1-[3-(2-[18 F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione: Design, Synthesis, and Radiosynthesis of a New [18 F]Fluoropyridine-Based Maleimide Reagent for the Labeling of Pept...

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1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione: Design, Synthesis, and Radiosynthesis of a New [^{18}F]Fluoropyridine-Based Maleimide Reagent for the Labeling of Peptides and Proteins

Béatrice de Bruin,[†] Bertrand Kuhnast,[†] Françoise Hinnen,[†] Loïc Yaouancq,[‡] Mohamed Amessou,[§] Ludger Johannes,[§] Alain Samson,[‡] Raphaël Boisgard,[†,||] Bertrand Tavitian,[†,||] and Frédéric Dollé*[,†]

Service Hospitalier Frédéric Joliot, Département de Recherche Médicale, CEA/DSV, 4 place du Général Leclerc, F-91401 Orsay, France, Bionexis Pharmaceuticals, Paris-Biotech, 4 Avenue de l'Observatoire, F-75006 Paris, France, Laboratoire "Trafic et Signalisation", UMR144 Curie/CNRS, Institut Curie, 26 rue d'Ulm, F-75248 Paris Cedex 05, France, and INSERM ERM 0103, Service Hospitalier Frédéric Joliot, Département de Recherche Médicale, CEA/DSV, 4 place du Général Leclerc, F-91401 Orsay, France. Received October 22, 2004; Revised Manuscript Received January 7, 2005

FPyME (1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione) was designed as a [^{18}F]fluoropyridine-based maleimide reagent for the prosthetic labeling of peptides and proteins via selective conjugation with a thiol (sulfhydryl) function. Its pyridinyl moiety carries the radioactive halogen (fluorine-18) which can be efficiently incorporated via a nucleophilic *hetero*aromatic substitution, and its maleimido function ensures the efficient alkylation of a free thiol function as borne by cysteine residues. [^{18}F]-FPyME (HPLC-purified) was prepared in 17−20% non-decay-corrected yield, based on starting [^{18}F]-fluoride, in 110 min using a three-step radiochemical pathway. The developed procedure involves (1) a high-yield nucleophilic *hetero*aromatic ortho-radiofluorination on [3-(3-*tert*-butoxycarbonylamino-propoxy)pyridin-2-yl]trimethylammonium trifluoromethanesulfonate as the fluorine-18 incorporation step, followed by (2) rapid and quantitative TFA-induced removal of the *N*-Boc-protective group and (3) optimized maleimide formation using *N*-methoxycarbonylmaleimide. Typically, 4.8−6.7 GBq (130−180 mCi) of radiochemically pure [^{18}F]FPyME ([^{18}F]-**1**) could be obtained after semipreparative HPLC in 110 min starting from a cyclotron production batch of 33.3 GBq (900 mCi) of [^{18}F]fluoride (overall radiochemical yields, based on starting [^{18}F]fluoride: 28−37% decay-corrected). [^{18}F]FPyME ([^{18}F]-**1**) was first conjugated with a small model hexapeptide ((*N*-Ac)KAAAAC), confirming the excellent chemoselectivity of the coupling reaction (CH_2SH versus CH_2NH_2) and then conjugated with two 8-kDa proteins of interest, currently being developed as tumor imaging agents (c-AFIM-0 and c-STxB). Conjugation was achieved in high yields (60−70%, isolated and non-decay-corrected) and used optimized, short-time reaction conditions (a 1/9 (v/v) mixture of DMSO and 0.05 M aq Tris NaCl buffer (pH 7.4) or 0.1 M aq PBS (pH 8), at room temperature for 10 min) and purification conditions (a gel filtration using a Sephadex NAP-10 cartridge or a SuperDex Peptide HR 10/30 column), both compatible with the chemical stability of the proteins and the relatively short half-life of the radioisotope concerned. The whole radiosynthetic procedure, including the preparation of the fluorine-18-labeled reagent, the conjugation with the protein and the final purification took 130−140 min. [^{18}F]FPyME ([^{18}F]-**1**) represents a new, valuable, thiol-selective, fluorine-18-labeled reagent for the prosthetic labeling with fluorine-18 of peptides and proteins. Because of its excellent chemoselectivity, [^{18}F]FPyME offers an interesting alternative to the use of the nonselective carboxylate and amine-reactive [^{18}F]reagents and can therefore advantageously be used for the design and development of new peptide- and protein-based radiopharmaceuticals for PET.

INTRODUCTION

Positron emission tomography (PET) is a high-resolution, sensitive, molecular, and functional imaging technique. It permits repeated, noninvasive assessment and quantification of specific biological and pharmacological processes and is the most advanced technology currently available for studying in vivo molecular interactions. PET plays an increasing role in both drug discovery and development of radiopharmaceuticals, by assessing (once labeled with a positron-emitter) their in vivo distribution, pharmacokinetics, and dynamics.

Complex high-molecular-weight bioactive chemical structures, such as single-stranded oligonucleotides, peptides, and proteins are more and more often proposed as radiopharmaceuticals, and their applications are rapidly gaining importance in nuclear medicine (*1*−*4*). Over the past decades, these agents have evolved from large proteins (e.g. intact polyclonal antibodies with low specificity) to monoclonal antibodies and fragments (with relatively higher specificity), to smaller molecular recognition units such as single-chain antigen-binding domain fragments, and finally to receptor-specific peptides, which have emerged as one of the most promising classes of radiopharmaceuticals for both scintigraphic applications and therapeutic purposes (*2*, *4*).

* Corresponding author. Tel: + 33 1 69 86 77 04; Fax: + 33 1 69 86 77 49; e-mail: frederic.dolle@cea.fr.
[†] Service Hospitalier Frédéric Joliot.
[‡] Bionexis Pharmaceuticals.
[§] Institut Curie.
[||] INSERM ERM 0103.

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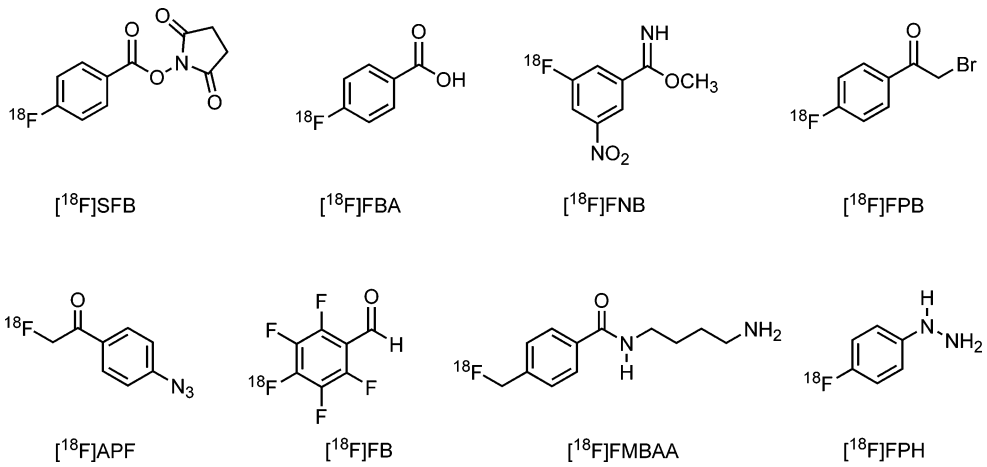


Figure 1. Chemical structures of *N*-succinimidyl 4-[18F]fluorobenzoate ([18F]SFB), 4-[18F]fluorobenzoic acid ([18F]FBA), 3-[18F]fluoro-5-nitrobenzimidate ([18F]FNB), 4-([18F]fluorophenacyl bromide ([18F]FPB), 4-azidophenacyl [18F]fluoride ([18F]APF), penta-[18F]-fluorobenzaldehyde ([18F]FB), 1-[4-([18F]fluoromethyl)benzoyl]-aminobutane-4-amine ([18F]FMBAA), and 4-[18F]fluorophenyl hydrazine ([18F]FPH),

Methods for radiolabeling these peptides and proteins have been of interest for several decades. Although many different radionuclides have been used to radiolabel proteins (*4*−*12*), the majority of labeled-protein studies have used radionuclides of iodine, principally iodine-125, iodine-123, and iodine-131. The latter radionuclides have properties which are adequate for a number of different applications (in vitro and in vivo imaging or therapy), are relatively easy to use, and are readily available at a nominal cost from commercial sources.

Peptides and proteins have also been labeled with positron-emitting radionuclides, even though fewer examples have been described in the literature. The short-lived positron-emitter fluorine-18 appears as the radionuclide of choice for the preparation of labeled bioactive peptides and proteins, as it is the case for conventional, low-molecular-weight radiotracers and radiopharmaceuticals (*13*−*16*). In regard of its physical and nuclear characteristics, fluorine-18 displays simple decay- and emission properties with a high positron abundance (97%). Compared with the other conventional short-lived positron-emitting radionuclides carbon-11, nitrogen-13, and oxygen-15, fluorine-18 has a relatively low positron emission energy (maximum 635 keV) and the shortest positron linear range in tissue (2.3 mm), resulting in the highest resolution in PET imaging. Its half-life (109.8 min) is long enough to give access to relatively extended imaging protocols compared to what is possible with carbon-11, therefore facilitating kinetic studies and high-quality metabolite and plasma analysis. Moreover, from a chemical point of view, it allows multistep synthetic approaches that can be extended over hours. These distinctive advantages, combined with its reliable multi-Curie production on widely implanted biomedical cyclotrons, have been effectively used for the labeling of various chemical structures (*13*−*16*).

Concerning peptides and proteins, the direct labeling of these macromolecules with fluorine-18 is, leaving aside some exceptions, not feasible and labeling is usually performed by conjugation of a prosthetic group, carrying the radioisotope, with a reactive function of the macromolecule. This strategy has the advantage of offering a wide choice of chemical routes, including drastic chemical conditions, for the preparation of the labeled prosthetic group entity, followed by the conjugation of the latter with a macromolecule using mild conditions needed to preserve the latter's integrity (*2*, *12*).

Several fluorine-18-labeled reagents have already been described in the literature (Figure 1). Most of them were designed for coupling to the peptide or protein via an amino function borne by an amino acid residue (*N*-terminus α-NH_2 or internal lysine ϵ-NH_2) or by an alkylamine linker. For example, the activated ester *N*-succinimidyl 4-[18F]fluorobenzoate ([18F]SFB) (*2*, *12*, *17*−*19*) is today probably the most often used prosthetic group introducing agent for the labeling of peptides and proteins via an acylation reaction, beside other carboxylic acids or esters, such as 4-[18F]fluorobenzoic acid ([18F]-FBA) (*20*), 2-[18F]fluoroacetic acid (*21*), or methyl 2-[18F]-fluoropropionate (*22*) (the latter two structures not shown). Other activated esters, such as *N*-succinimidyl 4-([18F]fluoromethyl)benzoate (*23*, *24*) and *N*-succinimidyl 8-[4′-[18F]fluorobenzyl)amino]suberate (*25*), have also been reported (structures not shown). Imidation reaction, using reagents such as 3-[18F]fluoro-5-nitrobenzimidate ([18F]FNB) (*26*), have been described. Finally, alkylation reactions using 4-[18F]fluorophenacyl bromide ([18F]FPB) (*26*) have been reported as well as photochemical conjugation using 4-azidophenacyl [18F]fluoride ([18F]APF) (*17*, *27*) and reductive amination using penta-[4-18F]fluorobenzaldehyde ([18F]FB) (*28*). A much smaller number of fluorine-18-labeled reagents for the coupling to the peptide or protein via another reactive function, the carboxylic acid function (*C*-terminus α-CO_2H or internal glutamic/aspartic acid α/β-CO_2H), have also been designed. To our knowledge, only two amines, 1-[4-([18F]-fluoromethyl)benzoyl]aminobutane-4-amine ([18F]FMBAA) (*29*) and 4-[18F]fluorophenylhydrazine ([18F]FPH) (*12*), have been described for these amidation reactions.

The free thiol function (also called sulfhydryl) is not very common in most peptides and proteins and is only present in cysteine residues (internal β-SH). Thiol-reactive agents have therefore been used to modify peptides and proteins at specific sites (*30*−*32*), providing a means of high chemoselectivity in contrast to the carboxylate and amine-reactive reagents described above. To our knowledge, to date, only two fluorine-18-labeled reagents have been described in an abstract by Shiue for the coupling to peptide or protein via alkylation of such a thiol function (*33*). In that work, the preparation of two *N*-substituted maleimides, 1-(4-[18F]fluorophenyl)pyrrole-2,5-dione ([18F]FPPD) and *N*-[3-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)phenyl]-4-[18F]fluorobenzamide ([18F]DDPFB),

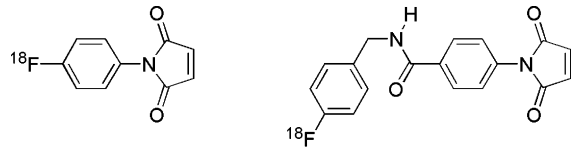


Figure 2. Chemical structures of 1-(4-[^{18}F]fluorophenyl)-pyrrole-2,5-dione ([^{18}F]FPPD) and *N*-[3-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-phenyl]-4-[^{18}F]fluorobenzamide ([^{18}F]DDPFB).

