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ORIGINAL ARTICLE

Site-selective radiolabeling of peptides by ^{18}F-fluorobenzoylation with [^{18}F]SFB in solution and on solid phase: a comparative study

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Abstract Peptides labeled with short-lived positron-emitting radionuclides are of outstanding interest as probes for molecular imaging by positron emission tomography (PET). Herein, the site-selective incorporation of fluorine-18 into lysine-containing peptides using the prosthetic labeling agent *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) is described. The reaction of [^{18}F]SFB with four biologically relevant resin-bound peptides was studied and optimized. For comparison, each peptide was ^{18}F-fluorobenzoylated in solution under different conditions and the product distribution was analyzed confirming the advantages of the solid-phase approach. The method's feasibility for selective radiolabeling either at the N-terminus or at the lysine side chain was demonstrated. Labeling on solid phase with [^{18}F]SFB resulted in crude ^{18}F-fluorobenzoylpeptides whose radiochemical purities were typically greater than 90% and that could be prepared in synthesis times from 65 to 76 min.

Keywords Fluorine-18 · Solid-phase synthesis · Regioselectivity · Positron emission tomography

Introduction

As peptides have the potential to address a huge number of biomolecular targets with high affinity and selectivity they represent an attractive basis for the development of probes for molecular imaging (Watt 2009). Positron emission tomography (PET) is a present-day imaging modality used for clinical diagnostics and research purposes routinely. Therefore, peptides labeled with positron-emitting radionuclides are of outstanding interest for the development of radiotracers that allow to image cellular functions and dysfunctions in vivo (Chen and Conti 2010; Tolmachev and Stone-Elander 2010; Kuhnast and Dolle 2010). The most widely used nonmetallic PET radionuclide is fluorine-18 (^{18}F $t_{1/2} = 109.8$ min) due to its favorable physical and chemical properties unparalleled by any other positron emitter. It can be conveniently produced on a small cyclotron and its low-positron energy of 0.64 MeV accounts for images of high-spatial resolution. Its half-life of 109.8 min enables multistep radiosyntheses of tracers and imaging studies up to 6 h after injection (Pimlott and Sutherland 2010). The conditions for the nucleophilic introduction of radiofluorine are characterized by temperatures around 100°C, aprotic water-free solvents and precursor molecules that preferably do not possess acidic hydrogens (Cai et al. 2008). To avoid the exposure of peptides to such harsh reaction conditions numerous ^{18}F-labeled prosthetic groups that allow the indirect radiofluorination of peptides have been developed (Wester and Schottelius 2007; Li and Conti 2010; Schottelius 2010). Among the various amine-reactive ^{18}F-labeled prosthetic groups the acylation agent *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB) is certainly the most commonly used. This is reflected by numerous radiosyntheses that have been developed for its automated production (Mäding et al. 2005; Vaidyanathan and Zalutsky 2006; Tang et al. 2008, 2010; Glaser et al. 2009; Bejot et al. 2011). Generally, amine-reactive prosthetic groups offer the advantage of broad applicability as most bioactive peptides exhibit an N-terminal amino group available for acylation.

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Furthermore, lysine residues bearing an amino group in the side chain are very abundant in peptide sequences. On the other hand, this implies the problem of site-selective ^{18}F-labeling in the presence of a free N-terminus and one or multiple lysine residues. This problem has been tackled by taking advantage of the slight difference between the pK_b values of the N-terminus and the ε-amino group of lysine (Wester et al. 1995; Hermanson 2008; Hoppmann et al. 2008) but the success of this strategy is limited and depends on the particular peptide or protein to be radiolabeled.

As an alternative to labeling by ^{18}F-fluoroacylation, methods based on chemoselective reactions such as Huisgen's [3 + 2] azide-alkyne cycloaddition were developed (Mamat et al. 2009; Glaser and Robins 2009; Ross 2010). However, this approach requires the introduction of artificial moieties into the peptide that can potentially impair its bioactivity and very often involves volatile ^{18}F-containing reagents. Furthermore, this method is not necessarily more efficient concerning the radiochemical yields of the labeled peptides compared to ^{18}F-fluoroacylation (Hausner et al. 2008; Schottelius 2010).

In a different prosthetic group-based approach the radiofluorine was directly introduced into peptides containing benzoyl moieties bearing a trimethylammonium or nitro leaving group by nucleophilic aromatic substitution (Becaud et al. 2009; Jacobson et al. 2011). However, this technique depends on the presence of electron-withdrawing groups in *ortho* position to the leaving group and thus requires a higher degree of modification of the parent peptide. Furthermore, by applying this methodology the separation of the radiolabeled product from the precursor can be sometimes difficult (Jacobson et al. 2011).

Due to the mentioned drawbacks of the different developed approaches the selective labeling of peptides with [^{18}F]SFB remains still a challenge. A site-selective modification with this reagent can be only achieved by reacting peptides at one reactive amino group while all others are protected. As fully protected peptides are often difficult to handle in solution, radiolabeling should be preferably carried out with peptides attached to a polymeric support. This is easy to realize because peptides bound to solid support by their C-terminus can be generated via Fmoc-based solid-phase synthesis. The ^{18}F-labeling of peptides by fluoroacylation on solid supports has been sporadically reported (Sutcliffe-Goulden et al. 2000, 2002; Marik et al. 2006) but not by employing [^{18}F]SFB and never by a direct comparison to fluoroacylation in solution. Therefore, the aim of this study was to evaluate the potential to label various lysine-containing peptides with [^{18}F]SFB at defined sites by solid-phase synthesis. The obtained results were systematically

