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Automated preparation of [^{18}F]AFP and [^{18}F]BFP: Two novel bifunctional ^{18}F-labeling building blocks for Huisgen-click

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ABSTRACT

A bioorthogonal labeling approach based on the Huisgen-click reaction including the one-step radiosynthesis of two novel versatile and bifunctional labeling building blocks ([^{18}F]AFP) **[^{18}F]12** and ([^{18}F]BFP) **[^{18}F]6** with the PET radionuclide fluorine-18 is described. Optimized reaction conditions for the fully automated synthesis procedure using the TRACERlab Fx_{FN} module gave both piperazine derivatives **[^{18}F]6** and **[^{18}F]12** with radiochemical yields of 31 ± 9% (S.D., n = 8, d.c.) and 29 ± 5% (S.D., n = 19, d.c.), respectively, within 40 min synthesis time and high specific activities after convenient purification using silica gel cartdridges. First biological studies of both building blocks revealed a remarkable *in vitro* stability in rat blood as well as rat plasma over more than 60 min. Sample ligation reactions of **[^{18}F]6** and **[^{18}F]12** with azide and alkyne functionalized amino acid derivatives yielded the corresponding labeled triazoles in good to high RCYs. Moreover, the azide functionalized peptide **17**, which is highly affine to the EphB2 receptor due to its SNEW sequence, was synthesized and successfully radiolabeled with [^{18}F]BFP **[^{18}F]6** under relatively mild conditions yielding the corresponding triazolyl-peptide **[^{18}F]18**.

1. Introduction

The chemoselective radiolabeling of biologically relevant high molecular weight compounds with fluorine-18 still remains an enormous challenge. The search and, in this regard, the development of novel bifunctional building blocks for the mild and convenient radiolabeling of biologically relevant biomacromolecules like peptides, proteins or antibodies is from considerable interest. Normally, these high molecular weight molecules cannot be labeled directly due to the harsh reaction conditions used for the incorporation of the positron emitting radionuclide fluorine-18 ($t_{1/2}$ = 109.8 min) with high specific activity (A_S). To fix this obstacle, various bifunctional labeling agents, also referred as radiolabeling building blocks, have been developed for an introduction under mild conditions. Linkage of these fluorine-18 containing building blocks to the respective molecules can be accomplished via various bioconjugation techniques [1], including acylation, imidation, photochemical conjugation, and thioether formation. However, every method has its advantages and limitations, and future work on the development of rapid, clean and mild synthesis techniques for fluorine-18-labeled high molecular weight derivatives like peptides and proteins is still needed.

To date, a wide range of small building blocks is accessible (Fig. 1). These are specific for appropriate functional groups of the biological active molecule in most of the cases. However, no universally applicable and bioorthogonal building block exist, and in this context, a labeling strategy for each peptide still needs to be evaluated. Approaches for the radiofluorination of e.g. lysine containing peptides with the well-established and most commonly used building block [^{18}F]SFB [2–5] and for cysteine containing peptides using [^{18}F]FBAM [6–8] as one of the maleimide based radiolabeling building blocks were recently described in the literature. Inappropriately, radiolabeling of peptides and proteins in water or buffer sometimes remains sophisticated [7] due to the high lipophilicity of aromatic building blocks like [^{18}F]FB-CHO, [^{18}F]SFB, [^{18}F]FBnA or [^{18}F]FBAM. Further, the selectivity of [^{18}F]SFB is lacking because of pH dependent labeling conditions. It is known that primary amines like lysine and arginine moieties were labeled with [^{18}F]SFB at the same time and, additionally, [^{18}F]SFB leads to decomposition under physiological pH [9]. A strategy for selective peptide labeling with [^{18}F]SFB at only one of the positions mentioned before was applied via solid phase peptide synthesis [9].

