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Radiosynthesis and bioconjugation of [^{18}F]FPy5yne, a prosthetic group for the ^{18}F labeling of bioactive peptides

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A new ^{18}F-based prosthetic group has been prepared for the labeling of azide-modified peptides for use in PET imaging. 2-[^{18}F]fluoro-3-(hex-5-ynyloxy)pyridine ([^{18}F]FPy5yne, [^{18}F]-1) was prepared via efficient nucleophilic heteroaromatic substitution of either the corresponding 2-nitro (2) or 2-trimethylammonium trifluoromethanesulfonate pyridine (3). Best radiochemical yield of [^{18}F]FPy5yne from 2 was 91% by radioTLC (15 min, 110°C, DMSO). From 3, best radiochemical yield by radioTLC was 93% (15 min, 110°C, MeCN). HPLC-purified [^{18}F]FPy5yne was ligated to model peptide N_3–$(CH_2)_4$–CO–YK-RI–OH by way of Cu^{I}-mediated Huisgen [3+2] cycloaddition in the presence of copper-stabilizing ligand tris(benzyltriazolylmethyl)amine (TBTA) and *N,N*-diisopropylethylamine (DIEA). Bioconjugate radiochemical yields were obtained in average yields of 89% ± 8.6% ($n = 4$), as judged by radioHPLC. Best non-decay-corrected, collected radiochemical yield of modified peptide from end-of-bombardment was 5.8% (18.7% decay-corrected), with a total preparation time of 160 min from start of synthesis.

Keywords: fluorine-18; peptide labeling; click; heteroaromatic substitution

Introduction

The growing popularity of the Huisgen 1,3-dipolar cycloaddition reaction[1] in bioconjugate chemistry owes much to the discovery that Cu^{I} accelerates the rate of this reaction and results in a diasterospecific 1,4-disubstituted 1,2,3-triazole linkage.[2,3] In this fashion, a number of peptide- and protein-based bioconjugates have been successfully prepared.[4–6] Recently, this quintessential 'click reaction'[7] was applied to the ^{18}F-labeling of model peptide sequences with 5-[^{18}F]fluoro-1-pentyne as a route to new PET imaging agents.[8] In addition, a number of small organic compounds, including sugars, amino acids, and nucleosides were efficiently coupled to aliphatic [^{18}F]fluoroalkynes.[9,10] [^{18}F]Fluoroaromatic prosthetic group 4-[^{18}F]fluoro-*N*-(prop-2-ynyl)benzamide was synthesized by Ramenda *et al.* for the radiolabeling of the neurotensin [8–13] peptide (NT[8-13]).[11] A successful ^{18}F-peptide preparation using 2-[^{18}F]fluoroethylazide has also been reported.[12] Most recently, a [^{18}F]fluorinated triethylene glycol derivative was bioconjugated to RGD peptide and subsequently used to image integrin $\alpha_V\beta_3$-specific tumours.[13] The aforementioned study represents the first use of 1,2,3-triazole linkers for *in vivo* PET imaging.

Efficient [^{18}F]fluorinations of 2-substituted pyridinyl moieties[14,15] have been utilized by the Orsay group and others to prepare a number of ^{18}F-based tracers and prosthetic tags.[16] Among the successful radiobioconjugations reported, the synthesis of bifunctional molecules 2-bromo-*N*-3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]acetamide ([^{18}F]FPyBrA) and 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME) are noteworthy in their capacity to both efficiently [^{18}F]fluorinate and ligate to biological molecules. [^{18}F]FPyBrA was originally used to radiolabel phosphorothiolate DNA;[17] later it was used to prepare ^{18}F-modified Spiegelmers,[18,19] short interfering RNA,[20] and polyamide nucleic acids.[21,22] [^{18}F]FPyME was used to successfully label variety of thiol-containing peptides and proteins.[23]

We sought to employ a 2-substituted pyridine [^{18}F]fluorination in the preparation of a general labeling agent for use with biomolecules of interest to PET. We reasoned that bifunctional precursor molecules containing 2-nitro (**2**) and 2-trimethylammonium triflate (**3**) leaving groups and robust terminal alkyne moieties could be used to prepare prosthetic label 2-[^{18}F]fluoro-3-(hex-5-ynyloxy)pyridine ([^{18}F]FPy5yne , [^{18}F]-**1**). It was anticipated that this compound could be synthesized in a *single radiochemical step* (Scheme 1), purified, then conjugated to biological molecules under chemospecific Huisgen [3 + 2] cycloaddition ligation conditions. Herein, we report the successful synthesis of [^{18}F]FPy5yne and, as proof-of-principle, its subsequent bioconjugation to an azide-modified peptide.

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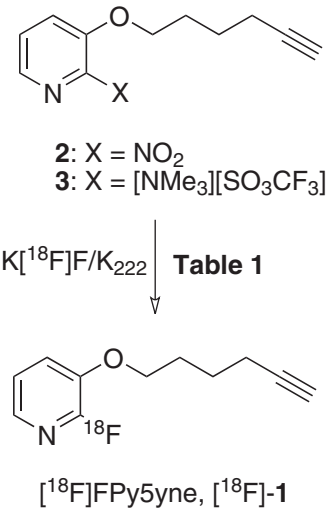
Results and discussion