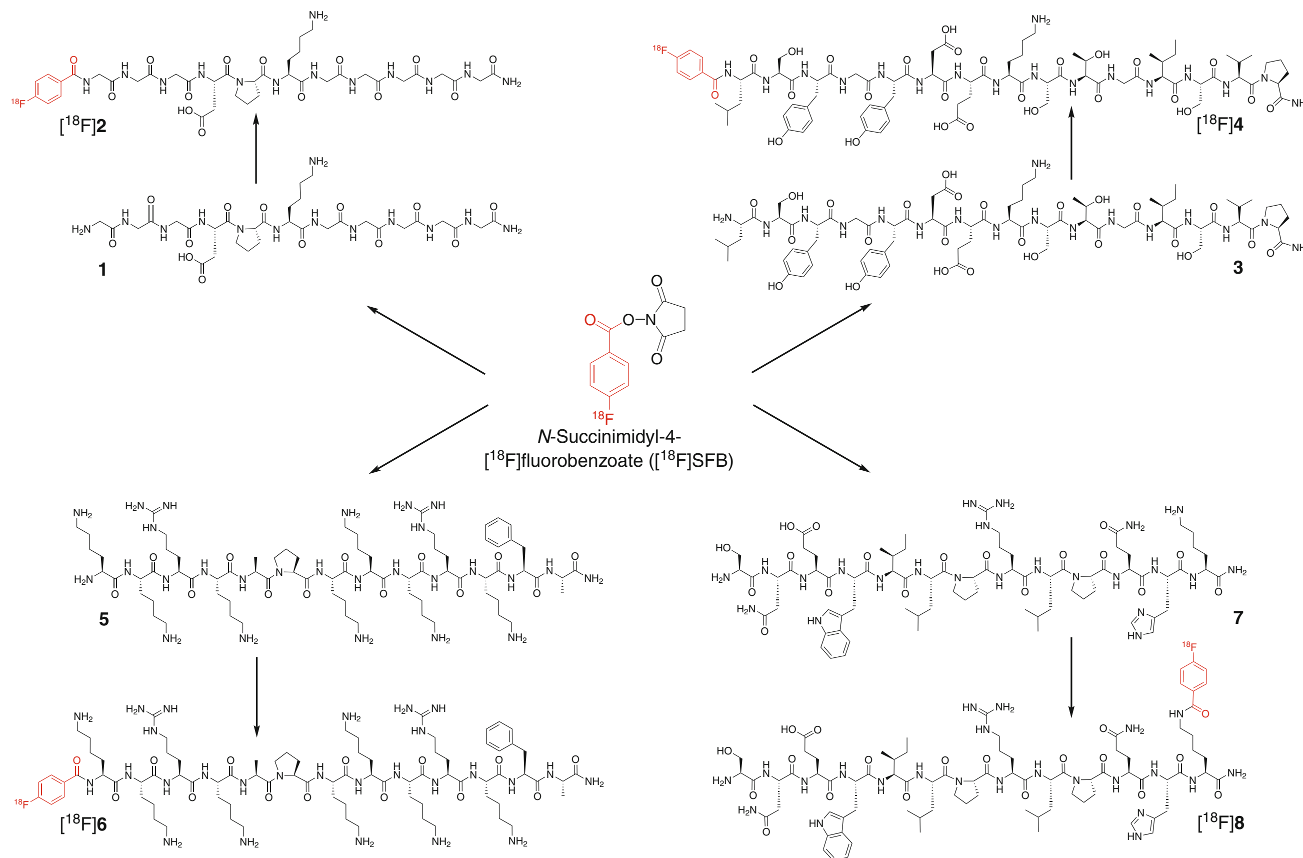


Fig. 1 Structures and desired sites of ^{18}F-fluorobenzoylation of the investigated peptides

Table 1 Sequences of investigated peptides

Peptide	Original sequence	Sequence modified for this study	
Derivative of α1(I)-N-telopeptide	GGGDPKGGGGG	GGGDPKGGGGG-NH_2	(**1**)
1(I)-N-telopeptide	pQLSYGYDEKSTGISVP	LSYGYDEKSTGISVP-NH_2	(**3**)
k7-fragment	KKRKAKKKRKFA	KKRKAKKKRKFA-NH_2	(**5**)
SNEW peptide	SNEWILPRLPQH	SNEWILPRLPQHK-NH_2	(**7**)

compared to those of ^{18}F-fluorobenzoylation in solution regarding radiochemical yield and radiochemical purity of the labeled peptides. The selection of peptides subjected to the radiolabeling experiments and the corresponding desired labeled products are shown in Fig. 1 and their sequences can be found in Table 1.

Materials and methods

General remarks

All chemical reagents and solvents were obtained from commercial suppliers and used without further purification. Fmoc-protected amino acids and coupling reagents were purchased from MultiSynTech (Witten, Germany) and Iris Biotech (Marktredwitz, Germany). Fmoc-Rink-Amide MBHA polystyrene resin was obtained from Multisyntech. Mass spectra were obtained on a Quattro/LC mass spectrometer (Micromass) equipped with an electrospray ionization source. Generally, mixtures of water and acetonitrile each containing 0.1% TFA were used for HPLC with the wavelength for UV detection at 220 nm. Gradient systems and flow rates are specified in Table 2 and Supplementary Material. Analytical HPLC and radio-HPLC were performed on an Agilent Technologies 1200 system equipped with automatic injector, UV detector and radioactivity detector (type Gabi, Raytest). Analyses were carried out with a Nucleosil 100-5 C18 Nautilus column (VP 250/10, Macherey–Nagel) operated by system A, Nucleosil 100-5 C18 PPN column (VP 250/4, Macherey–Nagel) operated by system C and Nucleosil 100-7 C18 EC (VP 250/4,6; Macherey–Nagel) operated by system F. Purification of nonradioactive peptides by semi-preparative HPLC was done using a Hewlett Packard 1050 system with automatic injector and UV detector at a volumetric flow rate of 4 mL/min and a Varian Prepstar system equipped with UV detector (Prostar, Varian) and automatic fraction collector Foxy 200 at a flow rate of 20 mL/min. Chromatographic purification of radiolabeled peptides was performed with a Jasco HPLC system equipped with UV detector (UV-2075) and radioactivity detector (type Gabi, Raytest). A Nucleosil 100-5 C18 column (VP 250/10, Macherey–Nagel) served as stationary phase using gradient systems B, D, E, or G.

[^{18}F]SFB was produced in an automated radiosynthesis using a remotely controlled synthesis module (GE TracerLab) in a hot cell as described (Mäding et al. 2005) with implemented refinements as reported recently (Kapty et al. 2011). The stability of [^{18}F]SFB was assessed by radio-TLC performed on Merck silica gel F-254 aluminum plates using a mixture of ethyl acetate and petroleum ether (1:1) as mobile phase. Detection was performed by radioluminography in a Fuji BAS 2000 scanner. The results were confirmed by radio-HPLC using system C. Nonradioactive SFB as standard for chromatography was synthesized according to Wester et al. (1996).

Table 2 HPLC systems

Peptide	Analytical HPLC system	Preparative HPLC system
Derivative of α1(I)-N-telopeptide	System A	System B
α1(I)-N-telopeptide	System C	System D
k7-fragment	System E	
SNEW peptide	System F	System G

Details can be found in Supplementary Material

Peptide synthesis

Peptides were synthesized by microwave-assisted, fully automated solid-phase peptide synthesis based on the Fmoc-protecting group strategy with appropriately side chain-protected L-amino acids as building blocks using a CEM Liberty Microwave Peptide Synthesizer combined with a CEM Discover microwave reactor. The Rink-amide MBHA-functionalized resin was Fmoc-protected and had a loading capacity of 0.6 mmol/g. A coupling cycle took 45 min and started with the deprotection using 0.1 M *N*-hydroxybenzotriazole (HOBt) in 20% piperidine/DMF (2 × 7 mL) followed by washing with DMF. Coupling of the amino acids was performed by addition of a 0.2 M solution of the corresponding Fmoc-protected amino acid in DMF (5 equiv., 2.5 mL), 0.45 M *O*-benzotriazolyl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) in DMF (1.0 mL), and 2 M diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (0.5 mL). The automated synthesis ended with a final deprotection of the N-terminal Fmoc group. Resins loaded with N-terminally deprotected peptides **1**, **3**, and **5**

were used as precursors for acylation with fluorobenzoyl chloride as well as for solid-phase radiolabeling with [^{18}F]SFB. In addition, peptides **1**, **3**, and **7** were synthesized with an Alloc-protected lysine side chain. In these cases, N-terminal Fmoc deprotection was followed by *tert*-butoxycarbonylation of the free N-terminus. As fully deprotected peptides were required for ^{18}F-labeling in solution as well as standards for HPLC, portions of the resins loaded with N-terminally deprotected peptides **1**, **3**, Alloc-**3**, **5**, and **7** were cleaved before further modification. Generally, TFA-mediated cleavage and deprotection was carried out in a syringe equipped with a frit filter at the bottom using 10 mL of 95% TFA/H_2O. Exceptionally, peptides **7**, **8**, and *iso*-**8** were released from the resin with 10 mL of reagent K (85% TFA, 5% H_2O, 5% thioanisole, 2.5% ethanedithiol) (King et al. 1990). The solutions containing the crude peptides were concentrated in a stream of nitrogen, precipitated by addition of cold diethyl ether, and the solids were collected by filtration. The peptides were purified by HPLC and characterized by mass spectrometry.