Besides, a single cysteine moiety in the biomolecule of interest is mandatory for the labeling using [^{18}F]FBAM with high selectivity. Further, it is known that the Michael addition is a

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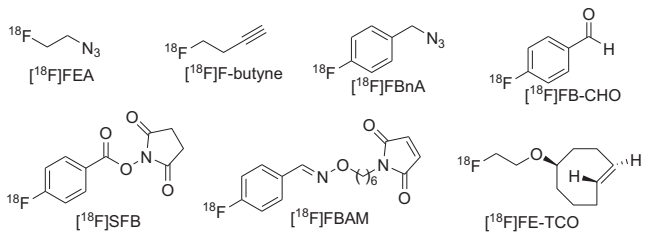


Fig. 1. Selected fluorine-18 containing building blocks.

reversible process even if using [^{18}F]FBAM [10]. There is also evidence of the formation of *E*- and *Z*-isomers due to the oxime group of [^{18}F]FBAM [8]. In addition, radiolabeling using [^{18}F]FE-TCO yielded two regioisomers [11] as well.

Bioorthogonal labeling strategies like the tetrazine-click [11], the traceless Staudinger Ligation [12–15] or the Huisgen-click reaction [16–19] were evaluated in the past. Azide or alkyne functionalized small organic building blocks were successfully applied [20,21] for the last two mentioned ligation reactions. Due to small differences in molar mass (e.g. in the case of [^{18}F]FEA), a separation of the starting peptide from the labeled product is sometimes intricate [20]. Moreover, the concomitant volatility of these small fluorine-18 building blocks like [^{18}F]FEA or [^{18}F]F-butyne frequently results in difficult handling during the radiofluorination process [22].

In this paper, we report the development and preparation of two novel and potent piperazine based building blocks 1-(3-azidopropyl)-4-(3-fluoropropyl)piperazine ([^{18}F]AFP) and 1-(but-3-ynyl)-4-(3-fluoropropyl)piperazine ([^{18}F]BFP) with beneficial properties like a high hydrophilicity or a medium molar mass. Both were prepared for the versatile and irreversible introduction of fluorine-18 into alkyne or azide functionalized bioactive macromolecules in aqueous solutions (without the use of organic solvents) using the Cu(I)-catalyzed Huisgen [3+2]cycloaddition. Both novel building blocks are easily amenable for this purpose due to the convenient two-step preparation of the non-radioactive references as well as the facile access to the precursors which was provided in a four-step synthesis from low priced starting material.

In preliminary publications, it was demonstrated, that the nucleophilic introduction of fluorine-18 succeeded facile and delivered high radiochemical yields with high specific activities in most of the cases [23,24]. Further, the chemical nature of spiro salt precursors allows the simple and adequate separation from the respective radiofluorinated building block by the use of RP-18 or silica gel cartridges, purifications via HPLC are not required. Moreover, radiodefluorination processes of the resulting building blocks were avoided as far as possible due to the use of the 3-[^{18}F]fluoropropyl moiety [25] in contrast to e.g. the fluoromethyl residue [26].

The access to these novel building blocks should be fast, facile and reproducible. To the best of our knowledge, only four publications were found describing remotely controlled preparations of building blocks for the Huisgen-click reaction [27–30]. All these facts prompted us to evaluate an automated synthesis procedure for [^{18}F]AFP as well as [^{18}F]BFP in a remotely controlled system to provide reproducible yields and high specific activities.