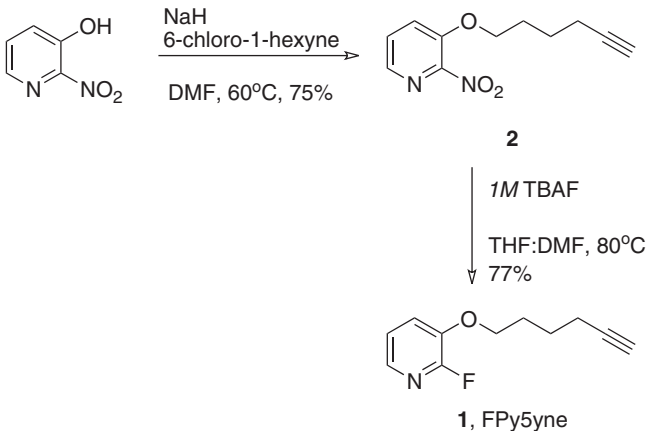
Chemistry

An attempt was made to prepare 3-(hex-5-ynyloxy)-2-nitropyridine (**2**) by way of Mistunobu condensation of commercially available 2-nitro-3-hydroxypyridine and hex-5-yn-1-ol, in a protocol similar to those reported for the synthesis of FPyBrA[17] and FPyME.[23] Unfortunately, **2** was found to elute closely with reaction by-product diisopropyl hydrazine-1,2-dicarboxylate, and the two compounds could not be separated in our hands using silica gel chromatography. Presumably, alternative Mitsonubu reagents with water-soluble by-products such as di-*tert*-butyl azodicarboxylate (DBAD)[24] and di-2-methoxyethyl azodicarboxylate (DMEAD)[25] could be employed. In our case, however, use of these expensive reagents proved unnecessary. Compound **2** was prepared by coupling 3-hydroxy-2-nitropyridine and 6-chlorohexy-1-yne under Wilkinson ether synthesis conditions (NaH, 60°C, DMF; Scheme 2). This approach resulted in a clean reaction and straightforward purification. A nearly identical procedure was used to prepare 2-dimethylamino-3-(hex-5-ynyloxy)pyridine (**5**), which was subsequently methylated to afford trimethylammonium triflate precursor **3** (Scheme 3). Cold standard [^{19}F]FPy5yne was obtained from the fluorination of 2-nitropyridine precursor **2** with commercial 1 M TBAF solution (Scheme 2). As observed previously, fluorinations of simple 2-substituted pyridines proceed smoothly with this reagent, despite the fact that it contains as much as 5% water.[26]

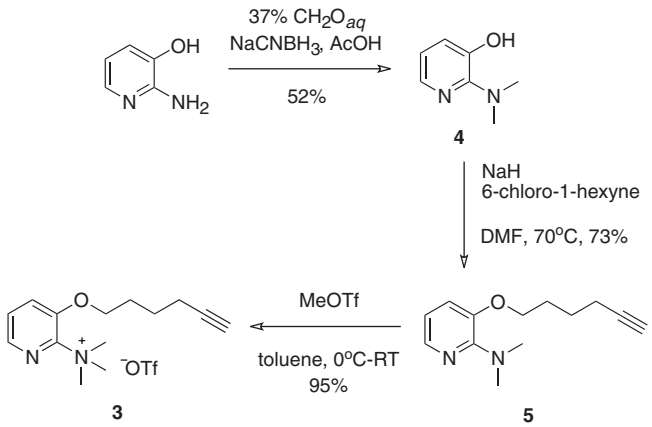
Model peptide **BG142**, consisting of the azide-modified four amino acid motif N_3–$(CH_2)_4$–CO–Tyr–Lys–Arg–Ile–OH, was synthesized using an automated system by way of standard Fmoc protocols. Cold peptide standard **^{19}F-BG142** was prepared using a Huisgen 1,3-dipolar cycloaddition ligation (Scheme 4). Acetonitrile-soluble complex $Cu(CH_3CN)_4PF_6$ was



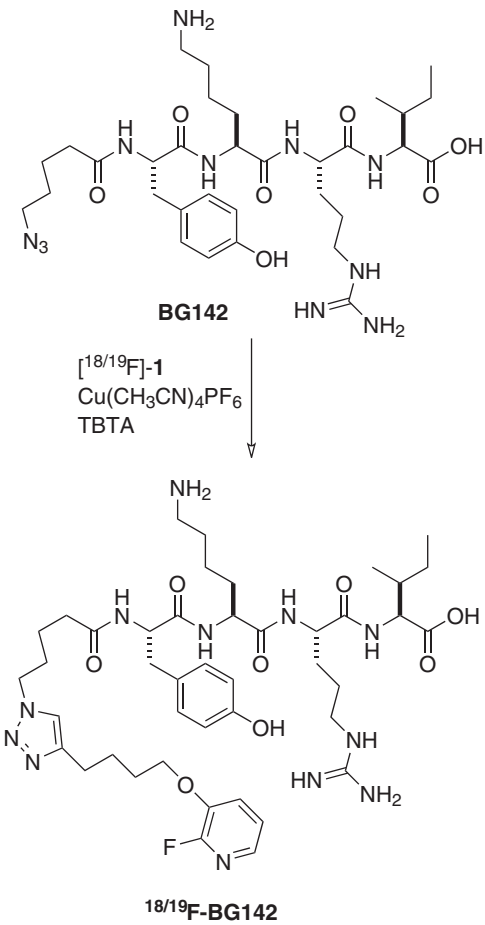
Scheme 1. Radiosynthesis of [^{18}F]Fpy5yne ([^{18}F]-**1**).



Scheme 2. Synthesis of 3-(hex-5-ynyloxy)-2-nitropyridine (**2**) and synthetic standard [^{19}F]-**1**.



Scheme 3. Synthesis of [3-(hex-5-ynyloxy)pyridin-2-yl]trimethylammonium trifluromethanesulfonate (**3**).



Scheme 4. Preparation of fluorine-labeled peptides **^{18}F-BG142** and **^{19}F-BG142**.

used as a source of Cu^{I}. Prior to contact with the peptide, the copper salt was complexed with Cu^{I}-chelating ligand *tris*-(benzyltriazolylmethyl)amine (TBTA). TBTA has been shown to preserve the oxidation state of the metal, while at the same time enhancing its catalytic role.[27] The bioconjugate was purified by semi-preparative HPLC and its identity confirmed by MALDI-TOF.

Radiochemistry

Bifunctional prosthetic group [^{18}F]FPy5yne ([^{18}F]-**1**) was synthesized by way of ^{18}F nucleophilic heteroaromatic substitution of either 3-(hex-5-ynyloxy)-2-nitropyridine (**2**) or [3-(hex-5-ynyloxy)-pyridin-2-yl]trimethylammonium trifluoromethanesulfonate (**3**; Scheme 1). Standard KF[^{18}F]-K_{222}/potassium carbonate conditions were used. Table 1 summarizes the various reaction conditions employed in this study, where radiochemical yields by radioTLC represent the percentage of the radioactive area representing ^{18}F-labeled product relative to the area of total radioactivity measured. Both precursors **2** and **3** resulted in satisfactory radiochemical yields of [^{18}F]-**1** at elevated temperatures in DMSO over 15 min. In the case of **3**, it was observed that raising the temperature to 120°C or lowering the reaction time to 10 min had a deleterious effect on radiochemical yield. In the small number of synthesis reported here, MeCN (0.2 mL) was comparable to that of DMSO (0.7 mL) at 110°C. However, while assessing bioconjugation methodologies, we often chose to use acetonitrile during [^{18}F]-**1** synthesis, since these reaction mixtures could more reliably be diluted and injected directly into an HPLC. In this way, we obviated the need for a manual solid-phase extraction prior to LC purification. Specific activity of [^{18}F]-**1** was determined to be 1650 mCi/μmol (61 GBq/μmol). A representative radiochromatogram of a [^{18}F]-**1** reaction mixture (MeCN, 110°C, 15 min) is shown in Figure 1. The chemical nature of radioproduct [^{18}F]FPy5yne was confirmed by way of HPLC co-injection of collected material with its corresponding ^{19}F standard (Figure 2).