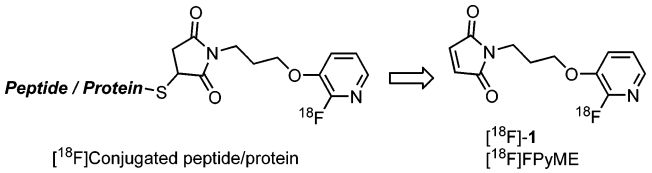


Figure 3. Fluorine-18-labeling design of peptide and protein via selective thiol alkylation using 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]FPyME, [^{18}F]-**1**).

was reported, and its coupling to proteins was briefly mentioned (Figure 2).

From a radiochemical point of view, all the above-mentioned fluorine-18-labeled reagents were prepared using the relatively restricted labeling strategies offered by *homo*aromatic and aliphatic nucleophilic substitution reactions (*14*, *15*). Recently, the scope of these nucleophilic radiofluorinations has been opened to *hetero*aromatic substitutions, particularly in the pyridine series (*34*). After evaluation and validation of the radiosynthesis of 2- and 4-[^{18}F]fluoropyridine as a model reaction (*35*, *36*), we and others have since applied successfully these nucleophilic *hetero*aromatic substitutions to the preparation of various PET radiotracers and radiopharmaceuticals (*34*), such as for example, the cholinergic $\alpha_4\beta_2$-subtype selective nicotinic radioligand 2-[^{18}F]fluoro-3-[2(*S*)-2-azetidinylmethoxy]pyridine (2-[^{18}F]F-A-85380) (*37*−*50*).

Considering chemical structures showing a fluoropyridinyl moiety, nucleophilic *hetero*aromatic substitution at the ortho-position with no-carrier-added [^{18}F]fluoride appears today as the more efficient method for the radiosynthesis of radiotracers and radiopharmaceuticals of high specific radioactivity when compared to *homo*aromatic or aliphatic nucleophilic radiofluorination. On the basis of these recent advances, we have designed a new [^{18}F]fluoropyridine-based maleimide reagent, [^{18}F]-**1** (1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione, coded [^{18}F]FPyME, Figure 3) as a new fluorine-18-labeled reagent for the highly specific conjugation to peptides and proteins via thiol alkylation.

We herein report the design, selection, and synthesis of the nonlabeled fluoropyridinyl reagent (**1**, FPyME) as a reference, as well as the synthesis of the required nitro- and trimethylammonium trifluoromethanesulfonate derivatives as precursors for labeling with fluorine-18. We also present the radiosynthesis of [^{18}F]FPyME ([^{18}F]-**1**) using an efficient three-step radiosynthesis, involving a nucleophilic *hetero*aromatic ortho-radiofluorination as the fluorine-18 incorporation-step. In addition, we describe the conjugation of this maleimide reagent ([$^{18/19}$F]FPyME, [$^{18/19}$F]-**1**) with a small hexapeptide, (*N*-Ac)KAAAAC, chosen as a model peptide in order to confirm the chemoselectivity of the coupling reaction (CH_2SH versus CH_2NH_2). Finally, we present as applications to protein labeling, (a) the preparation of [^{18}F]c-AFIM-0, a selected 75-amino acid (∼ 8.8 kDa) mini-protein, derived from the specific binding domain of annexins for phosphatidylserine and (b) the preparation of [^{18}F]c-STxB, the nontoxic homopentameric B subunit (monomer size ∼ 8.0 kDa) of the bacterial (*Shigella dysenteriae*) Shiga toxin, both proteins being developed as tumor imaging agents.

EXPERIMENTAL SECTION

1. General. *Chemicals.* Chemicals were purchased from standard commercial sources (Aldrich, Fluka, or Sigma France) and were used without further purification, unless stated otherwise. Oxygen-18-enriched water ([^{18}O]H_2O, >95%) was purchased from CortecNet (Paris, France). (*N*-Ac)KAAAAC was custom-synthesized by Neosystem (France).

Analytical Methods. Thin layer chromatographies (TLCs) were run on precoated plates of silica gel $60F_{254}$ (Merck). The compounds were localized (1) when possible at 254 nm using a UV-lamp and/or (2) by dipping the TLC-plates in a 1% ethanolic ninhydrin solution and heating on a hot plate. Radioactive spots were detected using a Berthold TraceMaster 20 automatic TLC linear analyzer. Flash chromatographies were conducted on silica gel 63−200 μm (Merck) at 0.3 bar (Ar). High-pressure liquid chromatographies (HPLCs) were performed on Waters or Shimadzu systems: [HPLC A]: Equipment: a Waters 600 Pump, a Waters 600 Gradient Controller, a Shimadzu SPD10-AVP UV-multiwavelength spectrometer, and a Geiger-Müller detector; column: preparative SiO_2, Lichrosorb, Merck (250 × 27.0 mm; porosity: 7 μm); solvents and conditions: isocratic elution with heptane/EtOAc; temperature: RT; absorbance detection at λ = 254 nm. [HPLC B]: Equipment: a Waters 600 Pump, a Waters 600 Gradient Controller, a Shimadzu SPD10-AVP UV-multiwavelength spectrometer, and a Geiger-Müller detector; column: preparative C18, DeltaPak, Waters (300 × 19.0 mm; porosity: 15 μm); solvents and conditions: 0.1% aq TFA (v/v) and acetonitrile containing 0.1% TFA (v/v); linear gradient in 30 min from 100/0 to 65/35, flow rate: 10 mL/min; temperature: RT, absorbance detection at λ = 214 nm. [HPLC C]: Equipment: a Waters 600 Gradient Controller, a Waters 490E UV-multiwavelength spectrometer, and a Geiger-Müller detector; column: preparative Gel Filtration SuperDex Peptide HR 10/30, Amersham Pharmacia Biotech (300 × 10 mm); solvents and conditions: isocratic elution with aq PBS (20 mM KH_2PO_4; 150 mM NaCl) at pH 7.4; flow rate: 1 mL/min; temperature: RT; absorbance detection at λ = 214 nm. [HPLC D]: Equipment: a Waters 510 pump, a Shimadzu SPD10-AVP UV-multiwavelength spectrometer and a Geiger-Müller detector; column: semipreparative SiO_2 Zorbax Rx-SIL, Hewlett-Packard (250 × 9.4 mm; porosity: 5 μm); solvents and conditions: isocratic elution with heptane/EtOAc: 60/40 (v/v); flow rate: 5 mL/min; temperature: RT; absorbance detection at λ = 254 nm. [HPLC E]: Equipment: a Waters 600 Pump, a Waters 600 Gradient Controller, a Hewlett-Packard Series 1100 multiwavelength spectrometer and a Flow One Scintillation Analyzer Packard equipped with a positron dedicated cell for radioactivity monitoring; column: semipreparative C18, μBondapak, Waters (300 × 7.8 mm; porosity: 10 μm); solvents and conditions: 0.1% aq TFA (v/v) and acetonitrile containing 0.1% TFA (v/v); linear gradient in 15 min from 80/20 to 40/60, flow rate: 4 mL/min; temperature: RT, absorbance detection at λ = 214 nm. [HPLC F]: Equipment: a Waters 600 Gradient Controller, a Hewlett-Packard Series 1100 multiwavelength spectrometer and a Flow One Scintillation Analyzer Packard equipped with a positron-dedicated cell for radioactivity monitoring; column: preparative Gel Filtration SuperDex Peptide HR 10/30, Amersham Pharmacia Biotech (300 × 10 mm); solvents and conditions: isocratic elution with aq PBS

(20 mM KH_2PO_4; 150 mM NaCl) at pH 7.4; flow rate: 1 mL/min; temperature: RT; absorbance detection at λ = 214 nm. NMR spectra were recorded on a Bruker AMX (300 MHz) apparatus and on a Bruker Advance (400 MHz) apparatus using the hydrogenated residue of the deuterated solvents (CD_2Cl_2, δ = 5.32 ppm; DMSO-d_6, δ = 2.50 ppm and CD_3OD, δ = 3.35 ppm) and/or TMS as internal standards for ^{1}H NMR as well as the deuterated solvents (CD_2Cl_2, δ = 53.8 ppm; DMSO-d_6, δ = 39.5 ppm and CD_3OD, δ = 49.3 ppm) and/or TMS as internal standards for ^{13}C NMR. The chemical shifts are reported in ppm, downfield from TMS (s, d, t, q, q^5, dd, td, m, b for singlet, doublet, triplet, quadruplet, quintuplet, doublet of doublet, triplet of doublet, multiplet (or multi sharp-peak system), and broad, respectively). The mass spectra (MS) were measured on a Nermag R10−10 spectrometer (DCI/NH_4^+), a Quantum TCQ Discovery spectrometer (ES+), or a Perseptive Biosystem DE-RP MALDI-TOF Voyager spectrometer (ES+).

Miscellaneous. Radiosyntheses using fluorine-18, including the HPLC purifications, were performed in a 7.5-cm-lead shielded cell using a computer assisted Zymate robot system (Zymark corporation, USA).

Radioisotope Production. No-carrier-added aqueous [18F]fluoride ion was produced via the [^{18}O(p,n)^{18}F] nuclear reaction by irradiation of a 2 mL [^{18}O]water (>97%-enriched) target on a CGR-MeV 520 cyclotron (17 MeV proton beam) or on a IBA Cyclone-18/9 cyclotron (18 MeV proton beam) and was transferred to the appropriate hot cell. Target hardware: CGR-MeV 520 cyclotron: home-made, large volume, three-port, keyhole-shaped stainless steel/silver target holder (a complete description of this target hardware and -operation can be found in ref 81); IBA Cyclone-18/9 cyclotron: commercial, large volume, two-port, stainless steel target holder equipped with a domed-end niobium cylinder insert. Target to hot cell liquid-transfer system: 60 m PTFE line (0.8 mm internal diameter; 1/16 in. external diameter), 2.0 bar He drive pressure, transfer time 3−6 min). Typical production of [18F]fluoride at the end of bombardment for a 20 μA, 30 min (10 μA·h) irradiation: CGR-MeV 520 cyclotron: 550−650 mCi (20.3−24.0 GBq) and IBA Cyclone-18/9 cyclotron: 750−800 mCi (27.7−29.6 GBq).

2. Chemistry. Preparation of [(2-Nitropyridin-3-yloxy)alkyl]carbamic Acid *tert*-Butyl Esters (3a−e). *General Procedure.* To a solution of the appropriate (*N*-Boc-amino)alcohol (**2a−e**, 9−12 mmol) in THF (100−150 mL) were added 0.8 equiv of triphenylphosphine (MW: 262.29), 0.8 equiv of 3-hydroxy-2-nitropyridine (MW: 139.01), and dropwise 1.38 equiv of diisopropylazodicarboxylate (DIAD, MW: 202.21, d: 1.027). After stirring at room temperature for 24−28 h, the reaction mixture was concentrated to dryness and the residue was purified by column chromatography on silica gel. Elution with EtOAc/heptane (60/40 (v/v)) gave >90% pure [(2-nitropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl ester (**3a−e**). For analytical purposes only, an aliquot was further purified by preparative HPLC [HPLC A].

*[2-(2-Nitropyridin-3-yloxy)ethyl]carbamic Acid tert-Butyl Ester (**3a**).* The procedure described above was used with 2-(*N*-Boc-amino)ethanol (**2a**, 2.00 g) to give 1.35 g of **3a** as a pale-yellow oil (38%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.74. t_R (HPLC A, EtOAc/heptane: 50/50 (v/v), flow rate: 25 mL/min): 10.3 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.07 (bs, $w_{1/2}$ = 9.0 Hz, 1H); 7.55 (bs, $w_{1/2}$ = 7.0 Hz, 2H); 5.12 (b, 1H); 4.19 (t, J = 5.4 Hz, 2H); 3.52 (q, J = 5.4 Hz, 2H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.9 (C); 149.8 (C); 147.3 (C); 139.9 (CH); 129.3 (CH); 124.4 (CH); 79.9 (C); 69.6 (CH_2); 40.1 (CH_2); 28.5 (3×CH_3). MS (DCI/NH_4^+) $C_{12}H_{17}N_3O_5$: 284 [M + H$^+$].

