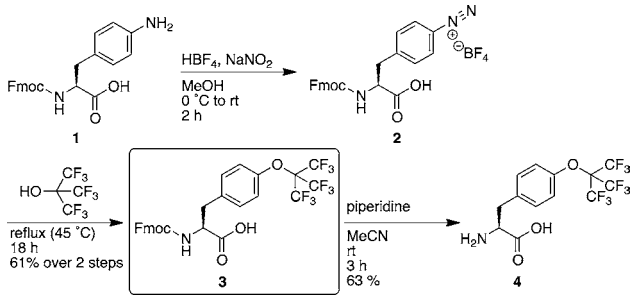
averaging despite the slow rotational correlation times that typically prevent standard NMR spectroscopy of proteins over 30 kDa. Similarly, isotropic averaging of trifluoromethylphenylalanine allowed the detection of a 98 kDa protein in living *E. coli* cells.[3d] These data suggest significant advantages of tyrosine-*tert*-butyl ethers in the NMR spectroscopy of large proteins. Therefore, we sought to develop an approach to the synthesis of the amino acid perfluoro-*tert*-butyl tyrosine, which could combine high signal sensitivity (nine equivalent fluorines) with the potential for future incorporation in expressed proteins and detection in high molecular weight complexes due to isotropic signal averaging.

There is only one previous synthesis of a molecule with a perfluoro-*tert*-butyl aryl ether, which was prepared via a diazonium coupling reaction with perfluoro-*tert*-butanol.[8] Therefore, we examined the synthesis of Fmoc-perfluoro-*tert*-butyl tyrosine using the commercially available amino acid Fmoc-4-NH_2-phenylalanine (**1**) as the starting material (Scheme 1).

Scheme 1. Synthesis of Perfluoro-*tert*-butyl tyrosine

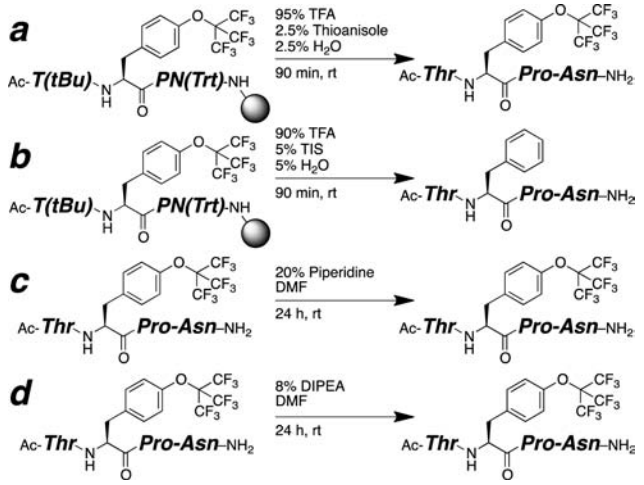


Diazotization of **1** using sodium nitrite in tetrafluoroboric acid generated the intermediate diazonium **2**, which has not previously been described and which was used in the subsequent step without purification. Diazonium coupling was achieved via heating **2** in perfluoro-*tert*-butanol at reflux, generating Fmoc-perfluoro-*tert*-butyl tyrosine **3** in good yield in two steps from commercially available starting material, with only a single purification step. This highly practical synthesis, which can be completed in 24 h, should encourage broad application of this amino acid. In addition, removal of the Fmoc group generated the free amino acid, for potential applications in protein expression and protein engineering. **Warning**: while tetrafluoroborate salts of diazoniums exhibit greater stability than other diazonium salts, these reactions inherently generate nitrogen gas, yielding a potential hazard from explosion. Reactions were conducted in glassware open to air, and the diazonium salt was used immediately after generation, without storage of this intermediate.