Analytical data for unmodified peptides:

1. HPLC retention times 15.4 min (system A), 18.0 min (system B); MS (ESI) *m/z* 815.1 ([M + H]$^+$), 408.3 ([M + 2H]$^{2+}$)
3. HPLC retention times 11.4 min (system C), 29.5 min (system D); MS (ESI) *m/z* 1617.1 ([M + H]$^+$) 809.2 ([M + 2H]$^{2+}$)
Alloc-**3**. HPLC retention time 37.6 min (modified-system C); MS (ESI) *m/z* 1698.5 ([M-H]$^-$), 848.5 ([M-2H]$^{2-}$)
5. HPLC retention time 21.5 min (system E), MS (ESI) *m/z* 808.3 ([M + 2H]$^{2+}$), 539.6 ([M + 3H]$^{3+}$)
7. HPLC retention times 3.3 min (system F); 22.8 min (system G); MS (ESI) *m/z* 1619.4 ([M + H]$^+$), 810.4 ([M + 2H]$^{2+}$).

Synthesis of N^{α}-4-fluorobenzoyl peptides

4-Fluorobenzoyl chloride (FBzCl) was reacted with the deprotected N-termini of peptides **1**, **3**, **5**, and **7** for the synthesis of peptides **2**, **4**, **6**, and *iso*-**8**. The preswollen resins (amount corresponding to 0.1 mmol of resin-bound peptide) loaded with peptides **1**, **3**, Alloc-**3**, **5**, and **7** were incubated with a solution of 4-fluorobenzoyl chloride (60 µL, 0.5 mmol) and triethylamine (71 µL, 0.5 mmol) in DMF (5 mL) for 1 h followed by washing the resin with dichloromethane (10 mL), DMF (10 mL) and ethanol (10 mL). The completion of the reaction was proven by the Kaiser test (Kaiser et al. 1970) before the fluorobenzoylated peptides were cleaved from the resin as described above.

Analytical data for N^{α}-4-fluorobenzoyl peptides:

2. HPLC retention times: 19.7 min (system A), 28.0 min (system B); MS (ESI) *m/z* 937.2 ([M + H]$^+$), 469.5 ([M + 2H]$^{2+}$)
4. HPLC retention times 17.0 min (system C), 42.5 min (system D); MS (ESI) *m/z* 1740.1 ([M + H]$^+$), 881.5 ([M + 2H]$^{2+}$)
Alloc-**4**. HPLC retention time 21.7 min (modified-system C); MS (ESI) *m/z* 909.0 ([M + 2H]$^{2+}$)
6. HPLC retention time 28.2 min (system E); MS (ESI) *m/z* 869.6 ([M + 2H]$^{2+}$), 580.4 ([M + 3H]$^{3+}$)
iso-**8**. HPLC retention times 11.0 min (system F), 30.5 min (system G); MS (ESI) *m/z* 1740.8 ([M + H]$^+$), 870.8 ([M + 2H]$^{2+}$).

Synthesis of N^{ε}-4-fluorobenzoyl peptides

The fluorobenzoylation of the side chain for the synthesis of peptides *iso*-**2**, *iso*-**4**, and **8** was carried out for peptidyl resins containing **1**, **3**, and **7** with Alloc-protected lysine side chains. The preswollen resins (amount corresponding to 0.1 mmol of resin-bound peptide) were incubated with di-*tert*-butyl dicarbonate (44 mg, 0.2 mmol) and triethylamine (28 µL, 0.2 mmol) in DMF (5 mL) for 3 h followed by washing as described above and deprotection of the lysine residue with diethylamine (21 µL, 0.2 mmol) $Pd(PPh_3)_4$ (58 mg, 0.05 mmol) in dichloromethane (5 mL) for 2 h. The Pd catalyst was removed by washing with DMF, 0.5% sodium *N*,*N*-diethyldithiocarbamate in DMF (m/v), dichloromethane (3 mL each, washing procedure was repeated three times) (Shepherd et al. 2004). A portion of peptidyl resin containing **7** treated in this manner was used as precursor for labeling with [^{18}F]SFB on resin. The nonradioactive fluorobenzoylations of the lysine side chains were performed in the same fashion as the N-terminal fluorobenzoylations described above. Cleavage of the peptides from the resin was done as described.

Analytical data for N^{ε}-4-fluorobenzoyl peptides:

iso-**2**. HPLC retention times 22.5 min (system A), 40.8 min (system B); MS (ESI) *m/z* 937.1 ([M + H]$^+$), 480.3 ([M + 2H]$^{2+}$)
iso-**4**. HPLC retention times 13.8 min (system C), 41.0 min (system D); MS (ESI) *m/z* 867.9 ([M-2H]$^{2+}$)
8. HPLC retention times 9.8 min (system F), 29.1 min (system G); MS (ESI) *m/z* 1741.3 ([M + H]$^+$), 871.4 ([M + 2H]$^{2+}$).

Synthesis of *N*-succinimidyl 4-[^{18}F]fluorobenzoate

[^{18}F]SFB was synthesized according to the procedure described in (Mäding et al. 2005). [^{18}F]Fluoride was

produced on a PET cyclotron Cyclone 18/9 (IBA, Belgium); [^{18}O]H_2O was irradiated with protons exploiting the ^{18}O(p,n)^{18}F nuclear reaction. After bombardment, the [^{18}O]H_2O containing [^{18}F]F^- was transferred from the cyclotron to an anion exchange cartridge (Sep-Pak® Light Waters Accell™ Plus QMA cartridge) placed in a commercially available synthesizer TRACERlab$_{FDG}$ for the nucleophilic fluorination (GE Medical Systems, Münster, Germany). The automated radiosynthesis of [^{18}F]SFB was performed as described in modified form in (Kapty et al. 2011). In a typical experiment starting from 16 GBq [^{18}F]fluoride 4 GBq [^{18}F]SFB could be obtained after final solid-phase extraction in radiochemical purity >95%. For determination of the radiochemical and chemical purity of the final product an HPLC System (Hewlett Packard series 1050) was used, including pump, injector and UV-detector (254 nm) coupled in series with a radioactivity detector GABI (raytest, Straubenhardt, Germany) and Nucleosil Nautilus C-10 column (5 µm, 250 × 4.6 mm + precolumn 4 × 4 mm). HPLC analyses were carried out at a flow rate of 0.5 mL/min with the following gradient of eluents (A = MeCN with 0.1% TFA; B = H_2O with 0.1% TFA): 0 min, 10% A/90% B; 10 min, 60% A/40% B; 14 min, 100% A: 25 min, 100% A.

Stability of *N*-succinimidyl 4-[^{18}F]fluorobenzoate

The stability of [^{18}F]SFB was evaluated in 0.15 M sodium phosphate at pH values of 7.0, 9.0, 9.2, 9.4, 9.6, 9.8, and 10.0 at 25°C as well as at 50°C. The composition of the [^{18}F]SFB solutions were analyzed by radio-TLC after 3, 10, 20, 30, and 60 min and for pH 7.0, 25°C in addition by radio-HPLC after 1 h using system C. Additionally, the aqueous [^{18}F]SFB obtained after radiosynthesis was analyzed immediately and after 5 h by radio-TLC as well as by radio-HPLC using system C. The results are shown in Table S1 of Supplementary Material.