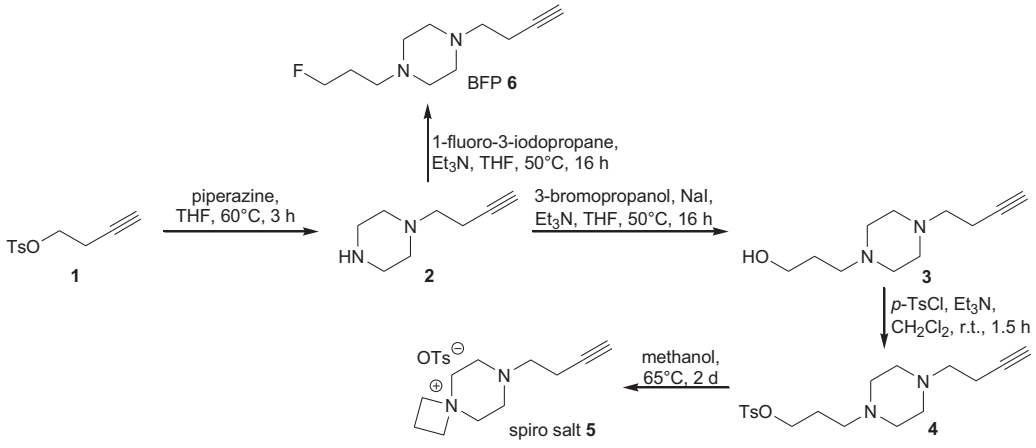
Functionalized amino acids, which were aditionally applied for the functionalization of biomolecules like peptides, were chosen as proof of concept for radiolabeling of biologically relevant molecules with [^{18}F]AFP and [^{18}F]BFP. Further, a peptide with SNEW key sequence, which is known to be highly affine to the EphB2 receptor, was selected and functionalized for this radiolabeling approach. This receptor is known to be dysregulated in different tumor entities, and therefore, it represents a notable and interesting target for specific tumor diagnosis [31].

2. Results and discussion

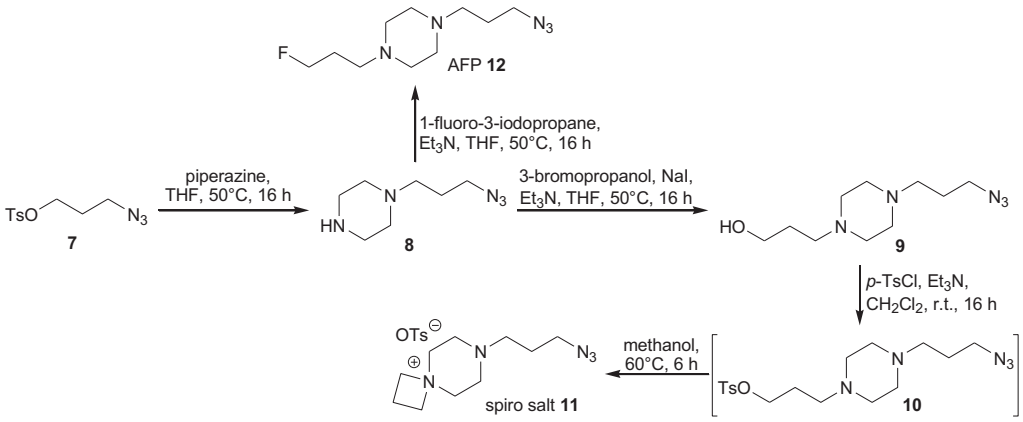
2.1. Synthesis of precursors and reference compounds

A successful and convenient approach for the preparation and radiofluorination of piperazine containing compounds as samples for the labeling of pyrido[2,3-*d*]pyrimidones was recently published [24]. Herein, it was demonstrated that precursors which contain an azoniaspirononane moiety are highly suitable and efficient for the nucleophilic introduction of [^{18}F]fluoride in excellent radiochemical yields (RCY). Based on these findings, two novel spiro precursors **5** and **11** with either azide or alkyne function were easily available in a four-step synthesis procedure whereas non-radioactive reference compounds **6** and **12** were prepared in two steps each starting from piperazine.

First, piperazine derivative **2** was synthesized from but-3-ynyl *p*-toluenesulfonate (**1**) [32] in a yield of 90%. Compound **2** was then reacted with 1-fluoro-3-iodopropane to give the first building block 1-(but-3-ynyl)-4-(3-fluoropropyl)piperazine – BFP (**6**) in 79% yield. Further, **2** was treated with 3-bromopropanol to obtain hydroxy derivative **3** (68%) which was next tosylated with *p*-toluenesulfonyl chloride to yield the open-chained piperazine **4**. Subsequently, spiro salt **5** was formed in 43% yield when **4** was dissolved in methanol and heated at 65 °C for 2 days. The synthesis path of **6** and corresponding spiro precursor **5** was exhibited in Scheme 1.



Scheme 1. Synthesis of spiro precursor **5** and BFP **6**.