Diazonium coupling is a versatile approach to the modification of aromatic rings, via direct coupling or via metal-mediated reactions, including Sandmeyer-type reactions and palladium-catalyzed (Heck, Suzuki, Stille, carbonylation) reactions.[9] Diazotization of the free amino acid 4-amino-phenylalanine (synthesized from 4-nitro-phenylalanine) has previously been applied to the synthesis of 4-chloro-, 4-azido-, 4-tetrazole-, 4-thiol-, and 4-selenol-phenylalanine derivatives, as well as phenylalanine derivatives containing nitrogen and sulfur mustards.[10] Diazonium salts may also be converted to azo compounds or may be directly reduced to hydrazines. The facile generation of the diazonium of Fmoc-4-NH_2-phenylalanine suggests that this approach may be highly practical and broadly applicable to the synthesis of diverse and functionally useful 4-substituted phenylalanines for direct application in Fmoc solid-phase peptide synthesis.

To examine the structural and electronic effects of the perfluoro-*tert*-butyl aryl ether, Fmoc-perfluoro-*tert*-butyl tyrosine **3** was incorporated via solid-phase peptide synthesis in the model peptide context Ac-TXPN-NH_2, where X = an aromatic amino acid.[11] These peptides exhibit *cis*−*trans* isomerism about the aromatic−proline amide bond due to a proline/aromatic C−H/π interaction, with the extent of *cis* amide bond (greater *cis* amide bond = reduced $K_{trans/cis}$) correlating with the electronics of the aromatic π face. This peptide was synthesized via standard solid-phase peptide synthesis, with optimization in yield achieved via PEG-polystyrene graft resin, 24 h amide coupling with **3**, and HATU as a coupling reagent. Subjection of the peptide to TFA cleavage/deprotection conditions using thioanisole and water as scavengers cleanly generated the peptide Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2 (Scheme 2a). In contrast, the use of triisopropylsilane (TIS)

Scheme 2. Stability of Tyr(C_4F_9) in a Peptide to Standard Conditions of (a, b) TFA Cleavage/Deprotection, (c) Fmoc Deprotection, and (d) Amide Coupling



as a scavenger resulted in product decomposition, generating the peptide with phenylalanine (elimination of the perfluoro-*tert*-butyl alcohol, Scheme 2b), presumably via an SNAr mechanism with the silyl hydride under acidic conditions. These results were reproduced using the purified peptide Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2, indicating the compatibility of perfluoro-*tert*-butyl tyrosine with TFA cleavage/deprotection conditions as long as hydride sources are avoided. In addition, the purified peptide was exposed to both 20% piperidine in DMF and 8% DIPEA in DMF for 24 h (Scheme 2cd). No significant decomposition was observed under either condition, confirming the compatibility of perfluoro-*tert*-butyl tyrosine with extended exposure to reagents employed in peptide synthesis.[12]

An aryl perfluoro-*tert*-butyl ether has only been described once previously,[8] and thus, no data exist on the aromatic electronic effects of a perfluoro-*tert*-butyl ether. Peptides with 4-substituted phenylalanines in the Ac-TXPN-NH_2 context (X = 4-Z-Phe) exhibit a Hammett correlation between log $K_{trans/cis}$ and the Hammett σ constant of the 4-substituent (ρ = 0.295 ± 0.017 across 13 neutral 4-substituents).[11c,e] Therefore, the $K_{trans/cis}$ of Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2 could be employed to provide an estimate of σ for the $-OC_4F_9$ group. The $K_{trans/cis}$ = 4.1 measured for this peptide (Figure 2) correlates to a σ of approximately

