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Synthesis of Tetrafluorinated Aromatic Amino Acids with Distinct Signatures in ^{19}F NMR

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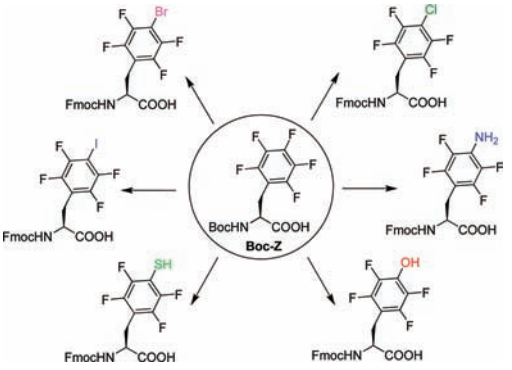
ABSTRACT



Fluorinated amino acids serve as powerful tools in protein chemistry. We synthesized a series of *para*-substituted tetrafluorophenylalanines via the regioselective S_{NAr} chemistry of the commercially available pentafluorophenylalanine Boc-Z. These novel unnatural amino acids display distinct ^{19}F NMR signatures, making them powerful tools for analyzing protein−membrane interactions with NMR spectroscopy.

Fluorination has become an increasingly popular strategy in medicinal chemistry and protein engineering due to the unique properties of fluorinated compounds.[1] For example, incorporating fluorinated amino acids into synthetic proteins often leads to improved stability owing to the added hydrophobicity by fluorination.[2] In addition, fluorinated amino acids have shown promise as supramolecular synthons to program protein assembly in both aqueous and membrane environments.[3] Another attractive feature of fluorinated compounds lies in their tractability by ^{19}F NMR, which is essentially blind to endogenous material in biological systems. Furthermore, the chemical shift of ^{19}F resonances is highly sensitive to the local environment; therefore, fluorinated amino acids serve as powerful tools for interrogating protein folding and protein−membrane interactions.[4] Ideally, multisite incorporation of fluorinated residues should allow one to monitor the behavior of a target protein with atomic resolution across the protein sequence. However, the assignment of individual ^{19}F resonances is often challenging[5]

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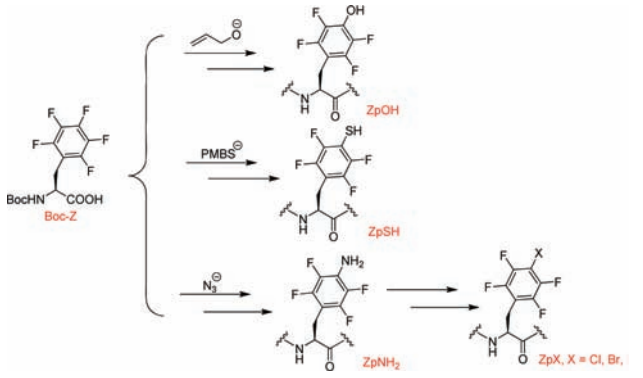
and would greatly benefit from amino acids with characteristic ^{19}F NMR signatures.

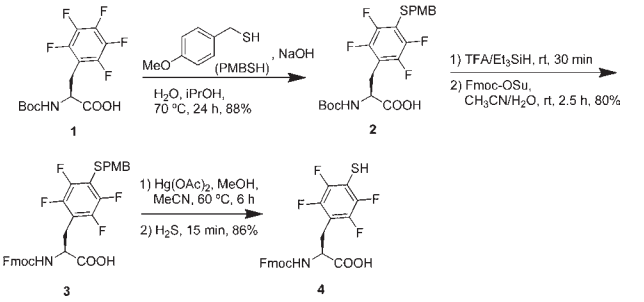
There are a limited number of fluorinated amino acids available from commercial sources as their synthesis is nontrivial. We recently reported a facile synthesis[6] of tetrafluorotyrosine via the regioselective S_{NAr} chemistry of pentafluorophenylalanine (named as Z for brevity). By using the starting material with the desired stereochemistry, the D- or L-isomer of tetrafluorotyrosine can be readily synthesized in gram quantities. Herein we show the S_{NAr} strategy can be extended to a series of *para*-X-tetrafluorophenylalanines (Scheme 1), where X represents a range of functionalities, including thiol, amine, and halogens. For ease of discussion, we name such a fluorinated amino acid as ZpX with X specifying the *para* substituent (e.g., ZpOH stands for tetrafluorotyrosine). Although structurally similar, these unnatural amino acids display easily distinguishable ^{19}F resonances, poised for multisite labeling and analysis of proteins. All the amino acids described in this report are in the L-configuration; however, the synthetic protocols should enable the synthesis of the D-isomers as well.