Scheme 2. Synthesis of precursor **11** and AFP **12**.

The access to the second fluorine containing building block 1-(3-azidopropyl)-4-(3-fluoropropyl)piperazine – AFP (**12**) and the appropriate precursor **11** is pursuant to the synthesis of **5** and **6**. In the first step, 3-azidopropyl tosylate (**7**) [33] was treated with piperazine to yield **8** (73%). Next, derivative **8** was reacted with 1-fluoro-3-iodopropane to give the non-radioactive reference **12** (74%). Further, **8** was treated with 3-bromopropanol to give compound **9** in nearly quantitative yield. Finally, **9** was tosylated with *p*-TsCl. In this case, it was not possible to isolate **10**. Thus, after removal of the solvent, the crude **10** was subsequently dissolved in methanol and heated at 60 °C for 6 h to give the desired spiro precursor **11** in a yield of 43% after purification via RP-18 column chromatography followed by lyophilization (Scheme 2).

2.2. Automated module syntheses of [^{18}F]AFP and [^{18}F]BFP

First of all, "hand preparations" with precursor **5** according to previous conditions ([^{18}F]fluoride/K 2.2.2., acetonitrile, 100 °C, 15 min) [24] were accomplished for the synthesis of **[^{18}F]6**. An [^{18}F]fluoride conversion of >98% was determined by radio-TLC analyses.

Subsequently, the reaction mixture had to be purified by means of cartridges. Initially, different RP-18 cartridges (CHROMAFIX® C18, Waters Sep-Pak® Light C18, Merck LiChrolut® RP-18, Phenomenex Strata® C18-T) were tested, on which the crude **[^{18}F]6** was trapped, washed with water to remove unreacted spiro precursor **5** and [^{18}F]fluoride and eluted with ethanol to obtain pure **[^{18}F]6**. All RP-18 cartridges resulted in a radiochemical purity (RCP) ranging from 84 to 99% but in a low A_S (<2 GBq/μmol). In addition, small amounts of unreacted [^{18}F]fluoride were found in the product fractions using Strata® C18-T and Sep-Pak® Light C18 cartridges. Thus, cartridges based on silica gel filling were tested. The reaction mixture containing **[^{18}F]6** was trapped on a LiChrolut® Si (Merck) cartridge, washed first with acetonitrile (3 mL) and then eluted from the cartridge (Fig. 3) with a mixture of water and acetonitrile (2 mL, v/v = 1:1). Unreacted spiro salt **5** and traces of [^{18}F]fluoride remained on the cartridge. Afterwards, the solvent was removed at 90 °C in a gentle stream of nitrogen within 4–5 min to obtain **[^{18}F]6** which is then readily available for following labeling procedures.

Based on these findings, an automated procedure was evaluated for the preparation of **[^{18}F]6**. The following radiosyntheses were