We employed all three reverse phase HPLC sorbents described in the experimental section below for the resolution of [^{18}F]-**1** from 3-(hex-5-ynyloxy)-2-nitropyridine (**2**). Of these, only the Luna PFP(2) column (Phenomenex, Inc.) afforded baseline separations. In the case of [^{18}F]fluorination of [3-(hex-5-ynyloxy)pyridine-2-yl]trimethylammonium triflate (**3**), some 2-dimethylamino-3-(hex-5-ynyloxy)pyridine (**5**) was observed, particularly in reactions employing DMSO. This is the result of competitive demethylation of the trimethylammonium triflate salt.[28] Nevertheless, this impurity could be completely and consistently separated from labeled [^{18}F]-**1** by HPLC and thus subsequent experiments were performed using precursor **3** exclusively.

Bioconjugation of [^{18}F]-**1** to model peptide sequence N_3–$(CH_2)_4$–CO–YKRI–OH (**BG142**) proceeded in a straightforward fashion. Radiochemical yields of 81–100% **^{18}F-BG142** (89% ± 8.6%, $n = 4$) as judged by radioHPLC were obtained in 10 min at 37°C by mixing the azide-modified peptide with [^{18}F]-**1** in the presence of tetrakis(acetonitrilo)copper(I) hexafluorophosphate, TBTA, and *N*, *N*–diisopropylethylamine (DIEA). These reagents were added in excess of the starting peptide. TBTA was only partially soluble in the solvent mixture employed (20 mM sodium phosphate buffer:DMF); thus, the reaction mixture was clarified with the addition of 50% acetonitrile or centrifuged to remove insolubles prior to HPLC purification. A representative radiochromatogram of a **^{18}F-BG142** reaction mixture is shown in Figure 3. The chemical nature of **^{18}F-BG142** was confirmed by way of HPLC co-injection of collected material with its corresponding ^{19}F standard (Figure 4).

Experimental

Chemicals

Oxygen-18-enriched water ([^{18}O]H_2O, >97% pure) was purchased from Rotem Industries (Beer Sheva, Israel). Fmoc-Lys(Boc)-OH and 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were obtained from Chem Impex International, Inc. (Wood Dale, USA). Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(*t*Bu)-OH and Fom-Ile-NovaSyn® TGA resin were purchased from Nova-Biochem. Succinimidyl 5-azidovalerate[29–31] and TBTA[27] were synthesized according to published procedures. All other chemicals were purchased from either Sigma-Aldrich or Alfa Aesar, and were classified ACS grade or better.

Analytical methods

TLC and radioTLC were performed on $60F_{254}$ silica gel plates purchased from Sorbent Technoligies (Atlanta, USA). TLCs were visualized using UV light or chemical stain (anisaldehyde or ninhydrin). RadioTLCs were obtained using a Bioscan System 200 Imaging scanner.

All reverse phase HPLC reported herein utilized a Waters 600 Controller in combination with a Waters 2487 dual λ absorbance

Table 1. Summary of reaction conditions for the preparation of [^{18}F]FPy5ynes

Precursor	Solvent	Time (min)	Temp (°C)	Radiochemical yield (%)[a]
2	DMSO[b]	15	120	91, 86
3	DMSO[b]	15	110	87, 88, 91 (89% ± 2.1%)[c]
3	DMSO[b]	10	110	68
3	DMSO[b]	15	80	72
3	DMF[b]	15	110	58
3	MeCN[d]	15	75	61, 59
3	MeCN[d]	15	110	90, 93, 88 (90% ± 2.5%)[c]

[a]Each value is an average of two chromatograms and represents a unique radiochemical synthesis.
[b]Reaction volume: 0.7 mL.
[c]Average yield ± SD.
[d]Reaction volume: 0.2 mL.

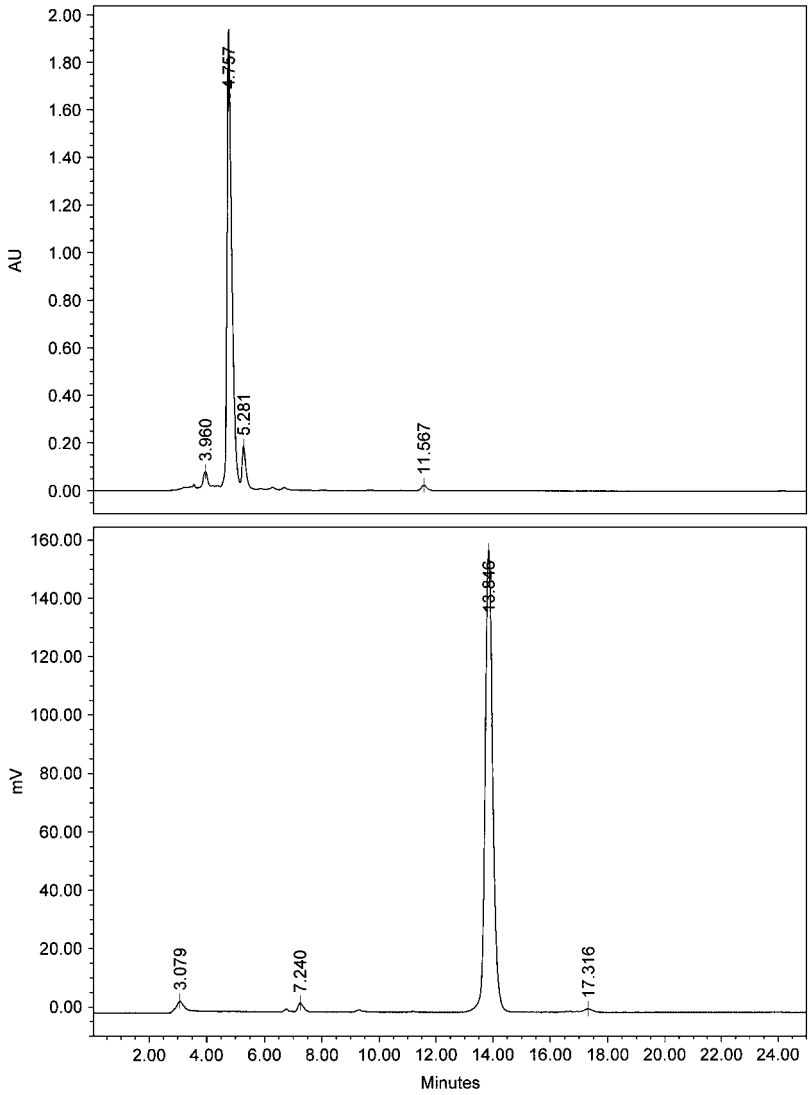


Figure 1. Preparative HPLC trace (HPLC B) of [^{18}F]Fpy5yne reaction mixture (MeCN, 110°C, 15 min). Top: UV (260 nm). Impurity **5** R_t = 11.567 min. Bottom: Rad. [^{18}F]-**1** R_t = 13.846 min.