Scheme 1. Scope of the S_{NAr} Reaction of Boc-Z



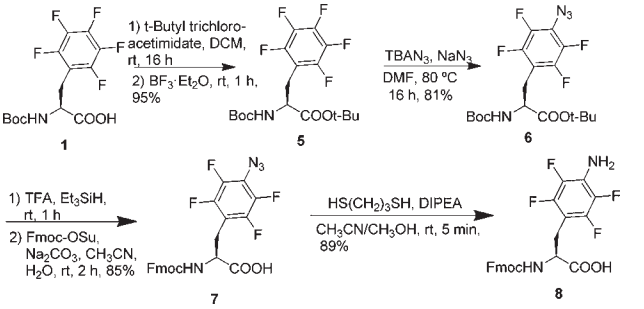
Similar to ZpOH, use of a sulfur nucleophile in the S_{NAr} reaction afforded the *para*-mercapto analogue ZpSH. Initially we treated Boc-Z with NaSH, which predominantly gave a thioether with two Boc-Z molecules cross-linked as a result of sequential S_{NAr} reactions. To prevent dimerization, we chose 4-methoxybenzenemethanethiol (PMBSH, Scheme 2) as an alternative, which reacted readily with Boc-Z to give Boc-ZpSPMB (**2**) in good yield. Protecting group conversion yielded Fmoc-ZpSPMB (**3**), which is compatible with solid phase peptide synthesis (SPPS) via Fmoc/tBu chemistry.[7] No epimerization was observed by Marfey's test (ref 8 and also see Supporting Information (SI)). The PMB protecting group was efficiently removed to give Fmoc-ZpSH (**4**) by using $Hg(OAc)_2$ under mild conditions. A similar protocol was previously used for removing the PMB group from peptides in solution.[9]

Scheme 2. Synthesis of ZpSH



The S_{NAr} chemistry of Boc-Z also applies to nitrogen nucleophiles. The *para*-amino analogue $ZpNH_2$ was synthesized via reduction of the azide precursor ZpN_3, for which a chemoenzymatic synthesis was previously reported by Ghadiri and co-workers.[10] In their work, a racemic mixture of ZpN_3 was made through the S_{NAr} chemistry and the L-isomer was obtained through kinetic resolution with *Aspergillus melleus* acylase-I. Our synthesis started with the pure L-enantiomer of Boc-Z (Scheme 3). We first protected the carboxyl group as a *t*Bu ester and then treated the product with Bu_4NN_3/NaN_3 in DMF. The S_{NAr} reaction proceeded smoothly without causing epimerization of the amino acid (see SI for details). Deprotection of the carboxyl group and conversion of Boc to Fmoc were accomplished through a one-pot process. The three-step synthesis gave Fmoc-ZpN_3 (**7**) with an overall yield of 65%. Reduction of Fmoc-ZpN_3 was best accomplished by using propylene dithiol[11] under mild conditions to afford Fmoc-$ZpNH_2$ (**8**) in excellent

Scheme 3. Synthesis of $ZpN_3/ZpNH_2$



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yield. Propylene dithiol treatment effectively converted ZpN_3 in a peptide into $ZpNH_2$ as well (see SI for details); in other words, ZpN_3 conveniently serves as a precursor for the synthesis of peptides containing $ZpNH_2$.