*[3-(2-Nitropyridin-3-yloxy)propyl]carbamic Acid tert-Butyl Ester (**3b**).* The procedure described above was used with 3-(*N*-Boc-amino)propan-1-ol (**2b**, 2.00 g) to give 1.37 g of **3b** as a pale-yellow oil (40%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.19. t_R (HPLC A, EtOAc/heptane: 50/50 (v/v), flow rate: 25 mL/min): 11.5 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.06 (t, J = 3.3 Hz, 1H); 7.55 (d, J = 3.0 Hz, 2H); 4.20 (t, J = 5.7 Hz, 2H); 3.28 (q, J = 6.3 Hz, 2H); 2.03 (q^5, J = 6.3 Hz, 2H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.3 (C); 149.2 (C); 147.3 (C); 139.5 (CH); 129.2 (CH); 124.0 (CH); 79.2 (C); 68.3 (CH_2); 37.9 (CH_2); 29.5 (CH_2); 28.4 (3×CH_3). MS (DCI/NH_4^+) $C_{13}H_{19}N_3O_5$: 298 [M + H$^+$].

*[4-(2-Nitropyridin-3-yloxy)butyl]carbamic Acid tert-Butyl Ester (**3c**).* The procedure described above was used with 4-(*N*-Boc-amino)butan-1-ol (**2c**, 2.00 g) to give 1.19 g of **3c** as a pale-yellow oil (36%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.42. t_R (HPLC A, EtOAc/heptane: 50/50 (v/v), flow rate: 25 mL/min): 11.4 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.04 (t, $w_{1/2}$ < 2.0 Hz, 1H); 7.53 (bd, $w_{1/2}$ < 2.0 Hz, 2H); 4.73 (b, 1H); 4.15 (t, J = 6.6 Hz, 2H); 3.14 (q, J = 6.6 Hz, 2H); 1.84 (q^5, J = 6.6 Hz, 2H); 1.63 (q^5, J = 6.6 Hz, 2H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.2 (C); 149.8 (C); 147.3 (C); 139.3 (CH); 129.0 (CH); 123.9 (CH); 79.1 (C); 69.8 (CH_2); 40.2 (CH_2); 28.4 (3×CH_3); 26.8 (CH_2); 26.6 (CH_2). MS (DCI/NH_4^+) $C_{14}H_{21}N_3O_5$: 312 [M + H$^+$].

*[5-(2-Nitropyridin-3-yloxy)pentyl]carbamic Acid tert-Butyl Ester (**3d**).* The procedure described above was used with 5-(*N*-Boc-amino)pentan-1-ol (**2d**, 2.00 g) to give 0.93 g of **3d** as a pale-yellow solid (29%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.35. t_R (HPLC A, EtOAc/heptane: 50/50 (v/v), flow rate: 25 mL/min): 10.0 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.03 (t, J = 3.0 Hz, 1H); 7.53 (bd, 2H); 4.70 (b, 1H); 4.13 (t, J = 6.3 Hz, 2H); 3.09 (q, J = 6.3 Hz, 2H); 1.82 (q^5, J = 6.3 Hz, 2H); 1.60−1.40 (bm, 4H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.2 (C); 149.3 (C); 147.3 (C); 139.2 (CH); 128.9 (CH); 123.8 (CH); 78.9 (C); 70.0 (CH_2); 40.6 (CH_2); 30.0 (CH_2); 28.7 (CH_2); 28.4 (3×CH_3); 23.3 (CH_2). MS (DCI/NH_4^+) $C_{15}H_{23}N_3O_5$: 326 [M + H$^+$].

*[6-(2-Nitropyridin-3-yloxy)hexyl]carbamic Acid tert-Butyl Ester (**3e**).* The procedure described above was used with 6-(*N*-Boc-amino)hexan-1-ol (**2e**, 2.00 g) to give 1.25 g of **3e** as a pale-yellow solid (40%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.17. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 30 mL/min): 8.6 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.03 (t, J = 3.0 Hz, 1H); 7.52 (bd, 2H); 4.65 (b, 1H); 4.12 (t, J = 6.6 Hz, 2H); 3.07 (q, J = 6.6 Hz, 2H); 1.81 (q^5, J = 6.6 Hz, 2H); 1.70−1.30 (bm, 15H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.2 (C); 149.6 (C); 147.4 (C); 139.2 (CH); 128.9 (CH); 123.8 (CH); 78.9 (C); 70.1 (CH_2); 40.7 (CH_2); 30.3 (CH_2); 28.9 (CH_2); 28.5 (3×CH_3); 26.6 (CH_2); 25.8 (CH_2). MS (DCI/NH_4^+) $C_{16}H_{25}N_3O_5$: 340 [M + H$^+$].

Preparation of [(2-fluoropyridin-3-yloxy)alkyl]-carbamic Acid *tert*-Butyl Esters (4a−e). *General Procedure.* To a solution of the appropriate (*N*-Boc-amino)alcohol (**2a−e**, 9−12 mmol) in THF (100−150 mL) were added 0.8 equiv of triphenylphosphine (MW: 262.29), 0.8 equiv of 2-fluoro-3-hydroxypyridine (MW: 113.09), and dropwise 1.38 equiv of diisopropylazodicarboxylate (DIAD; MW: 202.21, d: 1.027). After addition, the reaction mixture was stirred at room temperature for 24−28 h. The reaction mixture was concentrated to dryness, and the residue was purified by column chromatography on silica gel. Elution with heptane/EtOAc (60/40 (v/v)) gave >90% pure [(2-fluoropyridin-3-yloxy)-

alkyl]carbamic acid *tert*-butyl ester (**4a**−**e**). For analytical purposes only, an aliquot was further purified by preparative HPLC [HPLC A].

*[2-(2-Fluoropyridin-3-yloxy)ethyl]carbamic Acid tert-Butyl Ester (**4a**).* The procedure described above was used with 2-(*N*-Boc-amino)ethanol (**2a**, 2.00 g) to give 0.86 g of **4a** as a pale-yellow solid (27%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.55. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 25 mL/min): 8.1 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.71 (bs, $w_{1/2}$ = 9.0 Hz, 1H); 7.32 (t, J = 8.7 Hz, 1H); 7.13 (dd, J = 7.8 and 4.8 Hz, 1H); 5.05 (b, 1H); 4.09 (t, J = 8.7 Hz, 2H); 3.54 (q, J = 5.4 Hz, 2H); 1.42 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.1 (C); 154.0 (d, J^1_{F-C} = 235 Hz, C); 142.3 (d, J^2_{F-C} = 26 Hz, C); 137.8 (d, J^3_{F-C} = 13 Hz, CH); 123.2 (CH); 122.3 (CH); 79.7 (C); 68.9 (CH_2); 40.2 (CH_2); 28.4 (3×CH_3). MS (DCI/NH_4^+) $C_{12}H_{17}F_1N_2O_3$: 257 [M + H$^+$].

*[3-(2-Fluoropyridin-3-yloxy)propyl]carbamic Acid tert-Butyl Ester (**4b**).* The procedure described above was used with 3-(*N*-Boc-amino)propan-1-ol (**2b**, 2.00 g) to give 1.51 g of **4b** as a pale-yellow oil (49%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.44. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 25 mL/min): 10.2 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.68 (dt, J = 3.0 and 1.5 Hz, 1H); 7.28 (td, J = 7.8 Hz and 1.5 Hz, 1H); 7.10 (dd, J = 8.1 and 5.1 Hz, 1H); 4.07 (t, J = 6.3 Hz, 2H); 3.27 (q, J = 6.3 Hz, 1H); 1.98 (q^5, J = 6.4 Hz, 2H); 1.39 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.3 (C); 154.1 (d, J^1_{F-C} = 237 Hz, C); 142.5 (d, J^2_{F-C} = 27 Hz, C); 137.5 (d, J^3_{F-C} = 15 Hz, CH); 123.0 (CH); 122.2 (CH); 79.2 (C); 67.3 (CH_2); 38.0 (CH_2); 29.8 (CH_2); 28.5 (3×CH_3). MS (DCI/NH_4^+) $C_{13}H_{19}F_1N_2O_3$: 271 [M + H$^+$].

*[4-(2-Fluoropyridin-3-yloxy)butyl]carbamic Acid tert-Butyl Ester (**4c**).* The procedure described above was used with 4-(*N*-Boc-amino)butan-1-ol (**2c**, 2.00 g) to give 1.14 g of **4c** as a pale-yellow oil (38%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.36. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 20 mL/min): 5.9 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.69 (td, J = 1.5 and 3.0 Hz, 1H); 7.30 (t, J = 8.7 Hz, 1H); 7.11 (dd, J = 7.8 and 5.1 Hz, 1H); 4.76 (b, 1H); 4.05 (t, J = 4.5 Hz, 2H); 3.16 (q, J = 6.6 Hz, 2H); 1.84 (q^5, J = 6.9 Hz, 2H); 1.66 (q^5, J = 6.7 Hz, 2H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.8 (C); 153.9 (d, J^1_{F-C} = 235 Hz, C); 142.9 (d, J^2_{F-C} = 26 Hz, C); 137.3 (d, J^3_{F-C} = 13 Hz, CH); 123.0 (CH); 122.2 (CH); 79.5 (C); 69.3 (CH_2); 40.4 (CH_2); 28.5 (3×CH_3); 27.0 (CH_2); 26.6 (CH_2). MS (DCI/NH_4^+) $C_{14}H_{21}F_1N_2O_3$: 285 [M + H$^+$].

*[5-(2-Fluoropyridin-3-yloxy)pentyl]carbamic Acid tert-Butyl Ester (**4d**).* The procedure described above was used with 5-(*N*-Boc-amino)pentan-1-ol (**2d**, 2.00 g) to give 0.90 g of **4d** as a pale-yellow solid (31%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.43. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 20 mL/min): 5.4 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.68 (bs, J < 1.5 Hz, 1H); 7.28 (t, J = 8.1 Hz, 1H); 7.10 (dd, J = 5.1 and 7.8 Hz, 1H); 4.61 (b, 1H); 4.02 (t, J = 6.6 Hz, 2H); 3.10 (q, J = 5.7 Hz, 2H); 1.83 (q^5, J = 6.6 Hz, 2H); 1.60−1.40 (bm, 4H); 1.40 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 155.8 (C); 154.1 (d, J^1_{F-C} = 235 Hz, C); 142.3 (d, J^2_{F-C} = 25 Hz, C); 136.8 (d, J^3_{F-C} = 13 Hz, CH); 122.4 (CH); 121.8 (CH); 79.8 (C); 69.5 (CH_2); 40.3 (CH_2); 29.8 (CH_2); 28.6 (CH_2); 28.1 (3×CH_3); 23.1 (CH_2). MS (DCI/NH_4^+) $C_{15}H_{23}F_1N_2O_3$: 299 [M + H$^+$].

*[6-(2-Fluoropyridin-3-yloxy)hexyl]carbamic Acid tert-Butyl Ester (**4e**).* The procedure described above was used with 6-(*N*-Boc-amino)hexan-1-ol (**2e**, 2.00 g) to give 0.90 g of the **4e** as a pale-yellow oil (31%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.48. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 20 mL/min): 8.9 min. ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.68 (bs, 1H); 7.29 (t, J = 8.1 Hz, 1H); 7.11 (dd, J = 7.8 and 5.1 Hz, 1H); 6.82 (b, 1H); 4.02 (t, J = 6.6 Hz, 2H); 3.08 (q, J = 5.7 Hz, 2H); 1.83 (q^5, J = 6.6 Hz, 2H); 1.50 (bm, 6H); 1.41 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.0 (C); 154.4 (d, J^1_{F-C} = 235 Hz, C); 142.8 (d, J^2_{F-C} = 25 Hz, C); 137.2 (d, J^3_{F-C} = 13 Hz, CH); 122.9 (CH); 122.2 (CH); 79.0 (C); 70.1 (CH_2); 40.8 (CH_2); 30.3 (CH_2); 29.2 (CH_2); 28.5 (3×CH_3); 26.8 (CH_2); 25.9 (CH_2). MS (DCI/NH_4^+) $C_{16}H_{25}F_1N_2O_3$: 313 [M + H$^+$].

Preparation of [3-(2-Fluoro/nitro-pyridin-3-yloxy)]propylamines (5, 6). *General Procedure.* To a solution of the appropriate [3-(2- fluoro/nitro-pyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester (**3b** or **4b**, 0.5−5.0 mmol) in CH_2Cl_2 (1−10 mL) at 0−3 °C was added TFA (10−30% (v/v)). After stirring at room temperature for 1 h, the reaction mixture was concentrated to dryness to give the pure 3-(2- fluoro/nitro-pyridin-3-yloxy)propylamines (**5** and **6**, respectively) as their TFA salts (oils).