detector and a NaI detector. All HPLC solvents were filtered prior to use. HPLC A Column: Agilent Eclipse XDB-C18 (250 mm × 4.6 mm, 5 μm); Detector: 223 and 280 nm; Program: flow = 1 mL/min, 100% H_2O for 5 min, then 0–100% MeCN in H_2O over 30 min. HPLC B Column: Phenomenex Luna PFP(2) C18 (250 × 10 mm, 5 μm); Detector: 214 and 260 nm; Program: flow = 4 mL/min, 50:50 MeCN:0.1% TFA. HPLC C Column: Waters μBondapak C18 (100 × 7.8 mm, 10 μm); Detector: 214 and 280 nm; Program: linear gradient, flow = 4 mL/min, 0–100% MeCN in H_2O with 0.1% TFA over 25 min. HPLC D Column: Phenomenex Luna PFP(2) C18 (250 × 10 mm, 5 μm); Detector: 214 and 280 nm; Program: linear gradient, flow = 4 mL/min, 0–100% MeCN in H_2O with 0.1% TFA over 35 min.

^{1}H NMR and ^{13}C NMR were recorded on a Bruker 400 MHz apparatus. Chemical shifts are reported relative to the hydrogenated residue of the deuterated solvents. ^{19}F NMR was collected on a Bruker 300 MHz apparatus using $CFCl_3$ as internal standard. High-resolution electron impact mass spectroscopy was performed on a Kratos MS50 apparatus. High-resolution electrospray mass spectroscopy was performed on a Micromass LCT time-of-flight MS. MALDI time-of-flight mass spectroscopy of azide-modified peptide sequence **BG142** and bioconjugate **^{19}F-BG142** was carried out on a Micromass Tof Spec 2E instrument and an Applied Biosystems Voyager System 4311 apparatus, respectively. In both cases, α-cyano-4-hydroxycinnamic acid was used as matrix.

No-carrier-added [^{18}F]fluoride was produced by 13 MeV proton bombardment of [^{18}O] water [^{18}O(p,n)^{18}F] on the TRIUMF TR13 cyclotron. Typical production was 65–155 mCi of [^{18}F]F^- at end of bombardment for a 10 μA, 5 min irradiation.

2-Dimethylamino-3-hydroxypyridine (4)

Into a round-bottomed flask containing 2-amino-3-hydroxypyridine (0.50 g, 4.56 mmol) in acetonitrile (28 mL) was added 37% *aqueous* formaldehyde (3.4 mL), then sodium cyanoborohydride (5.84 g, 92.9 mmol). Over the course of 4 h, the reaction turned murky blue-black. Acetic acid (10 mL) was added and the reaction immediately clarified. The reaction was poured into 100 mL of 15% *aqueous* ammonia and extracted three times into CH_2Cl_2. The extract was dried over $MgSO_4$, concentrated and flash-chromatographed on a silica column with 1:1

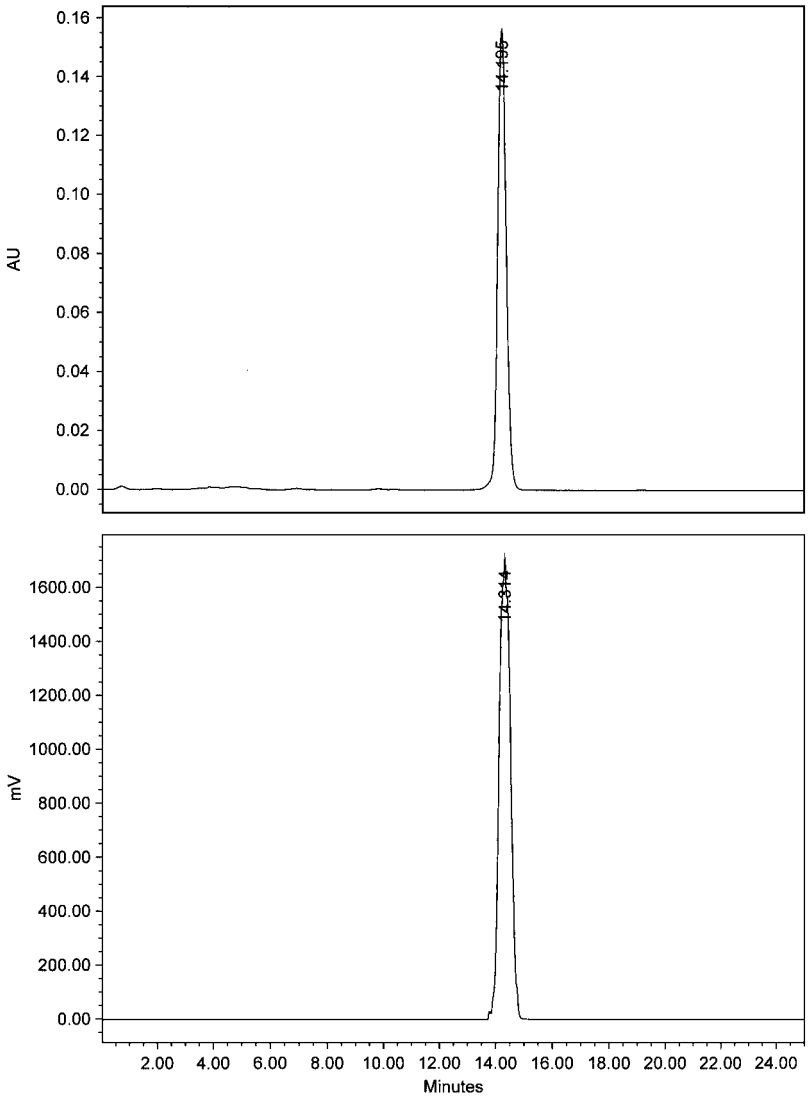


Figure 2. Co-injection of [^{18}F]-**1** with ^{19}F synthetic standard (HPLC B). Top: UV (260 nm). Bottom: Rad.