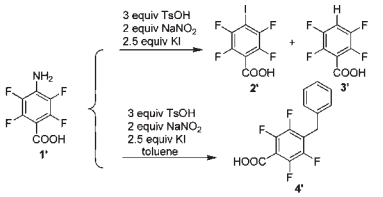
We hypothesized that $ZpNH_2$ could be converted into a variety of fluorinated phenylalanine analogues through the diazonium chemistry.[12] Here we describe the synthetic protocols for the *para*-halogenated (Cl, Br, I) tetrafluorophenylalanines (Scheme 4). Although widely used to access halogenated aromatics,[13] the diazonium reaction is less straightforward for heavily fluorinated anilines, as these electron-deficient anilines are less nucleophilic and more difficult to generate the diazonium species. Indeed, treating the model compound **1′** (*para*-carboxyltetrafluoroaniline) with the canonical conditions of diazonium iodination ($TsOH \cdot H_2O/NaNO_2/KI$) only afforded less than 60% conversion of the starting material, indicating inefficient formation of the diazonium species. In addition to the desired product **2′**, the reaction yielded a major byproduct, the *para*-hydrogenated compound **3′**. This byproduct is generated possibly because the diazonium intermediate and the subsequent phenyl radical are highly reactive and extract a hydrogen atom from the solvent molecules. This hypothesis is supported by the solvent dependence of byproduct formation: THF gives exclusively the hydrogenated byproduct, while CH_3CN favors the desired iodo product (Table 1), affording a mixture of **2′** and **3′** in a ratio of 3:1. Interestingly compound **4′** (with a *para*-benzyl substituent) is obtained as the sole product when toluene is used as a cosolvent. This is consistent with the radical mechanism of the diazonium chemistry, which leads to cross-coupling between the fluorinated phenyl radical and toluene. This observation also led us to suspect that the benzylic position of TsOH might participate in the radical chemistry and therefore should be avoided in the diazonium chemistry. The alternative conditions (tBuONO/I_2)[14] reduced the byproduct formation to ~5%. Fortuitously, these conditions also improved the diazonium formation to give complete conversion of the starting material.

Optimized conditions for the model reaction were investigated for the synthesis of the halogenated tetrafluorophenylanines. We initially subjected the Fmoc protected $ZpNH_2$ (**8**) to the iodination conditions, which generated a messy mixture. NMR analysis suggested possible degradation of the Fmoc group, presumably because the benzylic substructure of Fmoc was attacked by the radical species. Alternatively, we used the trifluoroacetyl protected $ZpNH_2$ (Compound **10**, Scheme 4) in the diazonium chemistry, which afforded ZpI (**11c**) in good yield. Similar to the model reaction, the byproduct ZpH was found to be ~5%.

Use of CuX_2/CuX (X: Br or Cl) in the diazonium chemistry gave ZpBr and ZpCl respectively in good yields. In comparison to iodination, the byproduct (ZpH) percentage was found to be similar for bromination and slightly higher for chlorination (~12%), consistent with the relatively low reactivity of chlorine in radical chemistry. The protecting group conversion was accomplished through a one-pot process to give Fmoc-ZpXs (**12a**–**c**), the form ready for peptide synthesis (see SI). The enantiopurity of these amino acids was confirmed by Marfey's test.

In order to utilize these novel amino acids as protein probes, we characterized their signatures in ^{19}F NMR. Despite their structural similarity, the tetrafluorinated phenylalanine analogues display a wide distribution of their ^{19}F resonances, ranging from −120 to −165 ppm (Table S1, SI). The electronegative substituents (−F, −OH, $-NH_2$) on the *para* position elicit significant upfield shift of the *meta* fluorine resonances, which appear around −163 ppm. In contrast, the bromo and

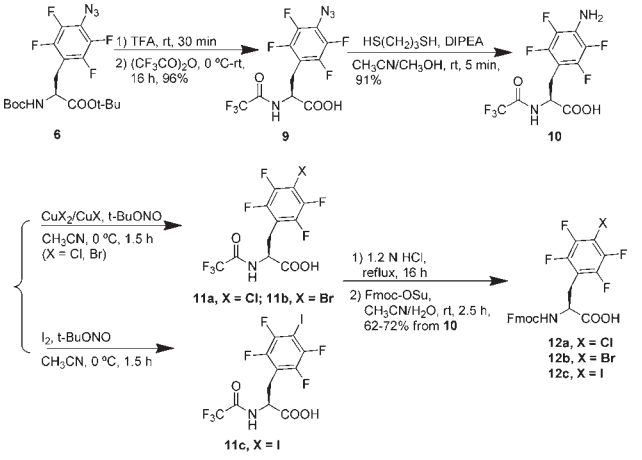
Table 1. Optimization of Diazonium Iodination Conditions with the Model Compound **1′**



solvent	2': 3' ratio	solvent	2': 3' ratio
H_2O	-[b]	DMSO	70:30
THF	0:100	CH_3CN	75:25
DMAc	25:75	CH_3CN[a]	95:5
DMF	30:70		