*[3-(2-Fluoropyridin-3-yloxy)]propylamine (**5**).* The procedure described above was used with [3-(2-fluoropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester (**4b**, 0.40 g) to give 0.50 g of **5** as a pale-yellow oil (quantitative). ^{1}H NMR (CD_3OD, 298 K): δ: 7.51 (bs, $w_{1/2}$ = 5.0 Hz, 1H); 7.36 (bt, $w_{1/2}$ = 25.0 Hz, 1H); 7.04 (bd, $w_{1/2}$ = 15.0 Hz, 1H); 4.03 (bt, $w_{1/2}$ = 15.0 Hz, 2H); 2.99 (bt, $w_{1/2}$ = 15.0 Hz, 2H); 2.01 (bq, $w_{1/2}$ = 15.0 Hz, 2H). ^{13}C NMR (CD_3OD, 298 K): δ: 155.3 (d, J^1_{F-C} = 237 Hz, C); 144.3 (d, J^2_{F-C} = 24 Hz, C); 138.5 (d, J^3_{F-C} = 13 Hz, CH); 125.1 (CH); 123.8 (CH); 67.9 (CH_2); 38.6 (CH_2); 28.4 (CH_2). MS (DCI/NH_4^+) $C_8H_{11}F_1N_2O_1$: 171 [M + H$^+$].

*[3-(2-Nitropyridin-3-yloxy)]propylamine (**6**).* The procedure described above was used with [3-(2-nitropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester (**3b**, 1.50 g) to give 2.00 g of **6** as a yellow oil (quantitative). ^{1}H NMR (CD_3OD, 298.0K): δ: 8.04 (d, J = 3.0 Hz, 1H); 7.82 (d, J = 9.0 Hz, 1H); 7.65 (dd, 9.0 and 3.0 Hz, 1H); 4.30 (t, J = 6.0 Hz, 1H); 3.12 (t, J = 6.0 Hz, 2H); 2.15 (q^5, J = 6.0 Hz, 2H). ^{13}C NMR (CD_3OD, 298.0K): δ: 149.8 (C); 148.5 (C); 140.8 (CH); 130.9 (CH); 126.6 (CH); 68.6 (CH_2); 38.7 (CH_2); 28.1 (CH_2). MS (DCI/NH_4^+) $C_8H_{11}N_3O_3$: 198 [M + H$^+$].

Preparation of 1-[3-(2-Fluoro/nitropyridin-3-yloxy)propyl]pyrrole-2,5-diones (1, 7). *General Procedure.* To a solution of the appropriate 3-(2-fluoro/nitropyridin-3-yloxy)propylamine (**5** or **6**, 3−5 mmol) in THF (100−170 mL) was added 5.0 equiv of *N*-methoxycarbonylmaleimide. The mixture was stirred at reflux overnight. A precipitate was formed and filtered. The obtained filtrate was concentrated to dryness, and the residue was purified by column chromatography on silica gel. Elution with heptane/EtOAc (50/50 (v/v)) gave pure 1-[3-(2-halogeno-pyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**,**7**) as a white powder.

*1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME).* The procedure described above was used with 3-(2-fluoropyridin-3-yloxy)propylamine (**5**, 0.50 g) to give 0.55 g of **1** (FPyME) as a white powder (75%). R_f (EtOAc/heptane: 50/50 (v/v)): 0.40. t_R (HPLC D): 10.2 min. ^{1}H NMR (CD_2Cl_2, 298.0K): δ: 7.69 (bd, J = 3.0 Hz, 1H); 7.27 (t, J = 9.0 Hz, 1H); 7.11 (dd, J = 9.0 and 3.0 Hz, 1H); 6.69 (s, 2H); 4.04 (t, J = 6.0 Hz, 2H); 3.72 (t, J = 6.0 Hz, 2H); 2.11 (q^5, J = 6.0 Hz, 2H). ^{13}C NMR (CD_2Cl_2, 298.0K): δ: 171.2 (2×C); 154.1 (d, J^1_{F-C} = 235 Hz, C); 142.4 (d, J^2_{F-C} = 25 Hz, C); 137.7 (d, J^3_{F-C} = 13 Hz, CH); 134.5 (2×CH); 123.2 (CH); 122.2 (CH); 67.5 (CH_2); 35.4 (CH_2); 28.4 (CH_2). MS (DCI/NH_4^+) $C_{12}H_{11}F_1N_2O_3$: 251 [M + H$^+$].

*1-[3-(2-Nitropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**7**).* The procedure described above was used with 3-(2-nitropyridin-3-yloxy)propylamine (**6**, 1.00 g) to give 0.40

g of **7** as a white powder (28%). R_f (CH_2Cl_2/AcOEt: 60/40 (v/v)): 0.40. ^1H NMR (CD_2Cl_2, 298.0K): δ: 8.05 (bd, $w_{1/2}$ = 9.0 Hz, 1H); 7.51 (bd, J = 3.0 Hz, 2H); 6.67 (s, 2H); 4.13 (t, J = 6.0 Hz, 2H); 3.69 (q, J = 6.0 Hz, 2H); 2.11 (q^5, J = 6.0 Hz, 2H). ^{13}C NMR (CD_2Cl_2, 298.0K): δ: 171.1 (2xC); 149.5 (C); 147.5 (C); 139.6 (CH); 134.5 (2×CH); 129.0 (CH); 124.0 (CH); 68.0 (CH_2); 35.1 (CH_2); 28.4 (CH_2). MS (DCI/NH_4^+) $C_{12}H_{11}N_3O_5$: 278 [M + H^+].

*[3-(2-Dimethylaminopyridin-3-yloxy)propyl]carbamic Acid tert-Butyl Ester (**8**).* To a solution of 3-(*N*-Boc-amino)-propan-1-ol (**2b,** 0.31 g, 1.8 mmol) in THF (20 mL) were added 0.47 g of triphenylphosphine (MW: 262.29, 1.8 mmol, 1.0 equiv), 0.25 g of 2-(dimethylamino)-3-hydroxypyridine (MW: 138.00, 1.81 mmol, 1.0 equiv) and dropwise 0.35 mL of diisopropylazodicarboxylate (DIAD; MW: 202.21, d: 1.027, 1.8 mmol, 1.0 equiv). After stirring at room temperature for 24 h, the reaction mixture was concentrated to dryness and the residue was purified by column chromatography on silica gel. Elution with heptane/EtOAc (70/30 (v/v)) gave 0.23 g of **8** as a yellow oil (43%). For analytical purposes only, an aliquot was further purified by HPLC. R_f (EtOAc/heptane: 50/50 (v/v)): 0.35. t_R (HPLC A, EtOAc/heptane: 40/60 (v/v), flow rate: 35 mL/min): 8.6 min. ^1H NMR (CD_2Cl_2, 298 K): δ: 7.77 (dd, J = 4.8 Hz, 1H); 7.00 (dd, J = 7.8 Hz, J < 1.5 Hz, 1H); 6.72 (dd, J = 7.8 and 4.8 Hz, 1H); 4.02 (t, J = 6.0 Hz, 2H); 3.30 (q, J = 6.0 Hz, 2H); 2.93 (s, 6H); 2.00 (q^5, J = 6.0 Hz, 2H); 1.42 (s, 9H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.6 (C); 153.7 (C); 146.2 (C); 139.0 (CH); 118.8 (CH); 115.9 (CH); 79.1 (C); 67.4 (CH_2); 41.1 (2×CH_3); 38.8 (CH_2); 32.3 (CH_2); 28.5 (3×CH_3). MS (DCI/NH_4^+) $C_{15}H_{25}N_3O_3$: 296 [M + H^+].

*[3-(3-tert-Butoxycarbonylaminopropoxy)pyridin-2-yl]trimethylammonium Trifluoromethanesulfonate (**9**).* To a solution of [3-(2-dimethylaminopyridin-3-yloxy)propyl]-carbamic acid *tert*-butyl ester (8, 0.4 g, 1.35 mmol) in toluene was added 0.2 mL of methanetrifluoromethylsulfonate (MW: 164.10, d: 1.45, 1.77 mmol, 1.3 equiv) at 0 °C. After 3 min, a precipitate was obtained and the reaction mixture was stirred at room temperature for 1 h. The precipitate was filtered and washed with Et_2O to give 0.58 g of **9** as a white powder (93%). ^1H NMR (CD_2Cl_2, 298 K): δ: 8.10 (bd, J = 3.3 Hz, 1H); 7.66 (d, J = 8.1 Hz, 1H); 7.60 (dd, J = 6.1 and 4.2 Hz, 1H); 4.31 (t, J = 6.3 Hz, 2H); 3.71 (s, 9H); 3.31 (q, J = 6.3 Hz, 2H); 2.12 (q^5, J = 6.3 Hz, 2H); 1.38 (s, 9H).^{13}C NMR (CD_2Cl_2, 298 K): δ: 156.6 (C); 147.7 (C); 142.6 (C); 139.0 (CH); 129.0 (CH); 124.6 (CH); 121.2 (q, J = 319 Hz, CF_3); 79.3 (C); 68.1 (CH_2); 37.5 (CH_2); 30.0 (CH_2); 28.4 (3×CH_3).

3. Peptide/Protein Chemistry: Conjugation with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (1, FPyME). Conjugation of (*N*-Ac)KAAAAC (P1) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (1, FPyME) at the Micromolar Scale. To 5.2 mg of (*N*-Ac)KAAAAC (**P1**, 9.0 μmol, MW: 575.69 g/mol), dissolved in a mixture of ethanol (450 μL), 50 mM aq phosphate buffer saline (PBS, at pH 6.5, 200 μL) and acetonitrile (450 μL), was added 4.5 mg of 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME, 18.0 μmol, 2 equiv). The reaction mixture was stirred at room temperature for 3 h and then purified by HPLC [HPLC B, t_R: 28.0 min]. The fractions containing the conjugated peptide, c-(*N*-Ac)KAAAAC (**c-P1**), were combined and then lyophilized, giving 2.0 mg of the conjugated peptide, c-(*N*-Ac)KAAAAC (**c-P1**). ^1H NMR (CD_3OD/D_2O, 50/50 (v/v)): δ: 7.65 (d, J: 4.8 Hz, 1H, Py); 7.53 (t, J: 9.6 Hz, 1H, Py); 7.27 (dd, J: 7.6 and 4.8 Hz, 1H, Py); 4.60 (m, 1H, α-CH-Lys); 4.31 (m, 1H, α-CH-Cys); 4.30−4.18 (4H, 4 × α-CH-Ala); 4.11 (m, 2H, CH_2O); 4.00 (ddd, J: 20.0 and 9.6 and 4.4 Hz, 1H, β-CH^1-Cys); 3.72 (m, 2H, CH_2N); 3.32 (m, 1H, β-CH^2-Cys); 3.25−3.10 (m, 2H, CH_2CO); 2.96 (m, 2H, ϵ-CH_2-Lys); 2.58 (m, 1H, CHCO); 2.09 (m, 2H, CH_2); 2.00 (s, 3H, *N*-Ac); 1.79 (m, 1H, β-CH^1-Lys); 1.74−1.60 (m, 3H, β-CH^2-Lys/δ-CH_2-Lys); 1.42 (m, 2H, γ-CH_2-Lys); 1.40−1.32 (m, 12H, 4 × β-CH_3-Ala). MS found: 826.3 (calcd: 825.91).

Conjugation of (*N*-Ac)KAAAAC (P1) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (1, FPyME) at the Submicromolar Scale. *(A) Preparation of (N-Ac)KAAAAC (**P1**) for Conjugation.* To 0.126 μmol (72.5 μg) of (*N*-Ac)KAAAAC (**P1**, MW: 575.69 g/mol, 36 μL of a 2.0 mg/mL or 3.5 μmol/mL solution) in aq Tris NaCl (50 mM Tris-HCl; 150 mM NaCl) at pH 7.4 was added 0.193 μmol of TCEP (1.5 equiv, 7 μL of a 7.9 mg/mL or 27.6 μmol/mL solution) in solution in the same buffer. The resulting solution was left standing at room temperature for 5 min.

*(B) Conjugation of (N-Ac)KAAAAC (**P1**) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME).* To 10 μL of DMSO containing 3−5 equiv of 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME, 190−315 μg) was added TCEP-treated (*N*-Ac)KAAAAC (**P1**, about 145 μg, without removal of the excess of TCEP) diluted in aq Tris NaCl, pH 7.4 (1 mL). The resulting solution was left standing at room temperature for 15 min, then the sample was purified by HPLC [HPLC B, t_R: 28.0 min].

Conjugation of AFIM-0 (P2) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (1, FPyME) at the Submicromolar Scale. *(A) Preparation of AFIM-0 (**P2**) for Conjugation.* To a solution of AFIM-0 (**P2**, 1.0 mg; MW: 8576.78 g/mol) in 1 mL of aq Tris NaCl (50 mM Tris-HCl; 150 mM NaCl) at pH 7.4 was added TCEP (0.3 mg, 10 equiv) in solution in the same buffer (500 μL). The resulting solution was left standing at room temperature for 15 min. The excess of TCEP was removed from the solution by gel filtration using a NAP-10 G25 Sephadex cartridge (Amersham Pharmacia Biotech). The TCEP-treated (reduction of S−S bond, regeneration of free SH function) AFIM-0 (**P2**) was eluted from the cartridge in 1.5 mL of aq Tris NaCl buffer (according to manufacturer's instructions) and used within 15 min after purification.