^{18}F-Fluorobenzoylation of peptides **1**, **3**, **5**, and **7** in solution

A solution of [^{18}F]SFB (50–100 MBq) in water (25 µL) as obtained by the radiosynthesis was added to a solution of the corresponding peptide (0.5–1.0 mg) in 0.15 M sodium phosphate (200 µL). The pH values of the phosphate-buffered peptide solutions were 7 and 9 (compounds **1**, **3**, and **7**) and 6, 7, and 9 (compound **5**). The reaction mixtures were incubated at 25°C and 50°C in a thermomixer for 30 min. The radiochemical yields of the formed ^{18}F-fluorobenzoylated peptides were determined by analyzing the reaction mixtures by radio-HPLC. The results are shown in Table 3 and all radio-chromatograms are included in Supplementary Material. Isolation of a radiolabeled peptide by semi-preparative HPLC was tried exemplarily for [^{18}F]**4** formed at pH 9 and 25°C.

^{18}F-Fluorobenzoylation of Alloc-**3** in solution

Radiolabeling was done as described above under the following conditions: pH 7, 50°C; pH 9, 25°C; pH 9, 50°C. After 30 min of incubation a mixture of 0.05 eq $Pd(PPh_3)_4$ (3.5 mg, 0.003 µmol) and morpholine (12 µL of a 0.1 M solution in THF) in THF (100 µL) was added and incubated for 20 min at the corresponding reaction temperatures. The reaction mixture was subjected to solid-phase extraction using a Chromafix C-18 cartridge (200 mg, Macherey–Nagel) preconditioned with 5 mL of THF followed by 5 mL of water). The product was eluted from the cartridge with THF (200 µL) and analyzed by radio-HPLC.

^{18}F-Fluorobenzoylation of resin-bound peptides

Reactions were carried out in a polypropylene syringe equipped with a frit at the bottom and a cartridge adapter on top. The peptidyl resin (preswollen in DMF) was suspended in a solution of [^{18}F]SFB (500–2500 MBq) in water (100 µL), DMF (200 µL) and 0.15 M sodium phosphate buffer pH 7 (100 µL). The reaction mixture was gently agitated in a heating bath at 50°C for 30–50 min ([^{18}F]**2** and [^{18}F]**4**: 30 min, [^{18}F]**6**: 40 min, [^{18}F]**8**: 50 min). The liquid phase was removed by filtration and the resin was washed with DMF and water (2 × 1 mL each) to remove unreacted [^{18}F]SFB. The resin-bound radioactivity was measured in an activity meter. The cleavage reagent ([^{18}F]**2**, [^{18}F]**4**, and [^{18}F]**6**: 400 µL 95% TFA/water; [^{18}F]**8**: 400 µL reagent K) was added to the resin and the resulting mixture incubated at 50°C for 20 min in a thermomixer. The product-containing solution was collected by filtration and concentrated in a stream of argon. The radioactivity of the filtrate and of the resin was determined. Aliquots of the crude products were analyzed by radio-HPLC. In the meantime, the products were purified by semi-preparative radio-HPLC followed by the evaporation of the eluent. Data are shown in Table 4.

Results and discussion

All peptide sequences considered in this study were assembled by microwave-assisted solid-phase peptide synthesis using Rink-amide linker-functionalized polystyrene-based resins to obtain C-terminal amides in quantitative yields. The purities of the crude peptides ranged between 72 and 94% and their identities were confirmed by ESI–MS. To enable an accurate analysis of labeling with [^{18}F]SFB in solution, the nonradioactive standards of both

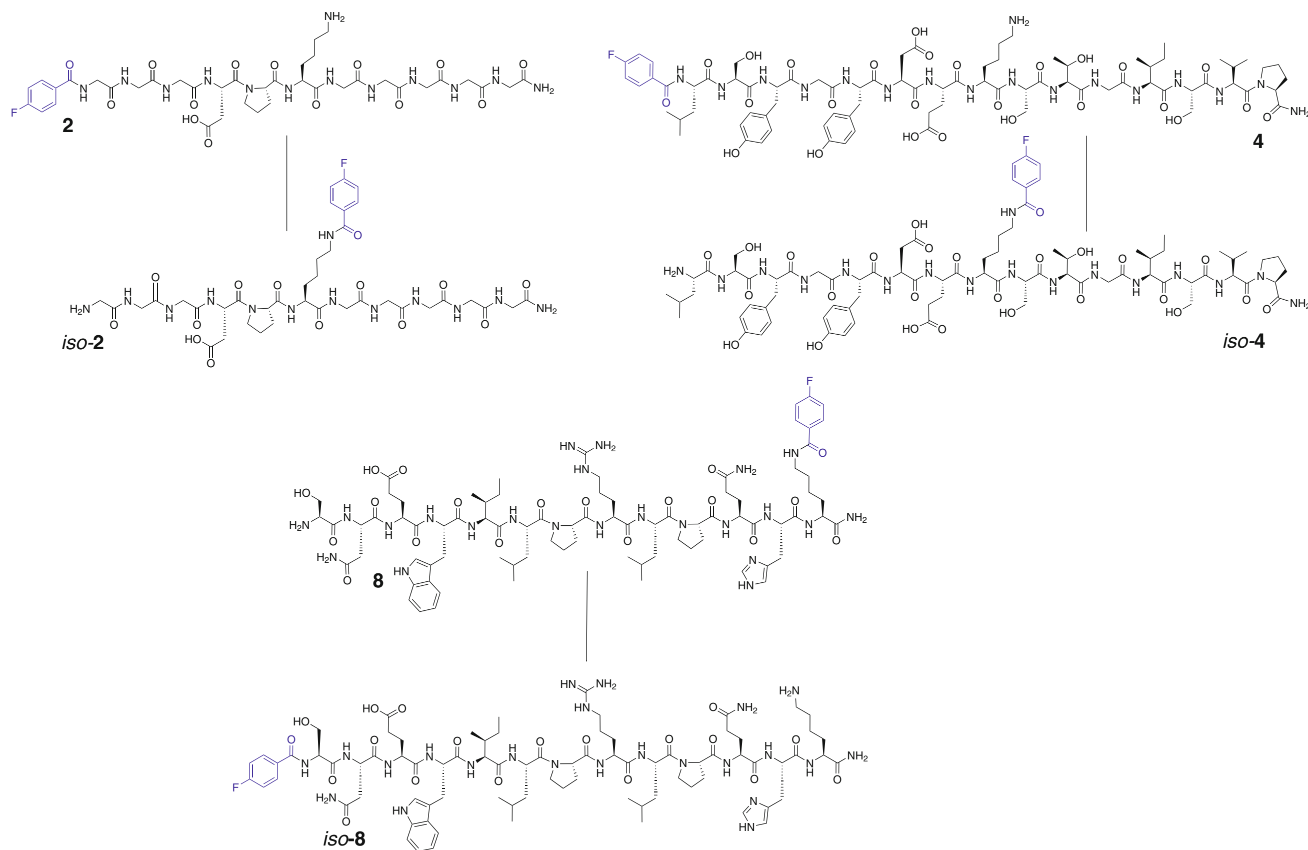


Fig. 2 Isomers of fluorobenzoylated peptides **2**, **4**, and **8**

the desired as well as the alternative ^{18}F-fluorobenzoylation products of the peptides **1**, **3**, and **7** were prepared. The pairs of isomeric fluorobenzoylated products are depicted in Fig. 2. The synthesis of the nonradioactive reference compounds **2**, **4**, **6**, and *iso*-**8** was accomplished by reacting the corresponding resin-attached peptides with 4-fluorobenzoyl chloride on their N-terminus. The side-chain fluorobenzoylated peptides *iso*-**2**, *iso*-**4**, and **8** were synthesized by incorporation of Fmoc-Lys(Alloc)-OH to allow a selective deprotection of the lysine ε-amino group. Protection of the N-terminus and deprotection of the ε-amino group was done as shown for [^{18}F]**8** in Scheme 3, fluorobenzoylation and cleavage/deprotection were carried out congruently to the N-terminally modified peptides.

