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Synthesis of Photoactive *p*-Azidotetrafluorophenylalanine Containing Peptide by Solid-Phase Fmoc Methodology

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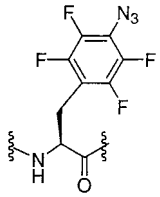
ABSTRACT



***N*-Fmoc-L-*p*-azidotetrafluorophenylalanine was prepared from achiral starting materials using an acetamidomalonate synthesis and enzymatic resolution. A photoactive peptide containing this fluorinated residue could be assembled using solid-phase Fmoc chemistry.**

The technique of photoaffinity labeling,[1] in which a reactive photogenerated intermediate cross-links to a protein, has been popular as a means for identification of ligand binding sites, regions of protein−protein interaction, and for affinity purification of receptors. Phenyl azides have been extensively employed as photoaffinity reagents, despite the disadvantage that on photolysis rapid ring expansion occurs to afford a comparatively long-lived ketenimine azepine intermediate[2] that will preferentially react with nucleophiles and consequentially may diffuse until it encounters a suitably reactive residue or solvent.

Fluorinated aryl azides have been proposed as new photoaffinity reagents[3] which offer the advantage of suppressing the ring expansion of the initially liberated nitrene intermediate, which in a hydrophobic environment may subsequently undergo insertion reactions even with unactivated CH bonds.[4] This offers the possibility for labeling of arbitrary residues at a binding site, and minimizes the opportunity for diffusion of the reactive intermediate. A number of reagents for convenient derivatization of proteins or their ligands,[5] and nucleic acids[6] with fluoroaryl azides have been described. However, in contrast to *p*-azidophe-

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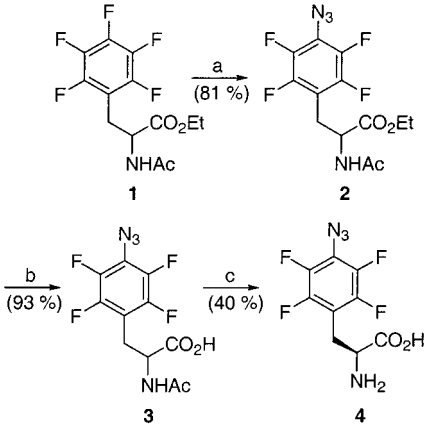
nylalanine (Pap), *p*-azidotetrafluorophenylalanine **4** (Fpap) had only been reported in racemic form[5e] and its incorporation into peptide affinity labels appears to be unexplored. To circumvent the shortcomings associated with the photochemistry of Pap and to take advantage of the preferential reactivity of fluorinated aryl azides we sought to develop a means for incorporation of **4** into peptides using conventional solid-phase synthesis. A diethylacetamidomalonate synthesis[7]

Scheme 1[a]

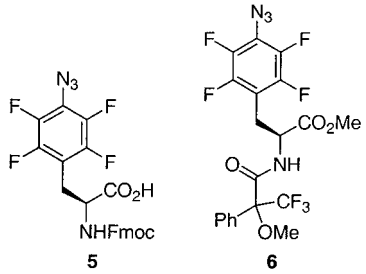


[a] Conditions: (a) NaN_3, 0.1 equiv of Bu_4NN_3, DMF, 80 °C; (b) NaOH, H_2O, MeOH; (c) Acylase-I, H_2O.

was used for preparation of protected racemic pentafluorophenylalanine derivative **1** with a view to introducing the azide group by nucleophilic substitution. Although fluorinated aromatics are activated toward nucleophilic attack, it was observed that **1** required comparatively forcing conditions to bring the reaction to completion. Treatment of **1** with NaN_3 in refluxing acetone for 7 h resulted in no reaction. Treatment under literature conditions[5e] of NaN_3 in DMF at 55 °C for 20 h afforded mostly starting materials, as judged by ^{19}F NMR. By increasing the temperature to 80 °C and addition of catalytic Bu_4NN_3 the reaction could be driven essentially to completion,[8] without excessive accumulation of impurities arising from thermolysis of the product **2**. Reaction of commercially available FmocPhe(F_5) under similar conditions resulted in significant loss of the Fmoc group, whereas unprotected Phe(F_5) afforded an intractable mixture of penta- and tetrafluoro species.