*(B) Conjugation of AFIM-0 (**P2**) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME).* To 100 μL of DMSO or acetonitrile containing 3−5 equiv of 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME, 42−71 μg) was added 750 μL of TCEP-treated AFIM-0 (**P2**, about 0.5 mg) in aq Tris NaCl, pH 7.4. The resulting solution was left standing at room temperature for 30 min, then the sample was purified by gel filtration using a NAP-10 G25 Sephadex cartridge (Amersham Pharmacia Biotech). The conjugated peptide, c-AFIM-0 (**c-P2**), was easily separated from nonreacted 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME) and was eluted from the cartridge in 1.5 mL of aq PBS (20 mM KH_2PO_4/150 mM NaCl; pH 7.4) according to manufacturer's instructions. For analytical purposes only, the conjugated peptide, c-AFIM-0 (**c-P2**), was further purified by chromatography on gel filtration [HPLC C, t_R: 14.5 min]. MS (ESI+): 8827.0 (theor.), 8830.5 (exp.).

*(C) Conjugation of STxB (**P3**) with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME).* To 10 μL of DMSO containing 1 equiv of 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (**1**, FPyME) was added 90 μL of STxB (**P3**, about 0.2 mg) in 0.1 M aq PBS at pH 8. The resulting solution was left standing at room temperature for 30 min, then the sample was purified by

dialysis against water. The conjugated peptide, c-STxB (**c-P3**), was characterized by RP-HPLC [HPLC E: t_R: 12.5 min] and gel filtration [HPLC F: t_R: 9.5 min]. MS (ESI+): 8043.0 (theor.), 8048.0 (exp.).

4. Radiochemistry. Preparation of the K[^{18}F]F-K_{222} Complex. To recover and recycle the [^{18}O]water target, 2 mL of aqueous [^{18}F]fluoride from the target holder was passed through an anion-exchange resin (Sep-Pak Light Waters Accell Plus QMA Cartridge in the chloride form, washed with 5 mL of 1M aq $NaHCO_3$, and then rinsed with 50 mL of water) by helium pressure (1.5−2.0 bar). Helium is blown through the column to maximally extract the [^{18}O]water. The [^{18}F]fluoride ion is then eluted from the resin using 1.0 mL of a 4.5 mg/mL aqueous K_2CO_3 solution into a Vacutainer tube containing 12.0 to 15.0 mg of Kryptofix222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane). The resulting solution was then gently concentrated to dryness at 145−150 °C under a nitrogen stream for 10 min to give no-carrier-added K[^{18}F]F-K_{222} complex as a white semisolid residue.

If desired, the [^{18}F]fluoride ion production batch on the cartridge can also be divided into 2 to 12 aliquots in order to perform parallel syntheses. To do this, the [^{18}F]fluoride ion is eluted from the resin using 1.0 mL of a 4.5 mg/mL aqueous K_2CO_3 solution into an empty Vacutainer tube. To distribute equally this activity over n tubes (Vacutainer tube, $n = 2-12$), the quantity of K_2CO_3 was first adjusted to n times 4.5 mg with a 50.0 mg/mL aqueous K_2CO_3 solution and second, the total volume of the solution was adjusted to 2.0 mL with water. This new aqueous [^{18}F]fluoride solution was then equally distributed over the n tubes each containing 12.0 to 15.0 mg of Kryptofix222. Finally, the volume of each fraction was adjusted to 1.0 mL with water. The resulting solutions were then independently gently concentrated to dryness at 145−150 °C under a nitrogen stream for 10 min to give no-carrier-added K[^{18}F]F-K_{222} complex as a white semisolid residue.

[(2-[^{18}F]Fluoropyridin-3-yloxy)alkyl]carbamic Acid *tert*-Butyl Esters ([^{18}F]4a−e): Incorporation Studies with K[^{18}F]F-K_{222} Complex. *(A) General Procedure Using Conventional Heating.* A 600 μL amount of DMSO containing 10 to 17 μmol of the appropriate [(2-nitropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl ester **(3a−e)** as precursor for labeling was directly added into the Vacutainer tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was thoroughly vortexed (15 s) and then placed in a heating block (at 145 °C for 1 to 30 min) without stirring the contents. The reaction vessel was then cooled using an ice−water bath, and the remaining radioactivity was measured. A 90% to 95% level of the initial radioactivity placed in the vessel was still present. The resulting often dark-colored reaction mixture was then analyzed by radiochromatography. The reaction yield was calculated from the TLC-radiochromatogram and defined as the radioactivity area of the [(2-[^{18}F]fluoropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl ester ([^{18}F]**4a−e**) over total fluorine-18 radioactivity area ratio. (SiO_2-TLC: (A) eluent: EtOAc/heptane: 50/50 (v/v), R_f: [^{18}F]**4a−e**: 0.40−0.60 and R_f: [^{18}F]fluoride ion: 0.0; (B) eluent: EtOAc, R_f: [^{18}F]**4a−e**: 0.75−0.80 and R_f: [^{18}F]fluoride ion: 0.0).

(B) General Procedure Using Microwave Activation. A 600 μL amount of DMSO containing 10 to 17 μmol of the appropriate [(2-nitropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl ester **(3a−e)** as precursor for labeling was directly added into the Vacutainer tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was thoroughly vortexed (15 s) and then placed in a dedicated microwave oven (at 100 W, for 0.5 to 3 min) without stirring the contents. The remainder of the synthesis used the same procedure as described above.

Preparation of 1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]-1, [^{18}F]FPyME). *(A) Procedure Using 3-(2-Nitropyridin-3-yloxy)propyl]carbamic Acid tert-Butyl Ester (**3b**) as Precursor for Labeling.* A 600 μL amount of freshly distilled DMSO containing 5.0 mg of 3-(2-nitropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester (**3b**) as the precursor for labeling was directly added into the Vacutainer tube containing the dried K[^{18}F]F-K_{222} complex. The tube (not sealed) was then thoroughly vortexed (15 s) and then placed in a heating block at 145 °C for 4 min without stirring the contents. The reaction mixture was diluted with 1 mL of water and transferred on a C18 Sep-pak cartridge (PrepSep R-C18, Fisher Scientific, activated beforehand with 2 mL of EtOH and then rinsed with 10 mL of water). The tube was rinsed twice with 1 mL of water which was also transferred and added to the diluted reaction mixture on the cartridge. After addition of another 2 mL of water, the whole was passed through the cartridge. The cartridge was washed with 1 mL of water and partially dried for 0.5 min by applying a nitrogen stream. The [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester ([^{18}F]-**4b**) was eluted from the cartridge with 3 mL of CH_2Cl_2 into a 5 mL reaction vial containing 0.1 mL of TFA. Twice 1 mL of CH_2Cl_2 was used to wash the cartridge and to completely transfer [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester ([^{18}F]-**4b**) (5% of the total radioactivity amount engaged in the fluorination process was left on the cartridge). The incorporation yield was also estimated after the C18 Sep-pak cartridge elution by the CH_2Cl_2 over total eluted radioactivity (DMSO/H_2O + CH_2Cl_2) ratio. The resulting CH_2Cl_2/TFA solution (50/1, v/v) was concentrated to dryness (at 65−75 °C under a gentle nitrogen stream for 4−6 min). The yield of deprotection was quantitative and no Boc-protected [^{18}F]-**4b** could be detected by radiochromatography (SiO_2-TLC, eluent: EtOAc, R_f: [^{18}F]-**4b**: 0.75 and R_f: [^{18}F]-**5**: 0.0). The above residue, containing 3-(2-[^{18}F]fluoropyridin-3-yloxy)propylamine ([^{18}F]-**5**) was redissolved in 2 mL of CH_2Cl_2 and concentrated again to dryness to minimize TFA presence (at 65−75 °C under a gentle nitrogen stream for another 2−3 min). The residue, containing [^{18}F]-**5**, was then redissolved in 0.25 mL of dioxane containing 25 mg of *N*-methoxycarbonylmaleimide. Aqueous sat. $NaHCO_3$ (0.75 mL) was then added to the solution, and the vessel was gently vortexed for 10 min at room temperature. The reaction mixture was diluted with 1 mL of aq 1M HCl and transferred onto a C18 Sep-pak cartridge (PrepSep R-C18, Fisher Scientific, activated beforehand with 2 mL of EtOH, rinsed with 10 mL of water, and loaded with 5 mL of water). The tube was rinsed twice with 1 mL of water which was also transferred and added to the diluted reaction mixture on the cartridge. The whole was passed through the cartridge. The cartridge was washed with 3 mL of water and partially dried for 0.5 min by applying a nitrogen stream. The 1-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]pyrrole-2,5-dione ([^{18}F]-**1**, [^{18}F]FPyME) was eluted from the cartridge with 3 mL of CH_2Cl_2. Twice 1 mL of CH_2Cl_2 was used to wash the cartridge and to completely transfer [^{18}F]FPyME ([^{18}F]-**1**) (10−25% of the radioactivity amount was left on the cartridge). The resulting CH_2Cl_2 solution was partially (not to dryness) concentrated (at 65−75 °C under a gentle nitrogen stream for 2−4 min). The residue was diluted up to a volume of 1.0−1.5

mL with CH_2Cl_2 and HPLC-purified [HPLC D, t_R: 10.0−10.5 min] to give pure labeled [18F]FPyME ([18F]-**1**).

*(B) Procedure Using [3-(3-tert-Butoxycarbonylaminopropoxy)pyridin-2-yl]trimethylammonium Trifluoromethanesulfonate (**9**) as Precursor for Labeling.* A 600 μL amount of freshly distilled DMSO containing 4.0 mg of [3-(3-*tert*-butoxycarbonylaminopropoxy)pyridin-2-yl]trimethylammonium trifluoromethanesulfonate (**9**) as the precursor for labeling were directly added into the Vacutainer tube containing the dried K[18F]F-K_{222} complex. The tube (not sealed) was then thoroughly vortexed (15 s) and then placed in a heating block at 145 °C for 2 min without stirring the contents. The remainder of the preparation used the same procedure as described above.

Conjugation of (*N*-Ac)KAAAAC (P1) with 1-[3-(2-[18F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([18F]-1, [18F]FPyME). Preparation of [18F]c-(*N*-Ac)KAAAAC ([18F]c-P1). To 50 μL of DMSO containing HPLC-purified [18F]FPyME ([18F]-**1**, freed from HPLC-solvents by concentration to dryness at 65−75 °C under a gentle nitrogen stream), was added TCEP-treated (*N*-Ac)KAAAAC (**P1**, about 72.5 μg, without removal of the excess of TCEP, see above) dissolved in aq Tris NaCl, pH 7.4 (1 mL). The resulting solution was left standing at room temperature for 10 min and then purified by HPLC [HPLC B, t_R: 28.0 min].

Conjugation of AFIM-0 (P2) with 1-[3-(2-[18F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([18F]-1, [18F]FPyME). *(A) Preparation of [18F]c-AFIM-0 ([18F]**c-P2**).* To 100 μL of DMSO containing HPLC-purified [18F]FPyME ([18F]-**1**, freed from HPLC-solvents by concentration to dryness at 65−75 °C under a gentle nitrogen stream), was added 750 μL of TCEP-treated AFIM-0 (**P2**, about 0.5 mg, see above) in aq Tris NaCl, pH 7.4. The reaction mixture was gently vortexed for 5 min and then purified by gel filtration on a NAP-10 G25 Sephadex cartridge (Amersham Pharmacia Biotech). The conjugated and labeled peptide, [18F]c-AFIM-0 ([18F]**c-P2**), was easily separated from nonreacted [18F]FPyME ([18F]-**1**), if any, and was eluted from the cartridge in 1.5 mL of aq 0.9% NaCl according to manufacturer's instructions.

*(B) Formulation and Quality Control of Conjugated and Labeled [18F]c-AFIM-0 ([18F]**c-P2**).* The cartridge-fraction containing the conjugated and labeled peptide, [18F]c-AFIM-0 ([18F]**c-P2**), was diluted with physiological saline and finally sterile-filtrated on a 0.22 μm GS-Millipore filter (vented). As demonstrated by HPLC analysis [HPLC C], radiosynthesized conjugated and labeled [18F]c-AFIM-0 ([18F]**c-P2**) coeluted with an authentic sample of reference compound (fluorine-19-synthesized c-AFIM-0 (**c-P2**)). The radiolabeled product was found to be >90% radiochemically pure. The radiopharmaceutical preparation was shown to be free of nonreacted [18F]FPyME ([18F]-**1**) and radiochemically stable for at least 240 min.