The separation of the isomeric fluorobenzoylated peptides by analytical HPLC required careful individual optimization regarding both the mobile and stationary phase. Details can be found in Supplementary Material.

Prior to the labeling experiments the stability of [^{18}F]SFB in aqueous solution at different pH and temperature was investigated. The ^{18}F-fluorobenzoylation agent revealed more stable at pH 7/50°C than at pH 9/25°C. These pH values were selected as pH 7 is known to favor radiolabeling at the N-terminus and pH 9 is considered to be advantageous for ^{18}F-fluorobenzylation of lysine ε-amino groups, however this pH is the upper limit beyond which substantial hydrolysis is taking place (Wester et al. 1995; Hermanson 2008). Indeed, at pH values above 9.5 a rapid degradation of the radiolabeling agent could be observed even at 25°C (see Supplementary Material).

Initial investigations were focused on the radiolabeling of peptides **1** and **3**. Compound **1** is based on a model sequence derived from the N-terminal telopeptide of the α1-chain of type I-collagen (Nagan and Kagan 1994) whereas **3** represents the authentic telopeptide (Helseth et al. 1979) whose N-terminal pyroglutamate residue was removed to allow replacement by a 4-fluorobenzoyl group. The central lysine residues of both derivatives undergo modification in vivo and, therefore, a radiolabeling that is selective for their N-terminus is desirable. These biologically relevant peptides containing only one lysine residue seemed to be suitable for systematic investigations concerning the outcome of ^{18}F-fluorobenzoylation in solution. Peptides **1** and **3** were subjected to reaction with [^{18}F]SFB in solution for 30 min at pH 7 and pH 9 each at 25 and 50°C. Labeling in solution of **1** resulted in the formation of [^{18}F]**2** as the main component independent of the employed conditions (Table 3). The highest yield of 70% for [^{18}F]**2**

Table 3 Results of radiolabeling with [^{18}F]SFB in solution

precursor	pH 7, 25°C					pH 7, 50°C					pH 9, 25°C					pH 9, 50°C				
	N$^{\alpha}$ (%)	N$^{\varepsilon}$ (%)	[^{18}F]SFB (%)	N$^{\alpha}$:N$^{\varepsilon}$	*n*	N$^{\alpha}$ (%)	N$^{\varepsilon}$ (%)	[^{18}F]SFB (%)	N$^{\alpha}$:N$^{\varepsilon}$	*n*	N$^{\alpha}$ (%)	N$^{\varepsilon}$ (%)	[^{18}F]SFB (%)	N$^{\alpha}$:N$^{\varepsilon}$	*n*	N$^{\alpha}$ (%)	N$^{\varepsilon}$ (%)	[^{18}F]SFB (%)	N$^{\alpha}$:N$^{\varepsilon}$	*n*
1	52 ± 2	11 ± 9	8 ± 1	83:17	2	54 ± 33	17 ± 9	–	76:24	2	70 ± 8	16 ± 12	2	81:19	2	56 ± 9	14 ± 5	–	80:20	4
3	19 ± 6	10 ± 4	50 ± 18	65:35	3	37 ± 14	28 ± 15	–	56:43	2	37 ± 11	28 ± 12	1	57:43	3	23 ± 14	49 ± 20	–	32:68	2
Alloc-**3**	–	–	–	–	1	8 ± 8	–	–	–	2	6 ± 3	–	–	–	2	6 ± 7	–	–	–	2
5	7	–	8	–	2	–	–	–	–	1	12	–	9	–	1	–	–	–	–	1
7	6 ± 3	12 ± 5	67 ± 16	33:67	4	24 ± 14	45 ± 20	–	35:65	3	14 ± 12	43 ± 27	1	25:75	4	15 ± 1	68 ± 6	–	18:82	2

Shown are the fractions of the radioactive components in the crude reaction mixture after 30 min

N$^{\alpha}$ and N$^{\varepsilon}$ refer to N-terminally and side-chain ^{18}F-fluorobenzoylated peptides, respectively

could be obtained at pH 9/25°C and the ratio of [^{18}F]**2** to [^{18}F]*iso*-**2** did not change much with the different conditions.

Compared to **1**, the conversion of [^{18}F]SFB with **3** was poor at pH 7 and 25°C with 50% of unconsumed acylation agent remaining. This emphasizes the dependence of the radiolabeling efficiency on the peptide sequence. The ratio of the isomeric ^{18}F-fluorobenzoylated peptides was at pH 7/25°C with 65:35 less favorable than in the case of **1**. Almost equal amounts of radiolabeled isomers were formed at pH 7/50°C and pH 9/25°C (Fig. 3) whereas the ratio reversed by increasing both pH and temperature. Attempts to isolate [^{18}F]**4** by semi-preparative HPLC failed due to poor separation of the fluorobenzoylated isomers. In conclusion, an efficient and selective radiolabeling of **3** in solution revealed as not feasible.

Labeling with [^{18}F]SFB on solid phase was optimized for peptides **1** and **3** regarding the volume of solvent and temperature. Concerning the amount of peptide-loaded resin, the precursor masses and solvent volumes that correspond to those that are typically employed for reaction of peptides with [^{18}F]SFB in solution were chosen (Richter et al. 2009), lower amounts led to less favorable results. The optimization of the reaction conditions was done on the basis of determining the ratio of the resin-bound radioactivity related to the applied activity of [^{18}F]SFB (see Table S2 in Supplementary Material). As acylations with *N*-hydroxysuccinimide esters are very often performed in aqueous media (Sewald and Jakubke 2002; Hermanson 2008) 0.15 M sodium phosphate at pH 7 was tested as reaction medium beside different organic solvents common for solid-phase organic synthesis on polystyrene-based resins. A pH of 7 was also preferable on basis of the results obtained in the [^{18}F]SFB-stability assays. Interestingly, performing the reaction with the preswollen peptide-loaded resin suspended in a mixture of aqueous phosphate-buffered solution and DMF revealed as superior to pure organic solvents. An elevated temperature of 50°C was clearly advantageous over lower temperatures. In the optimized procedure peptidyl resins (amounts corresponding to 2–3 μmol of resin-bound peptide) preswollen in DMF were incubated with a solution of [^{18}F]SFB in DMF/aqueous buffer pH 7 (1:1) at 50°C for 30 min (Scheme 1). After washing to remove unreacted acylation agent and TFA-mediated deprotection and cleavage from the resin [^{18}F]**2** and [^{18}F]**4** could be obtained in a radiochemical purity of 98–99 and 83–97%, respectively (Fig. 4).