hexanes:ethyl acetate to afford 326 mg (52%) of 2-dimethylamino-3-hydroxypyridine (**4**). Mp = 128°C [Lit.[32] = 128°C]. ^{1}H NMR (CD_3CN):δ2.83 (s, 6H); 6.75 (dd, J = 7.7, 4.8 Hz, 1H); 7.03 (dd, J = 7.7, 1.5 Hz, 1H); 7.75 (dd, J = 4.8 Hz, 1.5, 1H). HRMS (EI) calcd. for $C_7H_{10}N_2O$: 138.07931 [M^+]. Found: 138.07966.

3-(Hex-5-ynyloxy)-2-nitropyridine (2)

Into a dry flask containing NaH powder (107 mg, 4.46 mmol) was added DMF (12 mL) and the dispersion was cooled to 0°C. 3-Hydroxy-2-nitropryridine (500 mg, 3.57 mmol) was added, followed by 0.9 mL of 6-chloro-1-hexyne (8.02 mmol), dropwise. The reaction was held 0°C for 10 min, at room temperature for 2 h, and finally heated at 60°C for 45 h. The reaction was poured into water and extracted three times into ethyl acetate. The pooled organic portions were washed once with brine then dried over $MgSO_4$. Purification on a flash column of silica (1:1 hexanes:ethyl acetate) afforded 589 mg (75%) of **2**. ^{1}H NMR ($CDCl_3$):δ1.69–1.76 (m, 2H); 1.94–2.01 (m, 3H); 2.28 (td, J = 6.9, 2.6 Hz, 2H); 4.17 (t, J = 6.2, 2H); 7.49–7.54 (m, 2H); 8.08 (dd, J = 3.8, 2.0 Hz, 1H). ^{13}C NMR ($CDCl_3$):δ17.92 [CH_2]; 24.52 [CH_2]; 27.67 [CH_2]; 68.93 [CH_2]; 69.20 [CH]; 123.3 [CH]; 128.5 [CH]; 139.0 [CH]; 147.1 [C]; 149.0 [C]. HRMS (EI) calcd. for $C_{11}H_{12}N_2O_3$: 220.08479 [M^+]. Found: 220.08437.

2-Fluoro-3-(hex-5-ynyloxy)pyridine (FPy5yne, [^{19}F]-1)

3-(Hex-5-ynyloxy)-2-nitropyridine (**2**, 326 mg, 1.48 mmol) in 1:1 THF:DMF (12 mL) was treated with 5.4 mL of 1 M *tert*-butylammonium fluoride. The reaction was stirred for 24 h at 80°C, during which time the reaction turned dark red-orange. The reaction was poured into water and extracted twice into ethyl acetate. The organic portions were pooled, washed once with water and once with brine, and dried over $MgSO_4$. Purification on a flash column of silica (3:2 hexanes:ethyl acetate) afforded 221 mg (77%) of [^{19}F]-**1** as an oil. Bp = 69–70°C. ^{1}H NMR ($CDCl_3$):δ1.72–1.78 (m, 2H); 1.92–2.00 (m, 3H); 2.29 (td, J = 6.9, 2.6 Hz, 2H); 4.07 (t, J = 6.2 Hz, 2H); 7.09 (dd, J = 7.2, 4.9 Hz, 1H); 7.23–7.29 (m, 1H); 7.73 (d, J = 4.8 Hz, 1H). ^{13}C NMR ($CDCl_3$):δ18.07 [CH_2]; 24.78 [CH_2]; 27.98 [CH_2]; 68.77 [CH_2]; 68.81 [CH]; 83.80 [C]; 121.6 [$J_{F\text{-}C}$ = 4.3 Hz, CH]; 122.7 [$J_{F\text{-}C}$ = 4.4 Hz, CH]; 137.3 [$J_{F\text{-}C}$ = 13 Hz, CH]; 142.3 [$J_{F\text{-}C}$ = 26 Hz, C]; 153.9 [$J_{F\text{-}C}$ = 239 Hz, C]. ^{19}F NMR ($CDCl_3$):δ-84.38 (d). HRMS (EI) calcd. for $C_{11}H_{12}NOF$: 193.09029 [M^+]. Found: 193.09061.