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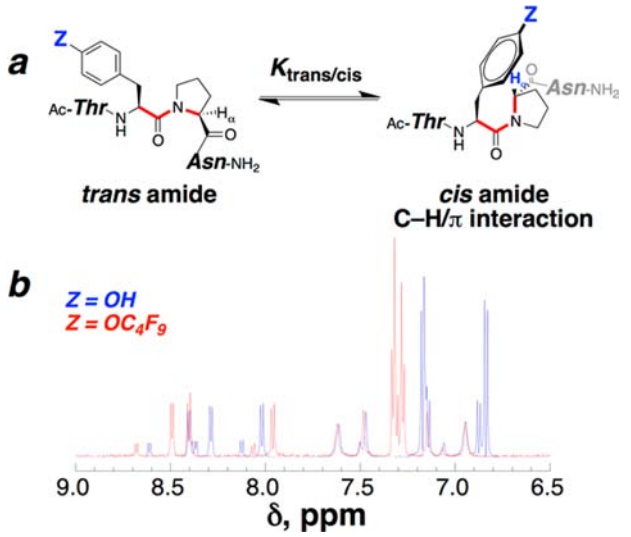


Figure 2. (a) *cis*–*trans* isomerism of the aromatic–proline amide bond (red) in Ac-TXPN-NH_2 peptides (X = aromatic amino acid) can be used to quantify aromatic electronic effects via $K_{trans/cis}$ and via the δ of proline H_α when in the *cis* conformation (with smaller $K_{trans/cis}$ and more upfield Pro_{cis} H_α δ indicating a more electron-rich aromatic and a stronger C–H/π interaction). (b) ^{1}H NMR spectra (amide-aromatic region, 90% H_2O/10% D_2O, 5 mM phosphate pH 4, 25 mM NaCl) of peptides with X = Tyr (Z = OH) (blue) and X = Tyr(C_4F_9) (Z = OC_4F_9) (red). Pro H_α for X = Tyr, δ_{trans} = 4.42 ppm, δ_{cis} = 3.83 ppm; Pro H_α for X = Tyr(C_4F_9), δ_{trans} = 4.40 ppm, δ_{cis} = 4.00 ppm.

+0.30, indicating a modestly electron-withdrawing effect for this group, despite nine fluorines. These results are consistent with other fluorinated ethers, which are electron-withdrawing in contrast to the analogous hydrocarbon ethers, but less electron-withdrawing than the equivalent fluorocarbons (Table 1).[13]

Table 1. Hammett σ_p Substituent Constants for Alkyl and Alkyl Ether Groups[a]

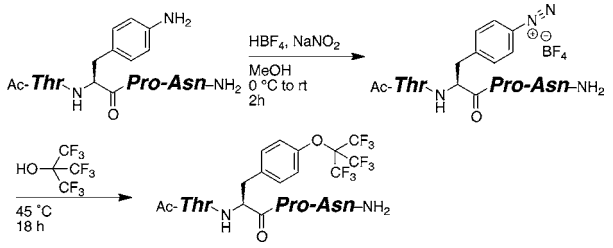
substituent	σ_p	substituent	σ_p
$-CH_3$	−0.07	$-OCH_3$	−0.27
$-CHF_2$	+0.32	$-OCHF_2$	+0.31
$-CF_3$	+0.54	$-OCF_3$	+0.35
$-CF_2CF_3$	+0.52	$-OCF_2CF_3$	+0.28
$-C(CH_3)_3$	−0.20	$-OC(CH_3)_3$	−0.29
$-C(CF_3)_3$	+0.55	$-OC(CF_3)_3$	+0.30[b]

[a]Values from ref 13, except as indicated. [b]Estimated from results herein.

Thus, the electronic properties of the perfluoro-*tert*-butyl aryl ether are affected by a balance of the electron-donating character of the ether and the electron-withdrawing character of the nine fluorines, whose effect is reduced because they are located four atoms away from the aromatic ring.

We also examined the solution-phase synthesis of perfluoro-*tert*-butyl tyrosine within peptides containing 4-NH_2-phenylalanine (Scheme 3). Diazotization of Ac-T***Phe(4-NH$_2$)***PN-NH_2 followed by heating with perfluoro-*tert*-butanol generated the peptide Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2, which was identical by NMR to the peptide synthesized using **3**. While this approach will not be compatible with all amino acids (e.g., potential side reactions with Tyr, Cys, Met),[14] it provides an alternative approach to the

Scheme 3. Solution-Phase Diazotization and Synthesis of Tyr(C_4F_9) within Peptides



synthesis of peptides containing Tyr(C_4F_9) and is also suggestive of the general synthesis of peptides containing diverse unnatural substituted phenylalanines via diazonium-coupling reactions.