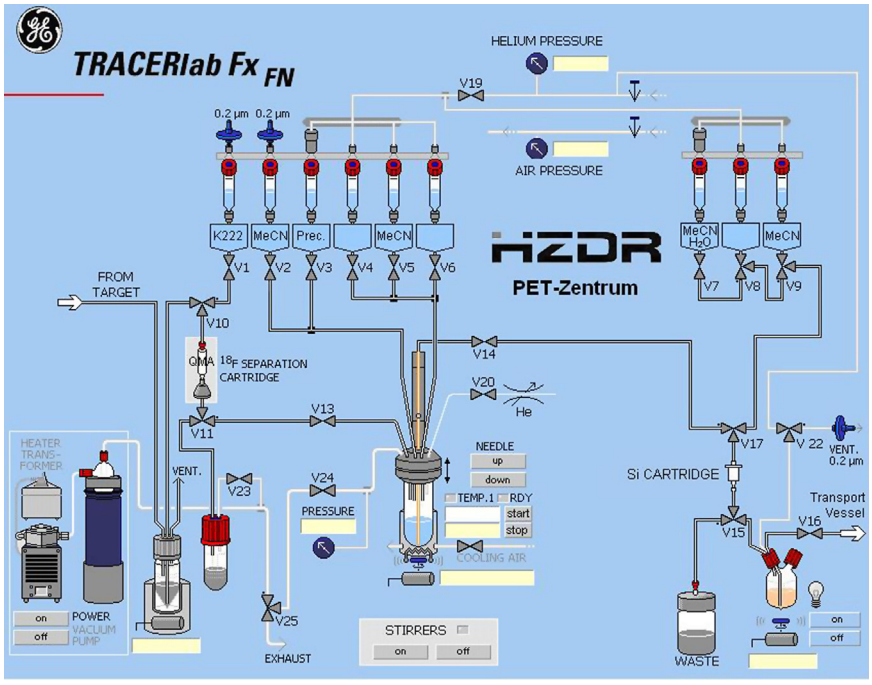


Fig. 2. Scheme of the synthesizer module for the automated radiosyntheses of **[^{18}F]6** and **[^{18}F]12**.

Scheme 3. Radiosyntheses of [^{18}F]BFP **[^{18}F]6** and [^{18}F]AFP **[^{18}F]12**.

carried out in a remotely controlled synthesis module (TRACERlab Fx_{FN}/GE Healthcare). Thus, spiro salt **5** was treated with dried [^{18}F]fluoride/K 2.2.2. in anhydrous acetonitrile at 100 °C for 15 min as demonstrated in Scheme 3.

TRACERlab Fx_{FN} was adapted in terms of program and hardware. The scheme of the synthesizer with modifications in the arrangement of tube lines and valves is outlined in Fig. 2. The major modification involved the installation of a direct connection between valve 14 and valve 17 for trapping of the reaction mixture onto the silica gel cartridge. Noteworthy, the drying of [^{18}F]fluoride (approx. 20 min) is the most time consuming step. The following reaction step proceeds within 15 min and the subsequent cleaning step takes 3 min. All reagent solutions, solvents and reaction mixtures within the module are transferred by nitrogen gas. The tempering of the reactor vessel was realized by compressed air.

The remotely controlled preparation was performed in a total synthesis time of approx. 40 min (starting from [^{18}F]fluoride drying). **[^{18}F]6** was obtained in 31 ± 9% RCY (S.D., n = 8, d.c.) after purification and drying and with a RCP (>98%) but with an increased A_S (7 ± 4 GBq/μmol) as verified by radio-HPLC analyses (Fig. 4). The sometimes low RCY of **[^{18}F]6** combined with the high [^{18}F]fluoride conversion can be explained with the loss of activity during washing, elution and drying. Nevertheless, the setting for **[^{18}F]6** is comparable with the synthesis of [^{18}F]FBAM (40 min, 29% RCY, d.c.) [34]. In contrast, [^{18}F]SFB was prepared within 70 min with 25–38% RCY (d.c.) [7]. Thus, a start activity of 2–6 GBq [^{18}F]fluoride yielded 1 ± 0.5 GBq **[^{18}F]6**.

The preparation and the settings of **[^{18}F]12** are similar to the procedure of **[^{18}F]6**. In this case, the reaction of spiro salt **11** with [^{18}F]fluoride/K 2.2.2. was performed at 100 °C for 15 min in anhydrous acetonitrile. Radio-TLC analyses of the reaction mixture from "hand preparations" showed again more than 95% conversion of [^{18}F]fluoride into **[^{18}F]12** after 15 min, thus, no further optimization was done. Subsequently, the reaction mixture was purified using the approved LiChrolut® Si cartridge (Fig. 5). Unreacted spiro salt **11** and traces of [^{18}F]fluoride remained on the solid phase. After washing with acetonitrile (4 mL), **[^{18}F]12** was simply eluted using a mixture of acetonitrile and water (1.5 mL, v/v = 1:1) followed by the removal of the solvent (90 °C, N_2 stream) with a high radiochemical purity.