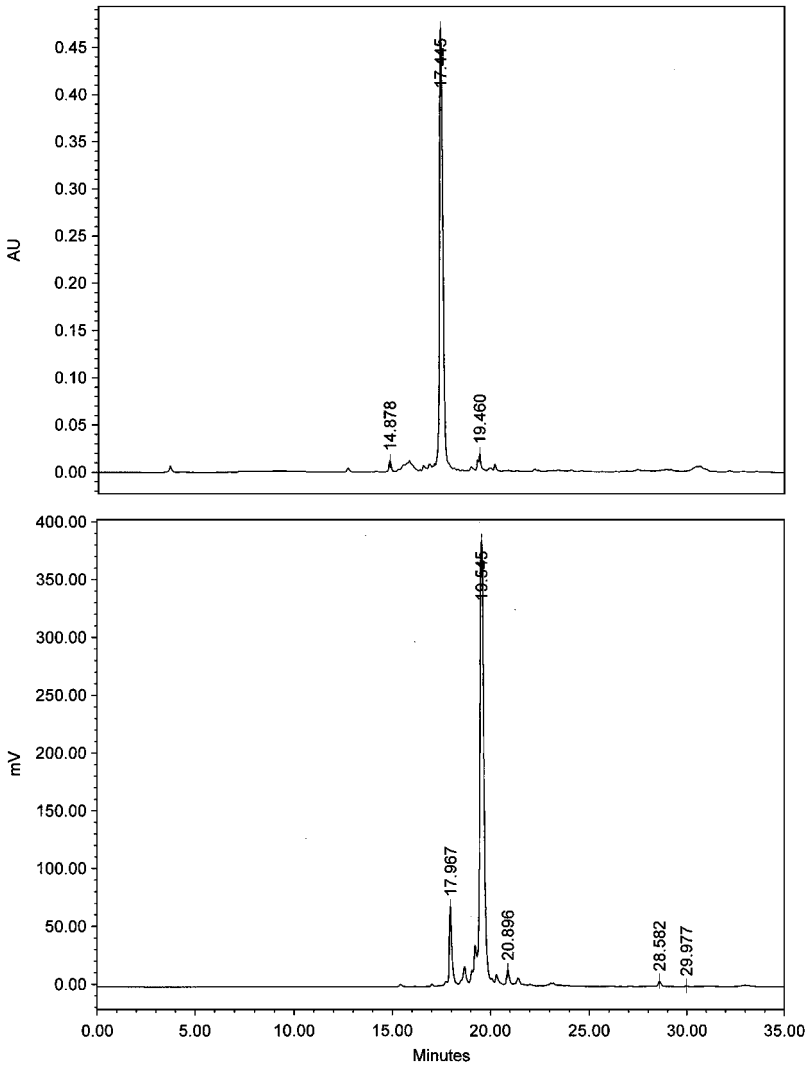


Figure 3. Preparative HPLC trace (HPLC D) of **^{18}F-BG142** reaction mixture [20 mM sodium phosphate (pH 8.5):DMF 2:1, 37°C, 10 min]. Top: UV (280 nm) **BG142** R_t = 17.455 min. Bottom: Rad. **^{18}F-BG142** R_t = 19.545 min. [^{18}F]-**1** R_t = 29.977 min.

2-Dimethylamino-3-(hex-5-ynyloxy)pyridine (5)

To a slurry of 95% sodium hydride (81.2 mg, 3.38 mmol) in dry DMF at 0°C was added 2-dimethylamino-3-hydroxypyridine (**4**, 286 mg, 2.07 mmol). The reaction was kept at this temperature for 20 min, then 6-chloro-1-hexyne (0.5 mL, 4.46 mmol) was added dropwise via syringe. The reaction was allowed to warm to room temperature over 30 min, then heated to 70°C for 22 h. The mixture was poured into water and extracted three times into ethyl acetate, and then the organic portions were combined and washed once with water and once with brine. The extract was concentrated and purified on a flash column of silica gel (1:1 hexanes:ethyl acetate) to afford 330 mg (73%) of **5** as a light yellow oil. ^{1}H NMR (CD_2Cl_2):δ1.73–1.77 (m, 2H); 1.91–1.98 (m, 2H); 2.00 (t, J = 2.7 Hz, 1H) 2.29 (td, J = 7.0, 2.6 Hz, 2H); 2.98 (s, 6 H); 3.98 (t, J = 6.3 Hz, 2H); 6.70 (dd, J = 7.8, 4.9 Hz, 1H); 7.00 (dd, J = 7.8, 1.1 Hz, 1H); 7.77 (dd, J = 4.9, 1.4 Hz, 1H). ^{13}C NMR (CD_2Cl_2):δ18.45 [CH_2]; 25.69 [CH_2]; 28.70 [CH_2]; 40.99 [CH_3]; 68.13 [CH_2]; 68.78 [CH]; 84.36 [C]; 115.5 [CH]; 118.5 [CH]; 138.7 [CH]; 146.0 [C]; 153.3 [C]. HRMS (EI) calcd. for $C_{13}H_{18}N_2O$: 218.14191 [M^+]. Found: 218.14217.

[3-(Hex-5-ynyloxy)pyridin-2-yl]trimethylammonium trifluoromethanesulfonate (3)

A solution of **5** (196 mg, 898 μmol) in dry toluene (3 mL) was cooled to 0°C and methyl trifluoromethanesulfonate (101 μL, 908 μmol) was added via syringe. The reaction was stirred at 0°C until fully mixed, then allowed to warm to room temperature over 75 min. The reaction was quenched with 5 mL of hexanes and quickly filtered. The resulting off-white precipitate (**3**, 327 mg, 95%) was washed with two 5 mL portions of hexanes and dried *en vacuo*. Mp = 66–67°C. ^{1}H NMR (CD_2Cl_2):δ1.70–1.77 (m, 2H); 2.07–2.11 (m, 3H); 2.33 (td, J = 6.8, 2.6 Hz, 2H); 3.72 (s, 9H); 4.31 (t, J = 6.5 Hz, 2H) 7.62 (dd, J = 8.3, 4.3 Hz, 1H); 7.66 (dd, J = 8.3, 1.5 Hz, 1H); 8.11 (dd, J = 4.3, 1.6 Hz, 1H). ^{13}C NMR (CD_2Cl_2):δ18.49 [CH_2]; 25.56 [CH_2]; 28.29 [CH_2]; 54.78 [CH_3]; 69.66 [CH_2]; 70.48 [CH]; 83.99 [C]; 121.5 [q, J = 321 Hz, CF_3]; 124.8 [CH]; 129.2 [CH]; 139.4 [CH]; 142.9 [C]; 147.8 [C]. HRMS (ESI) calcd. for $C_{14}H_{21}N_2O$: 233.1654 [M^+]. Found: 233.1655.

Preparation of azide-modified peptide N_3–$(CH_2)_4$–CO–Tyr–Lys–Arg–Ile–OH (BG142)

The peptide **BG142** was synthesized by continuous flow method on a Pioneer Peptide Synthesis System using 1 g of