Exemplary protocols of activity distribution for radiolabeling of **1** and **3** can be found in Table S2 of the Supplementary Material. Noteworthy, the resin-bound activity could be quantitatively released by TFA-mediated cleavage with no unconverted [^{18}F]SFB being detectable in the crude peptides indicating that unspecific binding of the ^{18}F-

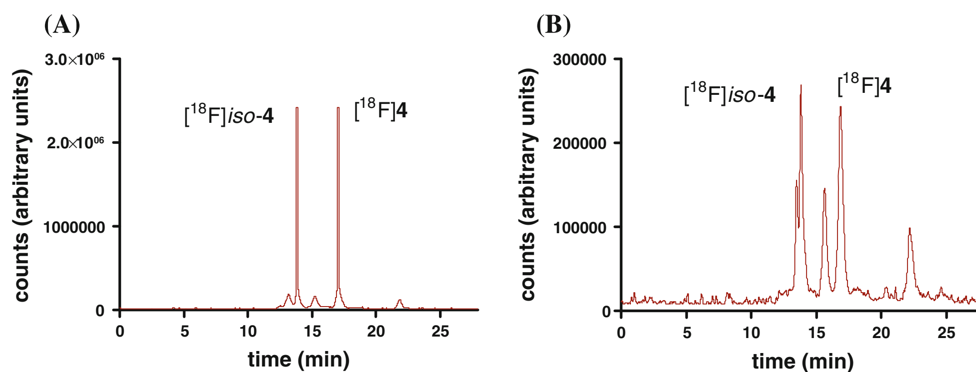


Fig. 3 Radiolabeling of **3** with [^{18}F]SFB in solution (0.15 M sodium phosphate buffer). Radio-HPLC-chromatograms of reaction mixtures after 30 min for **a** pH 7, 50°C; **b** pH 9, 25°C

Scheme 1 N-terminal ^{18}F-fluorobenzoylation of **1** on solid phase. Reagents and conditions: **a** [^{18}F]SFB, DMF/0.15 M sodium phosphate pH 7 (1:1), 50°C, 30 min; **b** TFA, 50°C, 20 min. Solid-phase radiolabeling of **3** and **5** was done accordingly

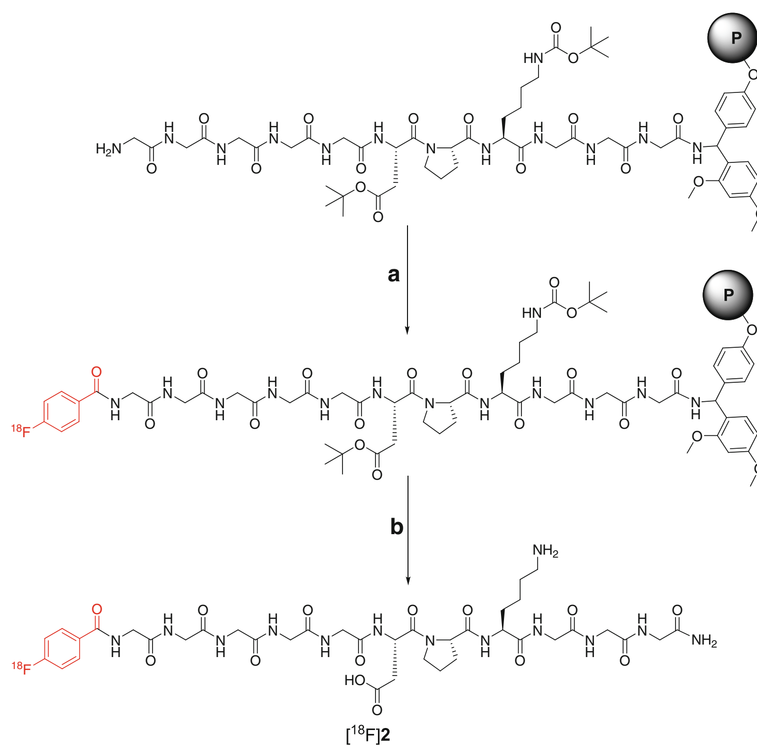


Fig. 4 Radiolabeling of **1** and **3** with [^{18}F]SFB on solid phase. Shown are the preparative radiochromatograms during purification of [^{18}F]**2** (**a**) and [^{18}F]**4** (**b**) cleaved from the resin

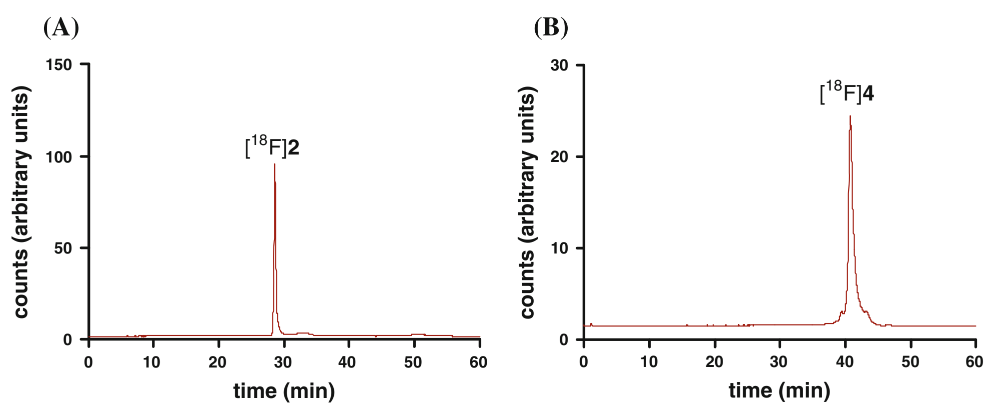


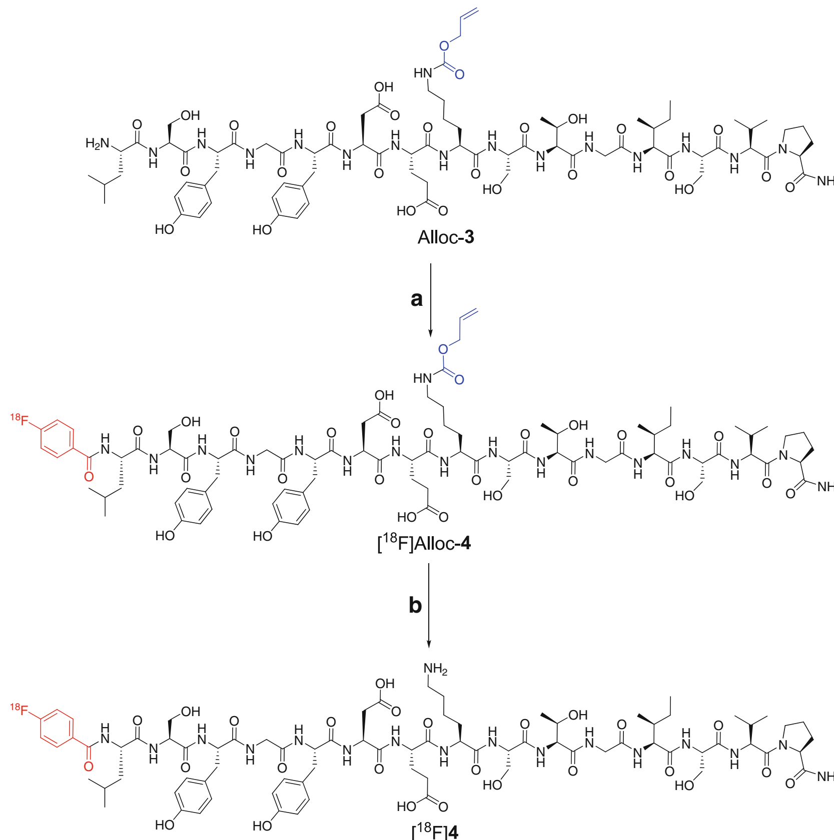
Table 4 Results of radiolabeling with [^{18}F]SFB on solid phase

Protected and resin-bound peptide precursor	Radiochemical purity (%)		Isolated + decay-corrected radiochemical yield (%)	Activity range of isolated product (MBq)	Synthesis time for crude product (min)	Total synthesis time (min)	*n*
	After cleavage	After purification					
1	98 ± 1	>99	14 ± 2	80–125	65 ± 5	135 ± 5	28
3	90 ± 7	98 ± 2	14 ± 4	70–172	64 ± 5	137 ± 3	11
5	99 ± 1	–	11 ± 1	30–35	73 ± 3	143 ± 3	4
7	54 ± 25	95 ± 1	5 ± 2	15–36	76 ± 6	153 ± 7	2

fluorobenzoylation agent to the polystyrene matrix does not occur.