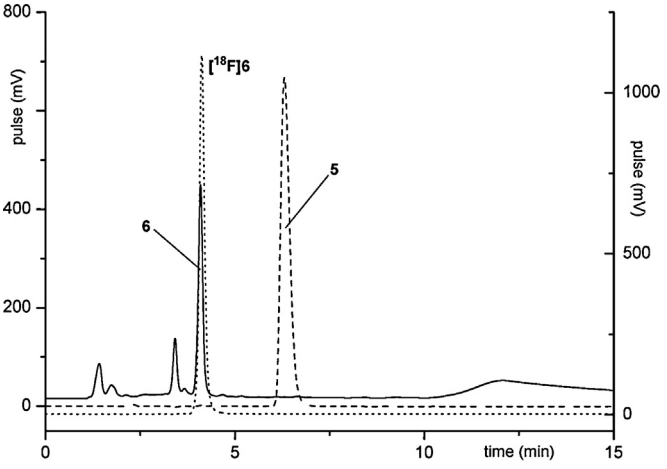


Fig. 4. Representative (radio-)HPLC chromatograms from the preparation of **[^{18}F]6** (dotted line, γ-trace, t_R = 4.2 min), reference **6** (solid line, UV trace, t_R = 4.1 min) and precursor **5** (dashed line, UV trace, t_R = 6.5 min).

Using the remotely controlled synthesis procedure, **[^{18}F]12** was obtained in a RCY of 29 ± 5% (S.D., n = 19, d.c.) in a total synthesis time of approx. 40 min (starting from [^{18}F]fluoride drying) and with a good RCP >97% after purification and drying as verified by radio-TLC and radio-HPLC analyses (Fig. 6). A start activity of 1–12 GBq [^{18}F]fluoride yielded 0.5–2.5 GBq **[^{18}F]12** (A_S = 10 ± 8 GBq/μmol).

2.3. Lipophilicity and in vitro stability tests of [^{18}F]AFP and [^{18}F]BFP

The liphophilicity of a pharmacologically active compound is a fundamental physicochemical parameter and, in this regard, logP/logD determinations are from high importance and relevance e.g. to evaluate structure-activity relationships in medicinal chemistry as well as in radiopharmacy [35]. Preferentially, peptide labeling should be performed under physiological condition to avoid decomposition, alteration or even loss of biological activity. In this context, the liphophilicity can be directed when choosing the respective labeling building block. For this purpose, the lipophilicity (logD) of **[^{18}F]6** and **[^{18}F]12** was determined at pH 7.47 using PBS buffer and n-octanol according to the shake-flask method

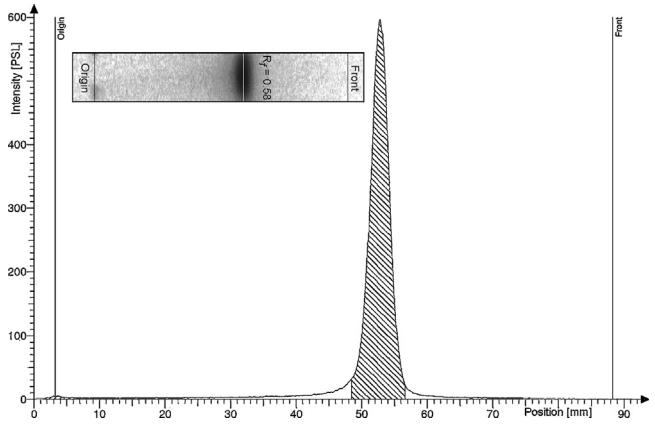


Fig. 3. Radio-TLC (eluent: methanol) of **[^{18}F]6** after purification using a LiChrolut® Si cartridge.