Conjugation of STxB (P3) with 1-[3-(2-[18F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([18F]-1, [18F]-FPyME). *(A) Preparation of [18F]c-STxB ([18F]**c-P3**).* To 100 μL of DMSO containing HPLC-purified [18F]FPyME ([18F]-**1**, freed from HPLC-solvents by concentration to dryness at 65−75°C under a gentle nitrogen stream), was added 330 μL of STxB (**P3**, about 1 mg) in aq 1.0 M PBS at pH 8. After addition of another 570 μL of 1.0 M aq PBS at pH 8, the reaction mixture was gently vortexed for 5 min and then purified by gel filtration on a NAP-10 G25 Sephadex cartridge (Amersham Pharmacia Biotech). The conjugated and labeled peptide, [18F]c-STxB ([18F]**c-P3**), was easily separated from nonreacted [18F]-FPyME ([18F]-**1**), if any, and was eluted from the cartridge in 1.5 mL of aq 0.9% NaCl according to manufacturer's instructions.

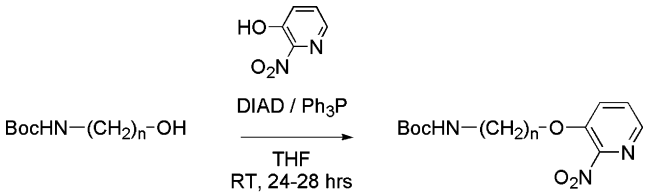
*(B) Formulation and Quality Control of Conjugated and Labeled [18F]c-STxB ([18F]**c-P3**).* The cartridge-fraction containing the conjugated and labeled peptide, [18F]c-STxB ([18F]**c-P3**), was diluted with physiological saline and finally sterile-filtrated on a 0.22 μm GS-Millipore filter (vented). As demonstrated by HPLC analysis [HPLC E and F], radiosynthesized conjugated and labeled [18F]c-STxB ([18F]**c-P3**) coeluted with an authentic sample of reference compound (fluorine-19-synthesized c-STxB (**c-P3**)). The radiolabeled product was found to be >95% radiochemically pure. The radiopharmaceutical preparation was shown to be free of nonreacted 1-[3-(2-[18F]fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([18F]-**1**, [18F]FPyME) and radiochemically stable for at least 400 min.

RESULTS AND DISCUSSION

Chemistry. The synthesis of FPyME (**1**) as well as that of the nitro derivatives **3b** and **7** and the trimethylammonium derivative **9** as the various selected precursors for fluorine-18 labeling is outlined in Schemes 1−4.

The [(2-nitropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl esters (**3a**−**e**) and [(2-fluoropyridin-3-yloxy)alkyl]-carbamic acid *tert*-butyl esters (**4a**−**e**) were all synthesized using the following general method. The nitro derivatives (**3a**−**e**) were prepared in 29−41% yield via a Mitsunobu coupling reaction (*51*) from the corresponding commercially available (hydroxyalkyl)carbamic acid *tert*-butyl esters (**2a**−**e**) and 2-nitro-3-hydroxypyridine (*37*, *40*), in THF at room temperature, using triphenylphosphine (0.8 equiv) and diisopropylazodicarboxylate (1.4 equiv) (Scheme 1).

Scheme 1. Preparation of [(2-Nitropyridin-3-yloxy)-alkyl]carbamic Acid *tert*-Butyl Ester (3a−e)



	n
2a	2
b	3
c	4
d	5
e	6

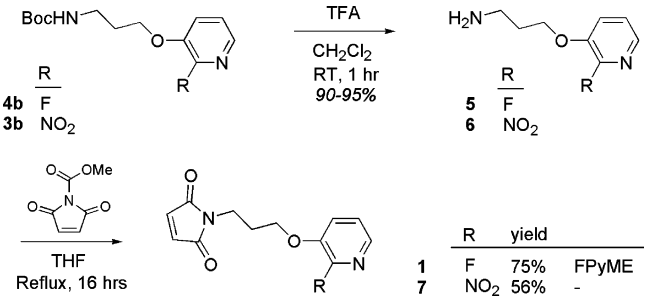
	n	yield
3a	2	38%
b	3	40%
c	4	40%
d	5	29%
e	6	41%

Analogously, the fluoro derivatives (**4a**−**e**) were prepared in 27−49% yield from the same corresponding (hydroxyalkyl)carbamic acid *tert*-butyl esters (**2a**−**e**) and 2-fluoro-3-hydroxypyridine (*37*, *40*) (Scheme 2).

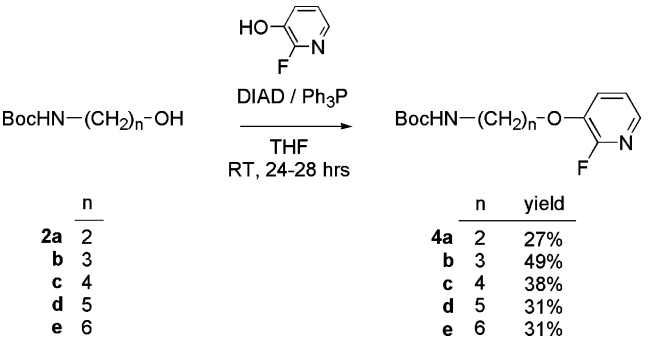
The identification of the propyl chain as the most appropriate alkyl spacer and therefore the selection of the fluorinated derivative **1** (FPyME) (Scheme 3) as well as the selection of the nitro derivatives **3b** and **7** and the trimethylammonium derivative **9** (Schemes 3 and 4) as possible precursors for fluorine-18 labeling will be discussed in the radiochemistry part below. The choice of these functions (nitro and trimethylammonium) as leaving groups in the radiofluorination process will also be discussed in that section.

Removal of the *tert*-butoxycarbonyl function of the propyl derivatives **4b** and **3b** was performed with trifluoroacetic acid (TFA) in dichloromethane at room temperature and gave the desired free amines **5** and **6** in 90−95% yield (Scheme 3). Treatment of **5** and **6** with

Scheme 3. Preparation of 1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione (1, FPyME) and the Corresponding Nitro Derivative 7 as Precursor for Labeling



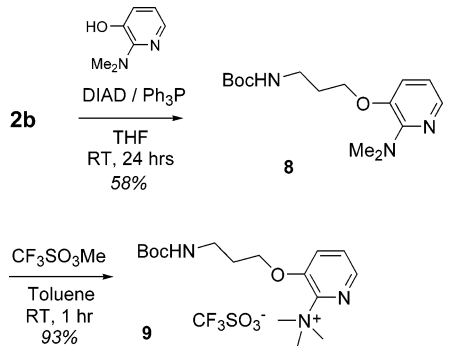
Scheme 2. Preparation of [(2-Fluoropyridin-3-yloxy)-alkyl]carbamic Acid *tert*-Butyl Ester (4a−e)



N-methoxycarbonylmaleimide in refluxing tetrahydrofuran gave the desired maleimides **1** (FPyME) and **7** in 75% and 56% yield, respectively (nonoptimized).

The dimethylamino derivative **8** was prepared in 58% yield, from the corresponding 2-(dimethylamino)-3-hydroxypyridine (*40*) under Mitsunobu conditions similar to those described above for the synthesis of **3a**−**e** and **4a**−**e** (Scheme 4). The trimethylammonium derivative **9** was cleanly obtained in 93% yield by methylation of the dimethylamine **8** using methyl trifluoromethanesulfonate (1.3 equiv) in toluene at 0 °C (Scheme 4).

Scheme 4. Preparation of [3-(3-*tert*-Butoxycarbonyl-aminopropoxy)pyridin-2-yl]trimethylammonium Trifluoromethanesulfonate (9)



The synthetic procedure of conjugation of peptides and proteins described below is based on the efficient and highly selective coupling reaction of free thiol functions (cysteine β-SH) and *N*-substituted maleimides reagents (*30*−*32*). The reaction involves addition of the thiol across the double bond of the maleimide to yield a thioether (see Figure 3). In contrast to haloacetamides, the most frequently class of thiol-reactive reagents used, maleimides do not react with functions of other amino acid residues such as methionine, histidine, or tyrosine (*30*−*32*). These reagents react rapidly (2 to 3 orders of magnitude higher in terms of rate constants (*52*) at near-neutral pH and usually can be coupled selectively in the presence of free amine function (*N*-terminus α-NH_2 or internal lysine ϵ-NH_2), which are relatively unreactive below pH 8.

Table 1. Analytical Data Obtained for Peptide/Protein Conjugation with 1-[3-(2-Fluoropyridin-3-yloxy)propyl]-pyrrole-2,5-dione (1, FPyME)

			MS	
peptide/protein	chemistry	HPLC[a,b]	calcd[a,g]	found[a,g]
P1/c-P1	(*N*-Ac) KAAAAC (-SH)	28.0 min[c]	825.9	826.3
P2/c-P2	AFIM-0 (-SH)	14.5 min[d]	8827.0	8830.5
P3/c-P3	STxB (-SH)	12.5 min[e,f]	8043.0	8048.0

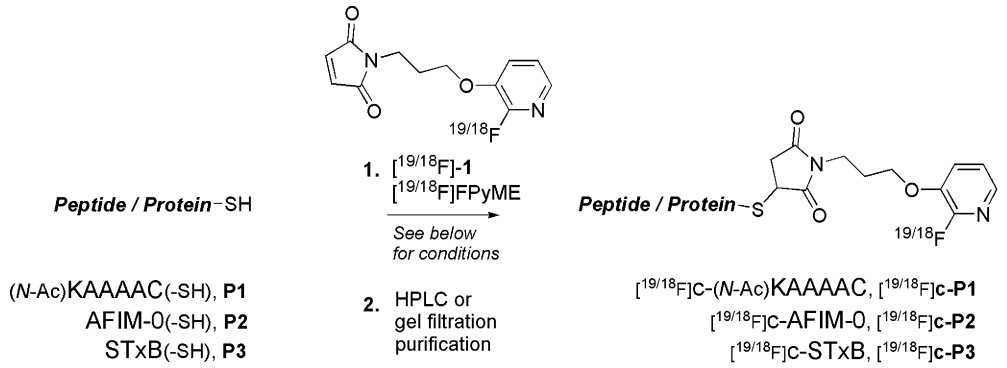
[a] Analytical data is given for the conjugated peptide/protein (**c-P1**, **c-P2**, and **c-P3**) only. [b] Analytical HPLC retention times. [c] HPLC B. [d] HPLC C. [e] HPLC E. [f] t_R: 9.5 min on HPLC F. [g] Mass spectrometry data (Perseptive Biosystem DE-RP MALDI-TOF Voyager spectrometer, ES+ mode).

Table 1 summarizes the principal characteristics of the peptide and proteins (**P1**, **P2**, and **P3**) used as starting material in the present study and the corresponding conjugated peptide and proteins (**c-P1**, **c-P2**, and **c-P3**) that we have synthesized. Analytical data obtained for these conjugated macromolecules are also indicated.

The (*N*-Ac)KAAAAC peptide (**P1**) was chosen as model peptide in order to confirm the chemoselectivity of the conjugation reaction of our maleimide reagent (**1**, FPyME). Conjugation of the (*N*-Ac)KAAAAC peptide (**P1**) at a micromolar scale was carried out using 3−5 equiv of FPyME (**1**) in a 1/9 (v/v) mixture of DMSO and 0.05 M aq Tris NaCl buffer (pH 7.4) at room temperature for 15−30 min (Scheme 5). The conjugation yields, based on starting peptide, were determined by HPLC (C18 Delta-Pak column, Waters) and were greater than 90%. Using preparative RP-HPLC (same column), the nonlabeled conjugated peptide (**c-P1**, t_R: 28.0 min) was easily separated from the nonreacted material such as the starting peptide (t_R: 13.0 min) and the maleimide reagent **1** (FPyME, t_R: 33.0 min). Mass spectrometry confirmed the identity of the conjugated peptide c-(*N*-Ac)KAAAAC (**c-P1**), giving the expected calculated mass (See Table 1). Higher molecular weight species could not be detected, clearly confirming the mono-conjugation with the maleimide reagent. ^{1}H NMR confirmed the chemoselectivity of the conjugation. Only the thiol (SH) function from the cysteine residue was conjugated with the maleimide reagent (**1**, FPyME), the amine (-ϵ-NH_2) function from the lysine residue being unaffected: in ^{1}H NMR experiments, the chemical shift of both β-CH_2 protons borne by the cysteine residue had moved from 3.00 and 3.35 ppm (CH_2SH) to 3.32 and 4.00 ppm (CH_2SR) respectively, after alkylation, whereas the chemical shift of both ϵ-CH_2 protons borne by the lysine residue were unchanged (2.96 ppm). The chemical shift of the α-CH proton borne by the cysteine residue was also slightly moved from 4.39 to 4.31 ppm.