In order to examine potential magnetic resonance applications of perfluoro-*tert*-butyl tyrosine, Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2 was examined by ^{19}F NMR spectroscopy. Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2 exhibited a sharp singlet peak (δ = −69.1 ppm) in water at 298 K (Figure 3a). Interestingly, the ^{19}F δ of the *cis* and *trans* rotamers

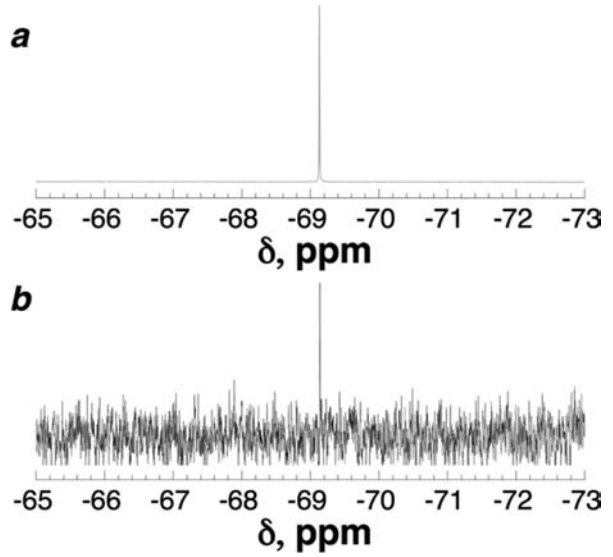


Figure 3. ^{19}F NMR spectra of the peptide Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2 in 90% H_2O/10% D_2O, 5 mM phosphate pH 4, 25 mM NaCl. (a) As in Figure 2b. (b) Peptide concentration = 500 nM, signal/noise = 10.1. This experiment was conducted with eight scans, two dummy scans, acquisition time = 0.8 s, relaxation delay = 2.0 s (28 s total experiment time).

were identical, consistent with the isolation of the fluorines from the backbone conformation of the peptide. To determine the sensitivity of perfluoro-*tert*-butyl tyrosine, the ^{19}F NMR spectrum of this peptide was examined at 500 nM and 200 nM peptide concentrations. Perfluoro-*tert*-butyl tyrosine was detected in 30 s (8 scans) at 500 nM [Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2] and in 5 min (128 scans) at 200 nM [Ac-T***Tyr(C$_4$F$_9$)***PN-NH_2] (Figure 3b, Figures S10–S12), suggesting broad potential applications of this amino acid to sensitively probe peptide and protein structure and function.

We have described the highly practical synthesis of Fmoc-perfluoro-*tert*-butyl tyrosine in two steps from the commercially available amino acid Fmoc-4-NH_2-phenylalanine. The synthesis proceeds via a diazonium coupling reaction. Ready access to this Fmoc-protected phenylalanine diazonium intermediate could provide highly practical access to a wide range of unnatural

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phenylalanine derivatives for applications in peptide synthesis. Perfluoro-*tert*-butyl tyrosine was detected at 500 nM peptide concentration in 30 s by ^{19}F NMR, promoting applications of this amino acid in the detection of protein function and protein interactions at physiologically relevant concentrations. Combined with the previously demonstrated ability of *tert*-butyl tyrosine to be encoded within proteins and to be a useful NMR probe even in extremely large proteins beyond the typical size limit of NMR spectroscopy; these results suggest broad potential future applications of perfluoro-*tert*-butyl tyrosine in NMR spectroscopy and MRI imaging.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02858.

Synthetic procedures, ^{1}H, ^{13}C, and ^{19}F NMR spectra for small molecules, synthesis and characterization data for peptides, and additional NMR data for peptides (PDF)

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Notes

The authors declare no competing financial interest.

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