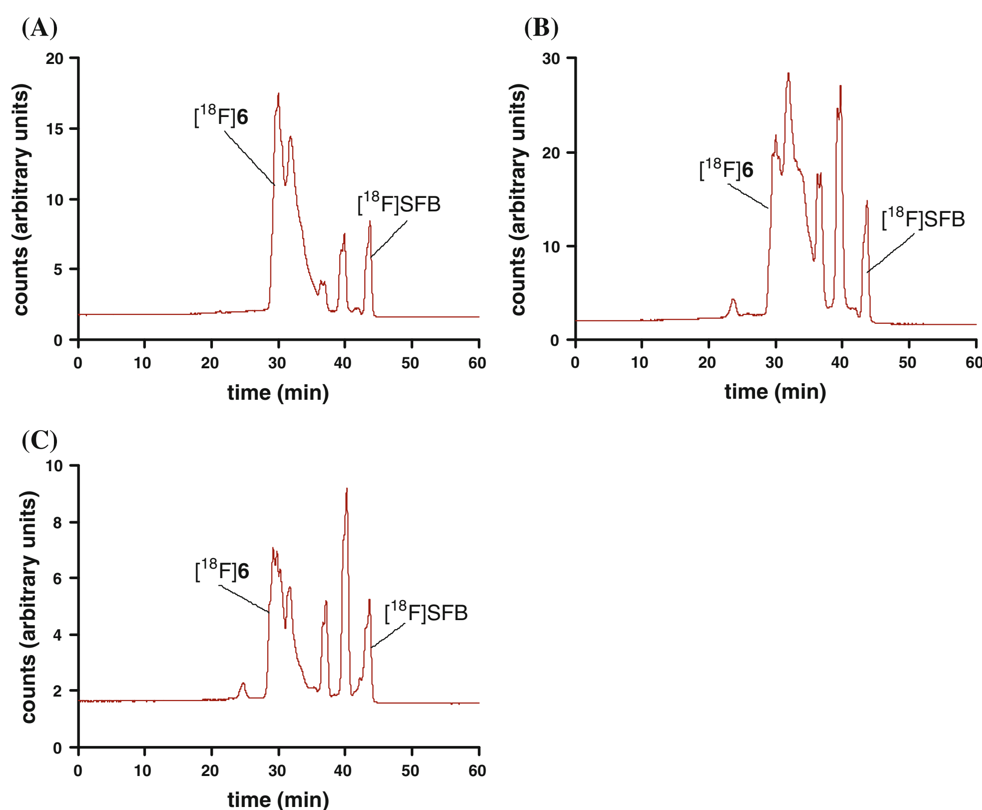
The following purification by semi-preparative HPLC to separate labeled and unlabeled peptide provided [^{18}F]**2** in a radiochemical purity of 98–99%, a high chemical purity and a decay-corrected radiochemical yield of 12–15%. Purification of [^{18}F]**4** led to a radiochemical purity of 96–99%, a high chemical purity and a decay-corrected radiochemical yield of 8–14% (Table 4, Fig. S7B of Supplementary Material).

To further evaluate the advantages of the solid-phase approach, it should be compared to the reaction of peptides containing a protected lysine side chain with [^{18}F]SFB in solution. This was done exemplarily for peptide **3** containing an Alloc-protected ε-amino group (Alloc-**3**; Scheme 2). The ^{18}F-fluorobenzoylation was performed under conditions identical to liquid-phase labeling of **1** and **3** except that reaction at pH 7 and 25°C was omitted. Subsequently to reaction with [^{18}F]SFB the reaction mixtures were treated with $Pd(PPh_3)_4$ and morpholine for side chain deprotection. Substantial formation of [^{18}F]fluorobenzoic acid could be observed in the radio-HPLC-chromatograms with analytical radiochemical yields of [^{18}F]**4** not exceeding 10%. Therefore, the potential of this approach for site-selective labeling with [^{18}F]SFB seems to be limited and clearly disadvantageous compared to ^{18}F-fluorobenzoylation on solid phase.



Scheme 2 Fluorobenzoylation of Alloc-protected **3** in solution. Reagents and conditions: **a** [^{18}F]SFB, 0.15 M sodium phosphate buffer pH 7 and 9, 30 min; **b** $Pds(PPh_3)_4$, morpholine, 20 min

Fig. 5 Radiolabeling of **5** with [^{18}F]SFB in solution (0.15 M sodium phosphate buffer). Radio-HPLC-chromatograms of reaction mixtures after 30 min at 25°C for **a** pH 7; **b** pH 9; **c** pH 6



Very often biologically active peptides comprise multiple lysine residues as exemplified with the k7-fragment **5**, a cell-penetrating peptide (Rennert et al. 2008). This seven lysine residues containing peptide was used for investigation towards labeling with [^{18}F]SFB. The acylation in solution was tried at 25°C, pH 7 and 9. As expected, the ^{18}F-fluorobenzoylation in solution resulted in a complex mixture of products (Fig. 5a, b) whose minor component was the desired product accompanied by side-chain labeled peptides represented by broad peaks in the chromatograms indicating the random distribution of the ^{18}F-label among the different lysine residues. To shift the product spectrum to the favor of the N-terminally labeled peptide, the reaction of **5** with [^{18}F]SFB was tried at pH 6. As obvious from Fig. 5c, this did not result in a more selective radiolabeling. Considering these results it was refrained from labeling of **5** at 50°C.

In contrast, ^{18}F-fluorobenzoylation of resin-bound protected **5** provided [^{18}F]**6** in an excellent radiochemical purity (Fig. 6) of 99% and an overall decay-corrected yield of 11 ± 1% ($n = 4$) (Table 4).

The examples of radiolabeled peptides discussed so far all contained a functional lysine residue. However, there are several peptides whose biological functionality is critically dependent on a free N-terminus. Examples are ligands of protease-activated receptors (Barry et al. 2006) or peptides containing the N-terminal sequence SNEW essentially binding to the extracellular domain of EphB2, a member of the Eph/ephrin tyrosine kinase receptor family (Koolpe et al. 2005; Chrencik et al. 2007). One peptide of the latter class was selected to demonstrate the feasibility of the solid-phase approach for selective radiolabeling at lysine side chains. For this purpose the parent sequence SNEWILPRLPQH was C-terminally extended by a lysine residue. To allow selective deprotection, lysine was incorporated with an Alloc-protected side chain. After protecting the N-terminus of the peptide with Boc, the side-chain amino group of the C-terminal lysine was deprotected by Pd(0)-catalyzed allyl transfer to diethyl amine. This