Conjugation of the (*N*-Ac)KAAAAC peptide (**P1**) at a submicromolar scale however required preactivation of the thiol function (S−S bond reduction using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 5 min at room temperature in 0.05 M aq Tris NaCl buffer at pH 7.4) prior to the formal coupling step with the maleimide reagent (**1**, FPyME). To this TCEP-treated peptide (**P1**) was then simply added FPyME (**1**, 3−5 equiv) in DMSO, and the mixture was left standing at room temperature for 15−30 min. Using these experimental conditions, RP-HPLC showed again almost com-

Scheme 5. Peptide/Protein Conjugation with 1-[3-(2-[19/18F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([19/18F]-1, [19/18F]FPyME)



Conditions :
^{19}F : [^{19}F]-**1** (3-5 eq), aq. 50 mM Tris NaCl (pH 7.4) or aq. 0.1M PBS (pH 8.0) / DMSO : 9/1 (v/v), RT, 15-30 min
^{18}F : [^{18}F]-**1**, aq. 50 mM Tris NaCl (pH 7.4) or aq. 0.1M PBS (pH 8.0) / DMSO : 9/1 (v/v), RT, 5-10 min

plete consumption of starting peptide (t_R: 13.0 min), and the conjugation yields estimated both by HPLC and mass spectrometry analysis was greater than 90%.

The nonlabeled conjugated peptide (**c-P1**) was purified using preparative RP-HPLC and was easily separated from the nonreacted material (starting peptide at t_R: 13.0 min and excess of maleimide reagent at t_R: 33.0 min) as well as a new side-product at t_R: 26.0 min identified with mass spectrometry as TCEP-reduced FPyME (pyrrolidine-2,5-dione derivative, structure not shown). Mass spectrometry confirmed again the identity of the conjugated peptide c-(*N*-Ac)KAAAAC (**c-P1**).

The above-developed methodology was applied to the preparation of c-AFIM-0 (**c-P2**), a selected 75-amino acid (~ 8.8 kDa) miniprotein, derived from the specific binding domain of annexins for phosphatidylserine (*53*, *54*). This protein and its derivatives show high thermodynamic stability, reversible folding, and a high affinity for phosphatidylserine exposed at the external surface of biological membranes. They could be an interesting alternative to the use of fluorine-18-labeled annexin-V (*55*−*58*) and are currently developed by Bionexis Pharmaceuticals as selective fluorine-18-labeled imaging agents for the early detection of apoptosis (*53*, *54*, *59*−*62*).

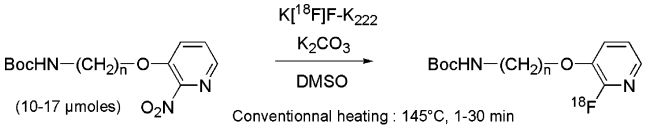
Conjugation of the selected protein AFIM-0 (**P2**) was carried out using similar conditions to those described above (including a TCEP-treatment of the starting protein) with conjugation yields estimated quantitative by mass spectrometry analysis. The nonlabeled conjugated protein (**c-P2**) was separated from the nonreacted maleimide reagent (**1**, FPyME) by gel filtration using a Sephadex NAP-10 cartridge. Size-exclusion liquid chromatography on a Gel Filtration SuperDex Peptide HR 10/30 column showed a unique peak (t_R: 14.5 min) and absence of starting maleimide reagent (**1**, FPyME, t_R: 25.0 min). Mass spectrometry confirmed the identity of the conjugated protein c-AFIM-0 (**c-P2**), giving the expected calculated mass (see Table 1). Higher molecular weight species could also be detected, clearly confirming the mono-conjugation with the maleimide reagent.

The above-developed methodology was finally applied to the preparation of c-STxB (**c-P3**), the nontoxic homopentameric B subunit (monomer size ~ 8.0 kDa) of the bacterial (*Shigella dysenteriae*) Shiga toxin (*63*−*66*). This toxin is composed of two subunits, a catalytic A-subunit that inhibits protein biosynthesis by modifying ribosomal RNA, and a nontoxic homopentameric B-subunit. STxB binds to the cellular receptor of Shiga toxin, the glycosphingolipid globotriaosyl ceramide (Gb_3), that is expressed by human cancers, including a restricted set of lymphomas, and a wide range of solid tumors such as breast and ovarian carcinomas and testicular seminomas (*63*, *66*). STxB is currently developed by the Institut Curie as a selective tumor-targeting agent.

Conjugation of the selected protein STxB (**P3**) was carried out in a 1/9 (v/v) mixture of DMSO and 0.1 M aq phosphate buffer saline (PBS) at pH 8 (without TCEP pretreatment of the protein). Conjugation yields were estimated quantitative by mass spectrometry analysis. The nonlabeled conjugated protein (**c-P3**) was separated from the nonreacted maleimide reagent (**1**, FPyME) by dialysis against water. RP-HPLC on a C18 μBondapak column and size-exclusion liquid chromatography on a Gel Filtration SuperDex Peptide HR 10/30 column showed a unique peak (t_R: 12.5 min and 9.5 min, respectively) and absence of starting maleimide reagent (**1**, FPyME, t_R: 9.0 min and 45.3 min, respectively). Mass spectrometry confirmed the identity of the conjugated protein c-STxB (**c-P3**), giving the expected calculated mass (See Table 1). Higher molecular weight species could not be detected, clearly confirming the mono-conjugation with the maleimide reagent.

Radiochemistry. In a first set of experiments, the [(2-nitropyridin-3-yloxy)alkyl]carbamic acid *tert*-butyl esters (**3a**−**e**) were all evaluated for their effectiveness as precursors for fluorine-18-labeling using nucleophilic *hetero*aromatic fluorinations (Scheme 6) in order to

Scheme 6. Preparation of 2-[18F]Fluoropyridin-3-yloxy)alkyl]carbamic Acid *tert*-Butyl Ester ([18F]-4a−e)



	n
3a	2
b	3
c	4
d	5
e	6

	n	yield*
[18F]-4a	2	07-13%
b	3	53-72%
c	4	41-57%
d	5	38-53%
e	6	17-35%

* Results at 5 min, 145°C, 17 µmoles of precursor (n=5)

determine the influence of the alkyl chain length on the incorporation of fluorine into the pyridine ring. These nucleophilic *hetero*aromatic substitutions, like the aliphatic nucleophilic radiofluorinations, only require a good

leaving group and there is no need for an additional strong electron-withdrawing substituent for activation of the aromatic ring such as in the *homo*aromatic nucleophilic radiofluorinations. The nitro function was therefore selected for its high potential as leaving group in comparison with the corresponding halo substituents (*35*, *36*) and for its ease of preparation from commercially available starting materials (see above). Introduction of the fluorine-18 as no-carrier-added naked [^{18}F]fluoride anion was performed in dimethyl sulfoxide (DMSO) with the activated K[^{18}F]F-Kryptofix222 complex (*67*) as the radiofluorinating reactant (Kryptofix222 (K_{222}): 4,7,13,16,-21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane) by either conventional heating (at 145°C for 1 to 30 min) or microwave activation (at 100 W for 0.5 to 3 min). The radiochemical yields of fluorine-18 incorporation were calculated from the TLC-radiochromatogram and defined as the [^{18}F]fluoropyridine derivatives [^{18}F]-**4a**−**e** over total fluorine-18 activity area ratio.

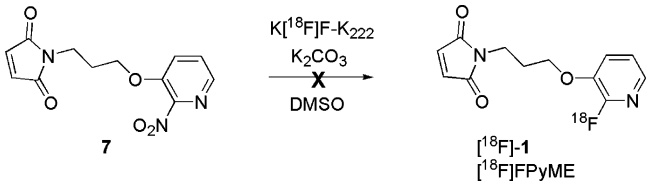
A DMSO solution (600 μL) containing 17 μmol (4.8−5.8 mg) of the appropriate precursor for labeling **3a**−**e** was transferred to 30−60 mCi of the dried K[^{18}F]F-K_{222} complex in a reaction vial (Vacutainer tube). The tube (not sealed) was then placed in a heating block at 145 °C for 1 to 30 min without stirring the contents or in a dedicated microwave oven at 100 W for 0.5 to 3 min.

Using conventional heating at 145 °C, all nitro derivatives (**3a**−**e**) were reactive under the conditions described above. Whatever the precursor used (**3a**−**e**), the incorporation yields increased with the reaction time up to 5 min and then almost leveled (very slowly decrease). In each run, the remaining radioactivity at the end of the experiment was measured and 85% to 95% of the initial radioactivity placed in the vessel was still present. At 5 min, excellent incorporation yields were observed for the propyl derivative **3b** (53−72% yield). Good incorporation yields were also observed for the butyl and pentyl derivatives (**3c** and **3d**), 41−57% and 38−53%, respectively, whereas moderate incorporation yields were observed for the hexyl derivative (**3e**, 17−35%). Only low incorporation yields were observed for the ethyl derivative (**3a**, 7−13%). At 15 min and 30 min for example, the incorporation yields observed for the propyl derivative **3b** were still high (50−65% and 45−55% yields, respectively). Using only 10 μmol of the nitro derivatives (**3a**−**e**) resulted in slightly lower incorporation yields. Using microwave irradiations at 100 W, comparable results to those described above were obtained for all nitro derivatives (**3a**−**e**) with the highest incorporation yields observed at 2 min and again the propyl derivative **3b** being the more reactive precursor for labeling (50−75% yield). No further efforts were made at this stage to optimize and further increase the observed yields.

On the basis of these results, the propyl alkyl chain was selected for the design of the maleimide final reagent (**1**, FPyME).

A first dedicated maleimide-containing precursor for fluorine-18-labeling, the nitro derivative **7** (1-[3-(2-nitropyridin-3-yloxy)propyl]pyrrole-2,5-dione), was therefore designed and synthesized (see above), expecting the use of a one-step radiochemical process. Introduction of fluorine-18 was attempted in DMSO (600 μL) containing 17 μmol (4.7 mg) of the nitro derivative **7** with the activated K[^{18}F]F−Kryptofix222 complex as the radiofluorinating reactant, by conventional heating only (at 145°C for 1 to 10 min). However, no incorporation of fluorine-18 could be detected by TLC or HPLC using these radiochemical conditions (similar to those described for the preparation of the [(2-[^{18}F]fluoropyridin-3-yloxy)-

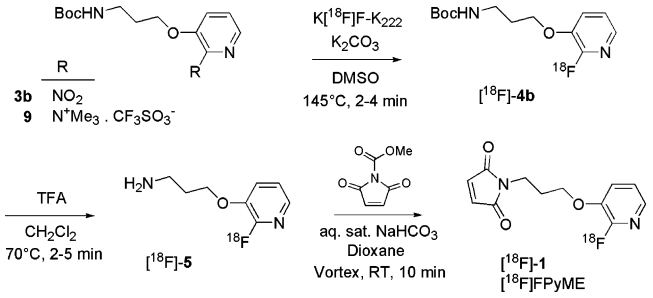
Scheme 7. Potential One-Step Process for the Preparation of 1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]-1, [^{18}F]FPyME)



alkyl]carbamic acid *tert*-butyl ester ([^{18}F]-**4a**−**e**)) (Scheme 7), and the expected [^{18}F]FPyME ([^{18}F]-**1**) could not be isolated. This is probably due to the rapid decomposition of the maleimide ring (base-labile) to a mixture of nonreactive maleamic acids using the strong alkaline conditions required for the introduction of [^{18}F]fluorine as described above. A similar unsuccessful direct radiofluorination of a preformed maleimide was also reported by others (*33*). In view of these failures, the preparation of the corresponding *N*-trimethylammonium trifluoromethanesulfonate derivative ([3-[3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)propoxy]pyridin-2-yl]trimethylammonium trifluoromethanesulfonate, structure not shown) was not attempted.

[^{18}F]FPyME ([^{18}F]-**1**) was therefore synthesized using the following three-step radiochemical process from the nitro derivative **3b** (Scheme 8).