[a] Optimized conditions: 0.05 M in CH_3CN, 2 equiv of I_2, and 1.5 equiv of ^{t}BuONO. [b] Neither **2′** nor **3′** was generated.

Scheme 4. Synthesis of ZpX (X = Cl, Br, I)



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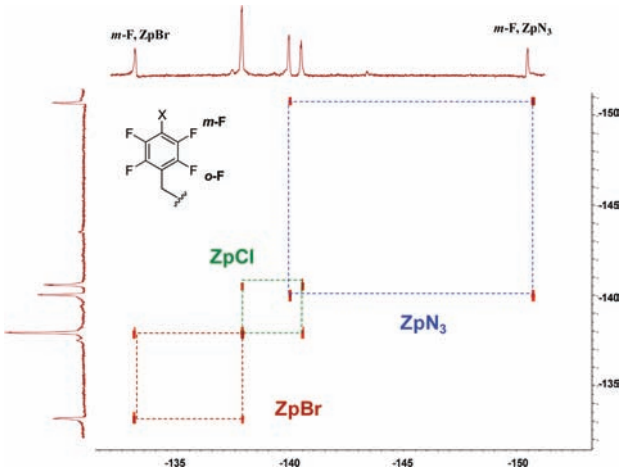

Figure 1. $^{19}F-^{19}F$ COSY spectrum of a magainin mutant incorporating three ZpX (ZpBr, ZpCl, ZpN3) residues. The highly dispersed ^{19}F resonances enabled facile assignment of all peaks in ^{19}F NMR.

iodo substituents on the *para* position cause a downfield shift of the *meta* fluorines, with ZpBr and ZpI displaying a resonance around −133 and −120 ppm respectively. These characteristic chemical shifts should allow facile assignment of ^{19}F resonances for peptides that incorporate multiple fluorinated residues. As a proof-of-concept experiment, we have incorporated three ZpX residues (ZpCl, ZpBr, and ZpN_3) into magainin 2, a well-known membrane-lytic peptide.[15] Due to their distinct chemical shifts, the *meta*-fluorine resonances of ZpBr and ZpN_3 were readily identified, which in conjugation with the $^{19}F-^{19}F$ COSY[16] data enabled unambiguous assignment of all ^{19}F peaks (Figure 1).

We further examined the environmental sensitivity of ^{19}F chemical shifts by using chloroform and methanol as solvents respectively. Consistent with previous reports,[5] the ^{19}F resonances are highly sensitive to environment: a polar solvent like methanol gives resonances upfield shifted by as much as 3 ppm in comparison to chloroform. On the other hand, the ionizable residues (ZpOH and ZpSH) show dramatic chemical shift changes in response to the environment pH (Table S1), presumably as a result of protonation/deprotonation of their side chains.

In summary, we have synthesized a series of novel fluoroaromatic amino acids that can be readily utilized in chemical synthesis of peptides and proteins. Our synthetic protocol builds on the regioselective S_{NAr} reaction of the pentafluorophenylalanine and the diazonium chemistry of $ZpNH_2$. The new amino acids exhibit distinct ^{19}F NMR signatures, which allow unambiguous assignment, and therefore enable multisite labeling of proteins for NMR characterization. Furthermore, these fluoroaromatic residues effectively sense the local environment by a significant shift of their ^{19}F resonances. Given the high abundance of aromatic residues at the protein−membrane interface,[17] we submit that the fluorinated aromatic residues described here are particularly useful for the study of protein−membrane interactions. Work along this direction is currently underway.

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Supporting Information Available. Experimental procedures and characterization of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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