As attempts at removal of the ethyl ester group of **2** using either α-chymotrypsin in water or DMF/water mixture,[9] or subtilisin in buffered DMSO/water were not fruitful,[10] we opted for a nonenzymatic basic hydrolysis of this group to acid **3**, followed by kinetic resolution via proteolytic cleavage of the acetamide. Compound **3** was unreactive to carboxypeptidase A catalyzed hydrolysis,[11] however *Aspergillus melleus* acylase-I treatment[12] while maintaining the pH over the range 7−8 by titration afforded L-*p*-azidotetrafluorophenylalanine **4** as a white precipitate with good ee (>95%, vide infra). Conversion to the Fmoc derivative **5** was effected by reaction with Fmoc-OSu.[13]

The enantiomeric purity of **4** was assessed by comparison of the ^1H NMR spectra of the Mosher amide derivatives, **6**, of the methyl ester of **4** with *R*-(+) and *S*-(−) α-methoxy-α-trifluoromethylphenylacetic acid (MTPA).[14] Both diastereomers of **6** gave distinct NMR spectra, but were indistinguishable by TLC. The chemical shifts of the methyl ether protons of the *R*-(+) and *S*-(−) MTPA derivatives of **4** were 3.34 and 3.49 ppm, respectively, consistent with the assignment of an L stereochemistry at the α-carbon, as would be predicted for this enzymatic resolution.[15] Contamination of **4** with the isomer with a D configuration at the α-carbon was not detectable in the NMR spectra of these derivatives.



To test the ease with which *p*-azidotetrafluorophenylalanine could be incorporated, via **5**, into peptides using conventional solid-phase methodology,[16] we prepared a 33 residue peptide, **7**, based on the GCN4 leucine zipper sequence where this residue was substituted at position 16. This sequence was chosen as a representative peptide to test the stability of the fluoroaryl azide group to a number of coupling/Fmoc deprotection cycles, and to the TFA deprotection byproducts of a range of side chain protecting groups commonly employed in Fmoc chemistry. Automated synthesis was performed using diisopropylcarbodiimide/hydroxybenzotriazole for couplings and 50% piperidine in

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N-methylpyrrolidone for Fmoc deprotection. Although there was a concern that scavengers used in the final TFA cleavage and deprotection of the peptide would reduce the azide group, in practice it was found that this was not a significant problem using a cleavage mixture with minimal quantities of thiol and avoiding the use of silanes.

AcRVKQLEDKVEELLSK-Fpap-YHLENEVARLKKLVGER-NH_2

7

Peptide **7** was analyzed by sonic spray ionization mass spectrometry,[17] a mild ionization technique similar to electrospray, which revealed an ion at *m*/*z* 1384.02, in good agreement with the calculated value of 1384.08 for $[\mathbf{7} + 3H]^{3+}$. In contrast, both **7** and a photoproduct arising from laser induced loss of N_2 were observed by MALDI TOF spectrometry. Peptide **7** displayed the characteristic UV absorption of the fluoroaryl azide group at 253 nm, and an IR absorption at 2126 cm^{-1} corresponding to the N_3 stretch. On exposure to 254-nm light from a handheld lamp, **7** was photolyzed in minutes, as monitored by the UV absorbance. Despite photolability, a CD spectrum of **7** could be measured without extensive photolysis, as judged by HPLC. Repeated acquisition of UV spectra of **7** in phosphate buffer also resulted in negligible photolysis. However, inclusion of 1 mM dithiothreitol (DTT) in a pH 8.0 solution of 10 μM **7** led to approximately 50% reduction of the azide moiety over 10 min as judged from the decreasing UV absorbance. At pH 7.0 the reaction proceeded at a lower rate, leading to approximately 20% depletion of the azide over the same time period, highlighting the need to avoid reducing buffers to maintain the integrity of the azide moiety.

In summary, we have developed a synthesis of Fmoc-L-*p*-azidotetrafluorophenylalanine and demonstrated its incorporation into a peptide using automated Fmoc synthesis methodology. The azide group is stable to the conditions required for peptide synthesis and deprotection, although it was rapidly reduced by dithiothreitol in aqueous buffer.

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Supporting Information Available: Experimental procedures and characterization for compounds **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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