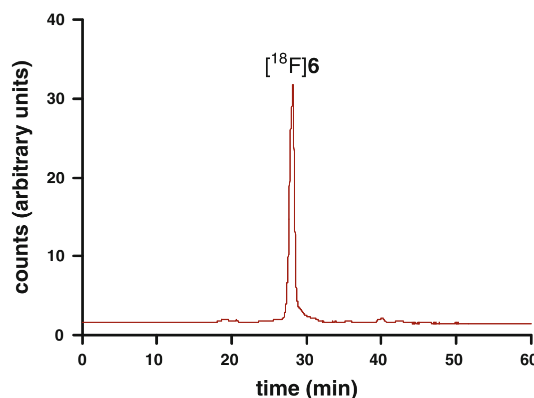
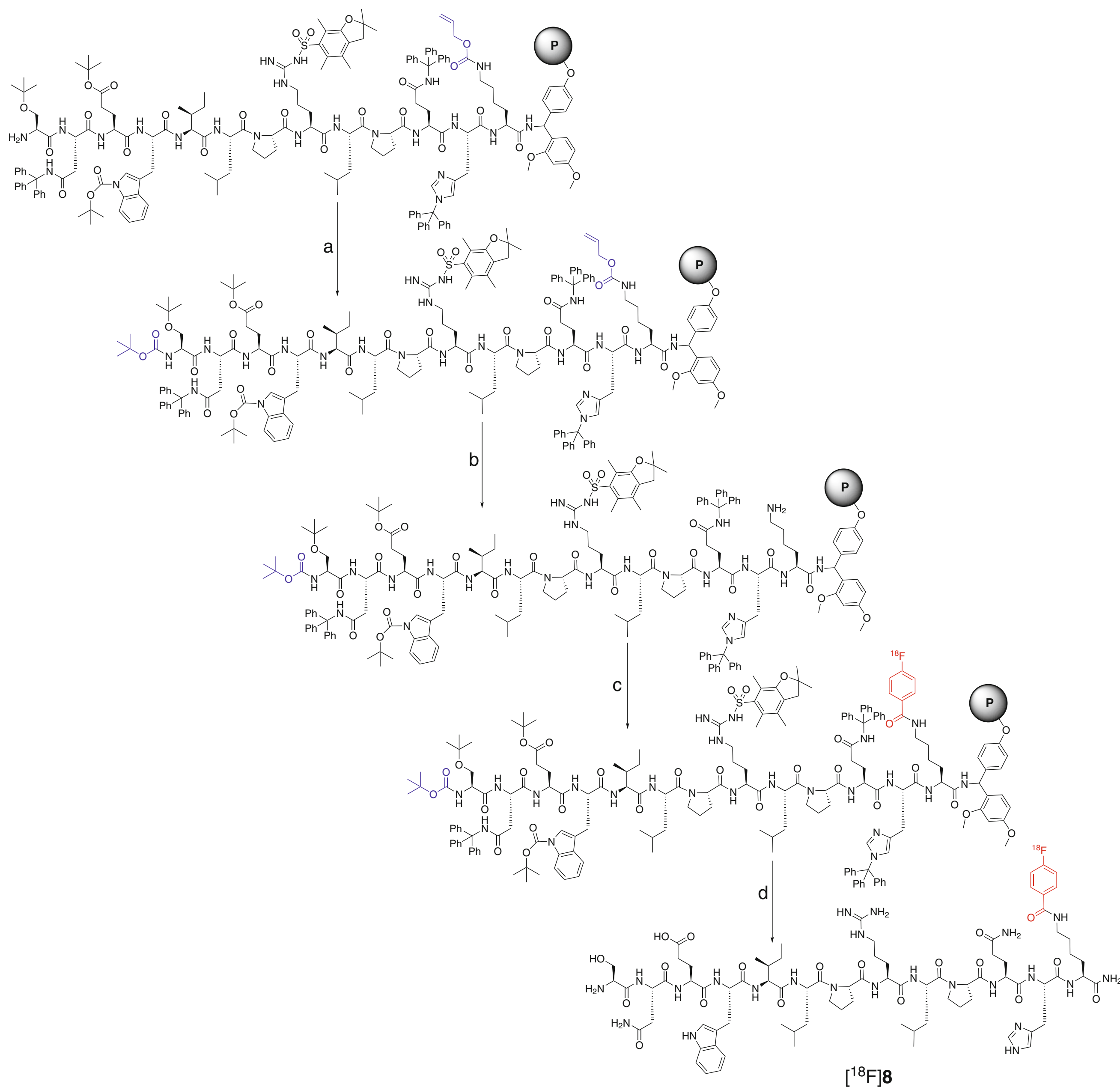


Fig. 6 Radiolabeling of **5** with [^{18}F]SFB on solid phase. Shown is the radio-HPLC-chromatogram of the crude product



Scheme 3 Side-chain ^{18}F-fluorobenzoylation of the ε-amino group of **7** on solid phase. Reagents and conditions: **a** (tBuOCO)$_2$O, DMF, TEA, rt, 2 h; **b** Pd(PPh$_3$)$_4$, diethylamine, DCM, rt, 2 h; **c** [^{18}F]SFB, DMF/0.15 M sodium phosphate buffer pH 7 (1:1), 50°C, 30 min; **d** reagent K, 50°C, 20 min

provided **7** attached to the solid phase ready for functionalization with [^{18}F]SFB and for preparation of the nonradioactive reference compound **8** (Scheme 3). It should be mentioned that the chromatographical separation of the isomeric fluorobenzoylated reference peptides **8** and *iso*-**8** revealed as cumbersome and required tedious optimization. This is indicated by the close retention times in the chromatograms (Supplementary Material, Fig. S11). Reaction of the SNEW peptide **7** with [^{18}F]SFB in solution had a slight tendency to the formation of [^{18}F]**8** over [^{18}F]*iso*-**8**, especially at pH 9, while there was a high variation between the single labeling experiments (Table 3). Reaction of resin-bound **7** with [^{18}F]SFB led to [^{18}F]**8** in varying radiochemical purities that were less satisfying compared to the other peptides investigated (Table 4) while the isolated radiochemical yield of the purified labeling products was similar to those of the other tracers.

To summarize the results of labeling in solution, the ^{18}F-fluorobenzoylation of **1** resulted in [^{18}F]**2** as main product nearly independent of the pH value. In contrast to this,

radiolabeling of **7** produced predominantly [^{18}F]**8** even at pH 7.

The influence of the chosen conditions on selectivity was most pronounced for peptide **3** while there was a tendency for an equal distribution of the radiolabeling products at pH 7/50°C and pH 9/25°C. Theses results show that the outcome of the radiolabeling reaction is unpredictable for any peptide and difficult to control by the pH value.

Conclusion

In this study a method for the site-selective labeling of peptides with fluorine-18 on solid support using [^{18}F]SFB, the most common prosthetic reagent for introduction of radiofluorine into biomolecules, was developed. Its advantages over the conventional radiolabeling in solution could be exemplarily demonstrated by systematic comparison of radiolabeling on solid phase and in solution for four different biologically relevant peptides. The elaborated methodology revealed especially advantageous for the radiolabeling of peptides containing multiple lysines such as k7-fragment **5** and can be also applied for labeling at sites distinct from the N-terminus as exemplarily shown for SNEW peptide **7**. Labeling on solid phase was in each case more reliable and efficient as a site-selective reaction with [^{18}F]SFB in aqueous solution revealed as impossible for every considered peptide despite systematic variation of pH and temperature. The benefits of the solid-phase approach become especially obvious if one considers the time-consuming optimizations that were necessary to separate the isomeric fluorobenzoylated peptides chromatographically. Labeling with [^{18}F]SFB on solid support has certainly the potential for straight-forward introduction of fluorine-18 into almost every peptide that is interesting as imaging probe for PET.

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Conflict of interest The authors declare that they have no conflict of interest.

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