Scheme 8. Preparation of 1-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione ([^{18}F]-1, [^{18}F]FPyME) Using a Three-Step Process



The first radiochemical step, the introduction of fluorine-18 into the pyridine ring, was performed as described above, in DMSO (600 μL) containing the nitro derivative **3b** (17 μmol) and the activated K[^{18}F]F-K_{222} complex, and by conventional heating at 145°C for 5 min, giving the desired [^{18}F]fluoropyridine derivative **4b** in 45−60% (decay-corrected) isolated radiochemical yields with respect to starting [^{18}F]fluoride after C-18 Sep-pak purification. The second radiochemical step, the removal of the *N*-Boc-protective group, was rapidly (2−5 min) performed in a mixture of TFA and dichloromethane (1/50 (v/v)) at room temperature, quantitatively yielding the free amine [^{18}F]-**5**. The third and final step, the maleimide ring formation, was first performed using maleic anhydride (25 mg to 50 mg) in *o*-xylene (at 180−190°C) for 10 to 20 min, based on literature procedures (see for example ref 68; over fifty references can be found in the literature via a bibliographic search using Beilstein Commander 2000 version 5.0, copyright 1995−2000, MDL Information Systems GmbH). On the basis of TLC analysis, the desired maleimide [^{18}F]-**1** ([^{18}F]FPyME) was obtained in 19−41% radiochemical yields. The use of other described solvents and experimental conditions using maleic anhydride, such as refluxing THF (*69*, *70*), acetic acid at 100 °C (*71*, *72*), refluxing dioxane containing triethylamine (*73*, *74*), or refluxing methanol containing sodium

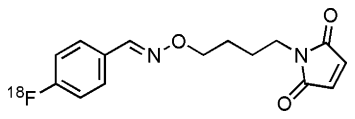
hydroxyde (*75*), gave systematically lower radiochemical yields when the desired maleimide [18F]-**1** ([18F]FPyME) could be detected at all.

Using instead of maleic anhydride, *N*-methoxycarbonylmaleimide (25 mg) in *o*-xylene (at 180−190°C) gave higher radiochemical yields (46−77%, based on TLC analysis) for 5 min of reaction only. However, HPLC purification (semipreparative SiO_2 Zorbax Rx-SIL, Hewlett-Packard) led to [18F]-**1** ([18F]FPyME) with radiochemical purity of only about 90% and with systematically lower isolated radiochemical yields that expected from the TLC analysis. The maleimido ring formation was then investigated again, and the following optimal conditions, based on literature procedures, were found (*76*−*78*): the free amine [18F]-**5** (3-(2-[18F]fluoropyridin-3-yloxy)propylamine) was treated with *N*-methoxycarbonylmaleimide (25 mg) in a strongly vortexed 3/1 (v/v) solution (1 mL) of aqueous saturated bicarbonate and THF at room temperature for 10 min. On the basis of TLC analysis, the desired maleimide [18F]-**1** ([18F]FPyME) was obtained in >90% radiochemical yields and was practically the only detectable product on the TLC chromatogram. C-18 Sep-pak trapping, followed by HPLC purification (semipreparative SiO_2 Zorbax Rx-SIL, Hewlett-Packard), gave radiochemically pure [18F]-**1** ([18F]FPyME) in 64−77% isolated yields, confirming the excellent radiochemical yields of maleimido formation obtained from the TLC analysis.

Finally, the three-step radiochemical process for the preparation of [18F]FPyME ([18F]-**1**) was further optimized (Scheme 8) using a more efficient and attractive precursor for labeling, the *N*-trimethylammonium trifluoromethanesulfonate derivative **9** ([3-(3-*tert*-butoxycarbonylaminopropoxy)pyridin-2-yl]trimethylammonium trifluoromethanesulfonate). This type of precursor for labeling has been used extensively already in nucleophilic *homo*- and *hetero*aromatic radiofluorinations (*13*−*15*). It is particularly suitable for its expected superior separation (using HPLC or SPE-cartridge) from the reaction product (the fluoro derivative [18F]-**4b** in this case) due to the large differences in the physicochemical properties. Moreover, the trimethylammonium function possesses higher potential as leaving group (*79*) in comparison with the corresponding nitro and especially halo derivatives, giving usually higher incorporation yields under milder conditions. Introduction of the fluorine-18 was therefore performed using only 8.7 μmol of the *N*-trimethylammonium trifluoromethanesulfonate derivative **9** (4 mg) in DMSO (600 μL) containing the activated K[18F]F-K_{222} complex by conventional heating at 145 °C for 2 min, giving the desired [18F]fluoropyridine derivative **4b** in 64−76% (decay-corrected) isolated radiochemical yields with respect to starting [18F]fluoride after C-18 Sep-pak purification. The remainder of the radiosynthesis used the same procedures as described above.

Typically, 4.8−6.7 GBq (130−180 mCi) of radiochemically pure [18F]FPyME ([18F]-**1**) could be obtained after semipreparative HPLC in 110 minutes starting from a 33.3 GBq (900 mCi) cyclotron production batch of [18F]-fluoride (overall radiochemical yields, based on starting [18F]fluoride: 28−37% decay-corrected and 17−20% non-decay-corrected).

According to the available literature, only one report exists on two fluorine-18-labeled maleimide reagents that have been prepared and used for prosthetic conjugation of proteins (*33*). In that abstract, the authors described the preparation of 1-(4-[18F]fluorophenyl)pyrrole-2,5-dione ([18F]FPPD, see Figure 2) and *N*-[3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)phenyl]-4-[18F]fluorobenzamide ([18F]-DDPFB, see Figure 2).



[18F]FBABM

Figure 4. Chemical structure of *N*-[4-[(4-[18F]fluorobenzylidene)aminooxy]butyl]maleimide ([18F]FBABM).

[18F]FPPD was synthesized in four radiochemical steps from 1,4-dinitrobenzene and cesium [18F]fluoride. It was obtained in 15% decay-corrected overall radiochemical yield in 100 min total synthesis time (*33*). [18F]DDPFB was synthesized in three radiochemical steps from 4-nitrobenzonitrile and cesium [18F]fluoride and was obtained in only 10% decay-corrected overall radiochemical yield in 70 min total synthesis time (*33*).

During the preparation of this manuscript, another fluorine-18-labeled maleimide reagent was published (*80*). *N*-[4-[(4-[18F]Fluorobenzylidene)aminooxy]butyl]-maleimide ([18F]FBABM, see Figure 4) was synthesized in only two steps, involving the preparation of 4-[18F]-fluorobenzaldehyde, followed by its aminooxy-aldehyde coupling reaction to the fluorine-18-labeled maleimide reagent, with an overall radiochemical yield of about 35% (decay-corrected) within 60 min of radiosynthesis time. This new fluorine-18-labeled maleimide reagent ([18F]-FBABM) is a valuable alternative to the use of our [18F]-FPyME ([18F]-**1**) and is the first reported example of an oxime-containing radiotracer for PET.

Conjugation of the (*N*-Ac)KAAAAC peptide (**P1**, TCEP-treated) with the HPLC-purified [18F]FPyME ([18F]-**1**) (Scheme 5) was carried out in a 1/9 (v/v) mixture of DMSO and 0.05 M aq Tris NaCl buffer (pH 7.4) at room temperature for 10 min. The labeled conjugated peptide [18F]**c-P1** was obtained in >90% yield, based on starting [18F]FPyME ([18F]-**1**), and determined both by radio-TLC analysis and radio-HPLC analysis (C18 DeltaPak column, Waters). [18F]c-(*N*-Ac)KAAAAC ([18F]**c-P1**) was purified by preparative HPLC (C18 DeltaPak column, Waters). It was easily separated from starting (*N*-Ac)-KAAAAC (t_R: 13.0 min), nonreacted [18F]FPyME ([18F]-**1**), if any (t_R: 33.0 min), and traces of a fluorine-18-labeled side product, associated with the TCEP-reduced-[18F]-FPyME species (t_R: 26.0 min, see above). [18F]c-(*N*-Ac)-KAAAAC ([18F]**c-P1**) coeluted with reference nonlabeled standard and was obtained with a radiochemical purity greater than 99%. Conjugation of AFIM-0 (**P2**) was carried out using similar conditions to those described above (including a TCEP-treatment, but including a removal step of this reducing agent by gel filtration prior to the use of the protein) with conjugation yields estimated quantitative by radio-TLC analysis. The labeled conjugated protein (**c-P2**) was separated from nonreacted [18F]FPyME ([18F]-**1**), if any, by gel filtration using a Sephadex NAP-10 cartridge. Size-exclusion liquid radiochromatography on a Gel Filtration SuperDex Peptide HR 10/30 column showed a unique peak (t_R: 14.5 min), which coeluted with reference standard. Conjugation of STxB (**P3**) was carried out in a 1/9 (v/v) mixture of DMSO and 0.1 M aq PBS at pH 8 (without TCEP-treatment of the starting protein), with conjugation yields estimated quantitative by radio-TLC analysis. The labeled conjugated protein (**c-P3**) was separated from nonreacted [18F]-FPyME ([18F]-**1**), if any, by gel filtration using a Sephadex NAP-10 cartridge. RP-HPLC on a C18 μBondapak column and size-exclusion liquid radiochromatography on a Gel Filtration SuperDex Peptide HR 10/30 column showed a unique peak (t_R: 12.5 and 9.5 min, respectively), which coeluted with reference standard.

Typically, 1.11−1.29 GBq (30−35 mCi) of pure conjugated [^{18}F]peptide or protein (specific radioactivity > 37 GBq/μmol) could be obtained in 25−30 min starting from 1.85 GBq (50 mCi) of [^{18}F]FPyME ([^{18}F]-**1**) (60−70% non-decay-corrected isolated yield). The whole synthetic procedure has been fully automated on our Zymate robot system and took 130−140 min. As demonstrated by LC analysis, radiosynthesized labeled conjugated peptide and protein ([^{18}F]**c-P1**, [^{18}F]**c-P2** and [^{18}F]**c-P3)** coeluted with the authentic synthesized reference compounds. The fluorine-18-labeled peptide and proteins were found to be more than 99%, 90%, and 97% radiochemically pure, respectively. The preparations were shown to be free of radioactive starting reagent ([^{18}F]FPyME, [^{18}F]-**1**) and radiochemically stable for at least 240 min.

The conjugation yields to the macromolecules obtained with [^{18}F]FPyME ([^{18}F]-**1**) are also higher than those obtained for the conjugation of the previously reported *N*-[3-(2,5-dioxo-2,5-dihydropyrrol-1-yl)phenyl]-4-[^{18}F]fluorobenzamide ([^{18}F]PPFB, see Figure 2). Therefore, about 50% yield could be obtained for the conjugation step with a protein part from a monoclonal antibody fragment (*33*). No complementary details, such as solvent, reagent, and conditions used for the conjugation, reaction time, and purification process, were given.

Conjugation to a protein of the recently published *N*-[4-[(4-[^{18}F]fluorobenzylidene)aminooxy]butyl]maleimide ([^{18}F]-FBABM, see Figure 4) has not been reported so far. Conjugation yields to a small tripeptide (GSH) were comparable to those obtained with [^{18}F]FPyME ([^{18}F]-**1**) (about 70% decay-corrected radiochemical yield).

CONCLUSION

The objective of the present work was to develop a [^{18}F]-fluoropyridine-based thiol (sulfhydryl)-selective reagent for the prosthetic labeling with fluorine-18 of peptides and proteins, offering a new alternative with improved chemoselectivity to the use of the nonselective carboxylate and amine-reactive [^{18}F]reagents already existing.

FPyME (**1**, 1-[3-(2-fluoropyridin-3-yloxy)propyl]pyrrole-2,5-dione) was designed as a radiochemically feasible reagent, its pyridinyl moiety carrying the radioactive halogen (fluorine-18) which can be efficiently incorporated via a nucleophilic *hetero*aromatic substitution, and its maleimido function ensuring the efficient alkylation of a free thiol function as borne by cysteine residues. [^{18}F]-FPyME ([^{18}F]-**1**, HPLC-purified) was efficiently prepared in 17−20% non-decay-corrected yield (based on starting [^{18}F]fluoride) in 110 min using a three-step radiochemical pathway. The developed procedure involves a high-yield nucleophilic *hetero*aromatic ortho-radiofluorination as the fluorine-18 incorporation step and uses a trimethylammonium trifluoromethanesulfonate as the most efficient precursor leaving group for labeling (when compared to the parallely tested nitro precursor). [^{18}F]FPyME ([^{18}F]-**1**) was first conjugated with a small hexapeptide ((*N*-Ac)-KAAAAC, **P1**), chosen as a model peptide, and the chemoselectivity of the coupling reaction (CH_2SH versus CH_2NH_2) was confirmed. The labeling strategy was then applied with success to the preparation of two 8-kDa proteins of interest, currently being developed as tumor imaging agents ([^{18}F]c-AFIM-0, [^{18}F]**c-P2**; [^{18}F]c-STxB, [^{18}F]**c-P3**). Conjugation was achieved in high yields (60−70%, isolated and non-decay-corrected yields) and uses optimized, short-time reaction and purification conditions, both compatible with the chemical stability of the proteins and the relatively short half-life of the radioisotope concerned. The whole radiosynthetic procedure, including the preparation of the fluorine-18-labeled reagent, the conjugation with the protein, and the final purification, took 130−140 min.

Given these high radiosynthetic yields, [^{18}F]FPyME ([^{18}F]-**1**) represents a new, valuable, thiol-selective, fluorine-18-labeled reagent and can advantageously be used for the design and development of peptide- and protein-based radiopharmaceuticals for PET.

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