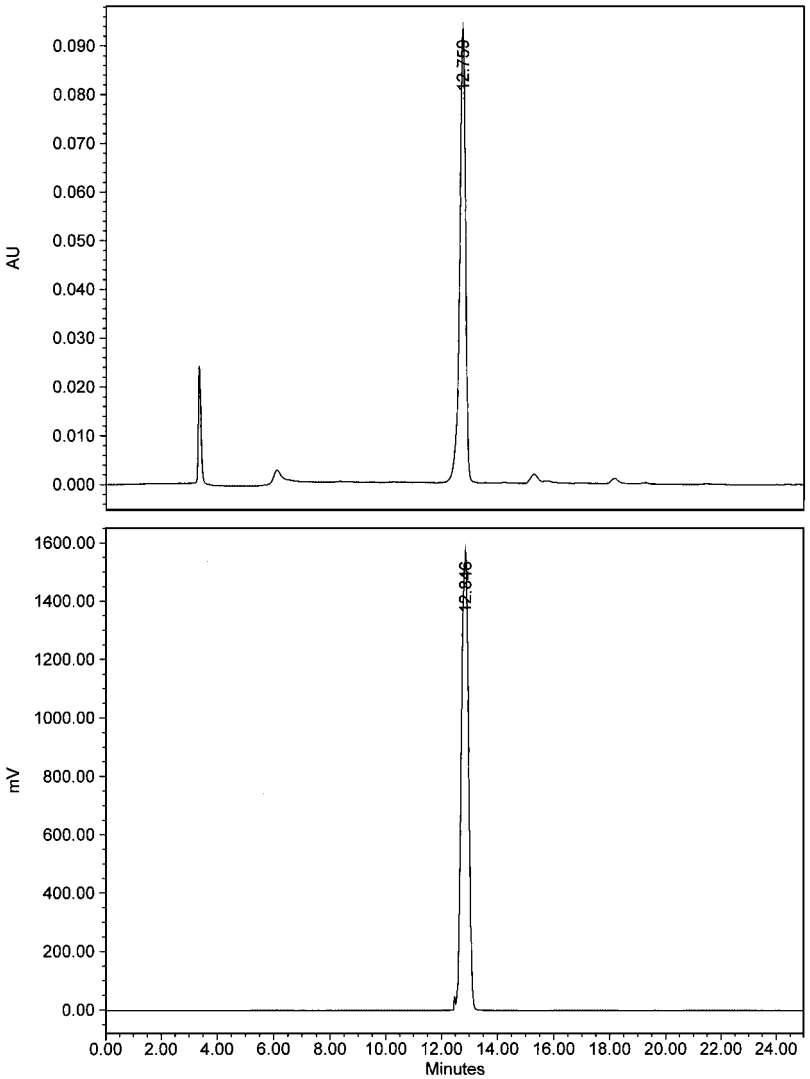


Figure 4. Co-injection of **^{18}F-BG142** with ^{19}F synthetic standard (HPLC C). Top: UV (280 nm). Bottom: Rad.

N-α-Fmoc-Ile-NovaSyn® TGA resin with a substitution rate of 0.27 mmol/g. Fmoc deprotection was performed in 20% piperidine in DMF and monitored through UV detection at 364 nm. A two-fold excess of Fmoc-protected amino acids over resin substitution rate was used for coupling. Synthesis was performed using amine-free DMF. Fmoc-protected amino acids are activated for coupling with an equimolar amount of HATU, and 2 equivalents of DIEA. After tyrosine Fmoc deprotection, a two-fold excess of 5-azidovalerate and 6-chloro-1-hydroxybenzotriazole in solution in DMSO were added to the partially protected peptide on resin. Mechanical agitation was maintained for 24 h at room temperature. The Fmoc deprotection and the coupling steps were followed by a Kaiser's test on resin; and the resulting colorimetric reaction between resin and ninhydrin indicated the presence of free primary amines after Fmoc deprotection (blue beads) and their absence after coupling (yellow beads). After coupling, the resin was washed with DMF (3 × 10 mL), MeOH (3 × 10 mL), DMF (3 × 10 mL), MeOH (3 × 10 mL), and DCM (3 × 10 mL). The azido peptide **BG142** was deprotected and cleaved from the polymer support by treatment with a cocktail of TFA:H_2O:thioanisole (92:2:6 v/v) for 4 h at room temperature with mechanical agitation. The resin was removed by filtration and washed with TFA. Combined filtrates were added dropwise to ethyl ether (1 mL of TFA/10 mL of ether). The precipitated peptide was centrifuged at 1200 rpm for 15 min and the ether solution was decanted. The crude peptide was dissolved in water and purified by Flash chromatography on a Biotage SP4 system (Charlottesville, VA) using a FLASH+® C18 Cartridge 25+M eluting with a linear gradient of 0–60% of acetonitrile in water for ten volumes of column. The peak corresponding to the desired peptide was collected, checked by HPLC (HPLC A) for purity prior to combination of collected fractions and lyophilization to give 58 mg (31%) of **BG142** as a white solid. Purity by HPLC was 97%; MALDI-TOF: *m/z* 705.4 ([MH+H]$^+$, 100%).

Cold peptide bioconjugation (^{19}F-BG142)

Peptide **BG142** (1.0 mg, 1.42 μmol) was dissolved in 200 μL of phosphate buffered saline (PBS, 75 mM, pH 7.2) and added to a screw-cap plastic vial (2 mL) containing [^{19}F]-**1** (2.7 mg, 14.0 μmol, 10 equiv.) in 50 μL DMF. Into a separate vial $Cu(CH_3CN)_4PF_6$ (5.3 mg, 14.2 μmol, 10 equiv.) and TBTA (7.9 mg, 14.9 μmol, 10.5 equiv.) were mixed together as powders

and dissolved in 50 μL DMF. This freshly prepared solution was added to the peptide and the reaction mixture was shaken vigorously for 3½ h. After this time, the reaction was centrifuged to remove undissolved materials and the decanted solution containing **^{19}F-BG142** was purified by semi-preparative HPLC (HPLC C) and concentrated *in vacuo*. Yield by HPLC was 96%. MALDI-TOF: *m/z* 1211.5 ([M]$^+$, 100%).

Radiosynthesis

2-[^{18}F]Fluoro-3-(hex-5-ynyloxy)pyridine ([^{18}F]FPy5yne, [^{18}F]-1)

From precursor ***3*** *in DMSO*: [^{18}F]fluoride (65.9 mCi) was immobilized on an ion exchange ^{18}F trap and release column (ORTG, Inc.), then eluted into a 5 mL conical vial with a 1 mL:0.3 mL MeCN:H_2O solution of Kryptofix 222 (14.1 mg, 37.5 μmol) and potassium carbonate (3.4 mg, 24.6 μmol). The solution was evaporated to dryness under a stream of helium (180 mL/min) at 110°C. Residual water was removed by azeotropic distillation with acetonitrile (2 × 1 mL). To this residue was added precursor **3** (3.5 mg, 9.2 μmol) in 0.7 mL DMSO, and the reaction was heated at 110°C for 15 min. Extent of ^{18}F incorporation was assayed by means of thin layer radiochromatography (SiO_2, ethyl acetate as eluent). R_f: 0.72. Afterwards, the solution was cooled to room temperature in a water bath, then diluted with 17 mL of H_2O and loaded onto a Waters Sep-Pak tC18 cartridge (400 mg, activated previously with 3 mL MeOH and 10 mL water). The column was washed with 2 × 5 mL of water and partially dried with a syringe filled with 5 mL of air. [^{18}F]-**1** (30.0 mCi) was eluted off of the column with 1 mL of acetonitrile. This solution was purified by semi-preparative HPLC (HPLC B). The collected eluent was poured into 20 mL of H_2O above a tC18 Sep-pak cartridge (activated as above) and the mixture was diluted to 50 mL total with H_2O, then loaded onto the column. The cartridge was then washed with 5 mL H_2O and partially dried using He flow (180 mL/min) for 3 min. Purified [^{18}F]-**1** (15.8 mCi) was eluted off of the column with 1 mL of acetonitrile. Non-decay-corrected, collected yield was 24% (42% decay-corrected) from start of synthesis

From precursor ***2*** *in DMSO*: A radiosynthesis starting from 3-(hex-5-ynyloxy)-2-nitropyridine (**2**; 4.2 mg, 19.1 μmol) were performed in a manner very similar to the above protocol, except the reaction temperature was set to 120°C and HPLC-purified [^{18}F]F-**1** (12.9 mCi) was eluted off a tC18 Sep-Pak with methylene chloride (2 mL). Non-decay-corrected, collected yield was 22% (50% decay-corrected) from start of synthesis.

From precursor ***3*** *in acetonitrile*: Starting from 71.8 mCi, K[^{18}F]F-K_{222} complex was prepared as above. To this 'dried' [^{18}F]fluoride residue, precursor **3** (3.3 mg, 8.6 μmol) was added in 0.2 mL acetonitrile, and the reaction was heated at 110°C for 15 min. The solution containing [^{18}F]-**1** was diluted with 0.5 mL of acetonitrile and injected directly into the HPLC (HPLC B). The collected eluent was diluted to 50 mL with H_2O and trapped on a tC18 Sep-pak cartridge (activated as above). The cartridge was then washed with 5 mL H_2O and partially dried with a flow of helium (180 mL/min). Purified [^{18}F]-**1** (16.8 mCi) was eluted off the column with diethyl ether (2 mL). Non-decay-corrected, collected yield was 23% (34% decay-corrected) from start of synthesis.

Preparation of ^{18}F-labeled peptide [^{18}F]-BG142

A solution containing 30.0 mCi of prosthetic label [^{18}F]-**1** in methylene chloride was placed under a stream of helium and *carefully* dried down at 50°C. To this residue was added a 50 μL aliquot of Cu^I-TBTA complex (5.3 equiv.) in DMF. This solution was prepared immediately before addition to the reaction mixture and had a concentration of 56 mg/mL $Cu(CH_3CN)_4PF_6$ and 80 mg/mL TBTA. DIEA was then added (2 μL, 8.1 equiv.), followed by peptide **BG142** (1.0 mg, 1.4 μmol, 1 equiv.) in 100 μL of sodium phosphate buffer (20 mM, pH 8.5). The reaction was heated at 37°C for 10 min, then diluted with 400 μL of 1:1 acetonitrile:water and purified by semi-preparative HPLC (HPLC D). The collected eluent was evaporated azeotropically following the addition of 2 × 1 mL of acetonitrile, then formulated in 600 μL PBS (150 mM, pH 7.2). Collected material (5.65 mCi) represents a non-decay-corrected yield of 5.8% from end-of-bombardment (18.7% decay-corrected).

Conclusion

We prepared bifunctional ^{18}F-bearing prosthetic molecule [^{18}F]FPy5yne ([^{18}F]-**1**) from precursor molecules 3-(hex-5-ynyloxy)-2-nitropyridine (**2**) and [3-(hex-5-ynyloxy)pyridin-2-yl]trimethylammonium trifluoromethanesulfonate (**3**) by way of nucleophilic heteroaromatic [^{18}F]fluorination at the 2-position of the pyridine ring. Both precursors afforded the desired molecule in a single, high yielding radiochemical step, but reaction mixtures starting from **3** proved easier to purify by HPLC. Excellent radiochemical yields of [^{18}F]FPy5yne from **3** (87–93%) were recorded over 15 min in either DMSO or MeCN at 110°C. As for the bioconjugation step, we incorporated a terminal alkyne into our precursors for use in diastereospecific Huisgen 1,3-dipolar cycloaddition ligations. Of particular importance to our strategy was the chemical inertness of the alkyne, which is tolerant of the high temperatures and basic environments typically used to facilitate nucleophilic aromatic [^{18}F]fluorinations. After HPLC purification, [^{18}F]FPy5yne was efficiently conjugated to an azide-modified (though otherwise unprotected) peptide sequence.

It should be noted that the advantages of this new ^{18}F-based prosthetic group (e.g. excellent ^{18}F incorporation and ease of synthesis) are mitigated somewhat by product losses suffered during evaporation of the solution containing purified [^{18}F]FPy5yne. Careful drydown from a low boiling point solvent such as methylene chloride or diethyl ether is essential. Presumably, the volatility of [^{18}F]-**1** (bp = 69–70°C) would allow for a modified protocol where [^{18}F]FPy5yne is concentrated by way of distillation. However, one disadvantage of this approach is that radiochemical distillations are often difficult to automate and reproduce. Out of the five currently published protocols known to us for preparing ^{18}F-based bifunctional molecules for use in Huisgen [3+2] cycloaddition ligations, two include distillations,[8,12] while three (including ours) do not.[11,13]

Many pharmaceuticals bearing aliphatic fluorines can be stripped of fluorine *in vivo* by way of α-hydroxylation and subsequent elimination of hydrofluoric acid. Thus, they are typically considered more susceptible to metabolism than fluoroaromatics.[33] To our knowledge, the only other reported azide/alkyne-based prosthetic group bearing an aromatic [^{18}F]fluorine is 4-[^{18}F]fluoro-*N*-(prop-2-ynyl)benzamide.[11] This compound was prepared by coupling *N*-succinimidyl

4-[^{18}F]fluorobenzoate ([^{18}F]SFB) with an excess of propargyl amine. Bioconjugation to NT[8-13] (yield by radioHPLC = 66%) resulted in a radiopeptide whose ^{19}F standard showed nanomolar receptor binding *in vitro*. As an alternative, [^{18}F]FPy5yne can be prepared in a single radiochemical step (versus four for [^{18}F]fluoro-*N*-(prop-2-ynyl)benzamide) and couples efficiently (89% ± 8.6%, $n = 4$) in 10 min at 37°C.

The ultimate value of this new ^{18}F prosthetic group depends on further enquiries designed to test the effect of a [^{18}F]FPy5yne-based side chain on the distribution and targeting efficiency of biological imaging agents. To this end, experiments designed to expand the utility of [^{18}F]FPy5yne for the labeling and *in vivo* assessment of a novel peptide sequence targeting breast cancer are currently underway.

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