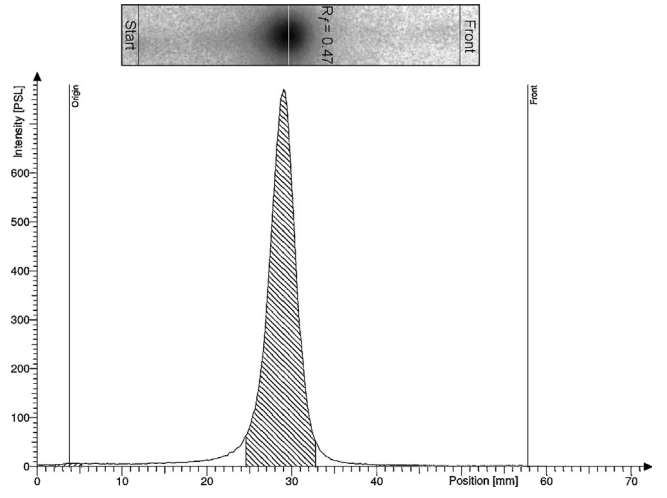


Fig. 5. Radio-TLC (eluent: methanol) of **[^{18}F]12** after purification using a LiChrolut® Si cartridge.

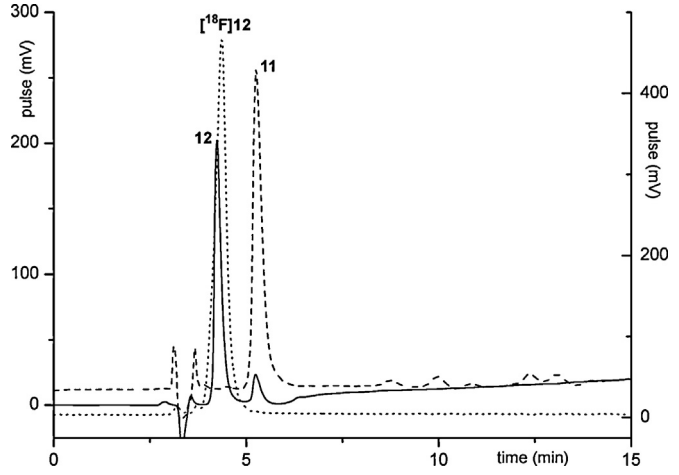


Fig. 6. Representative (radio-)HPLC chromatograms from the preparation of **[^{18}F]12** (dotted line, γ-trace, t_R = 4.4 min), reference **12** (solid line, UV trace, t_R = 4.2 min) and precursor **11** (dashed line, UV trace, t_R = 5.3 min).

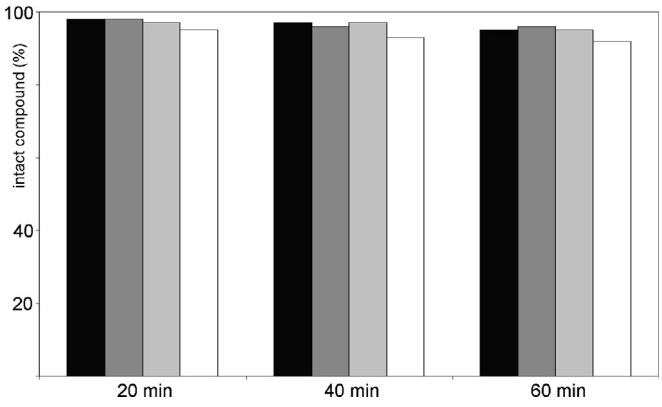


Fig. 7. Results of *in vitro* stability determinations of **[^{18}F]6** in rat plasma □ and in rat blood ■ as well as **[^{18}F]12** in rat plasma ■ and in rat blood ■ after 20, 40 and 60 min, respectively.

(OECD guideline) [36] being 0.31 ± 0.03 (S.D., n = 3) for **[^{18}F]6** and 0.70 ± 0.01 (S.D., n = 3) for **[^{18}F]12**. On the basis of these values, radiofluorinations are facilitated in water or buffer as well as in organic solvents.

Next, the stability of both **[^{18}F]6** and **[^{18}F]12** was elaborated *in vitro*. Therefore, a solution of **[^{18}F]6** in PBS buffer was treated with rat plasma and rat blood (erythrocytes), respectively, and was shaken for at least 60 min at 37 °C. Afterwards, radio-TLC as well as radio-HPLC analyses showed no decomposition of **[^{18}F]6**. The same procedure was applied for the determination of the stability of **[^{18}F]12**. Supplementary, no radiodefluorination was observed for both novel building blocks as well. The results were outlined in Fig. 7.

2.4. Radiolabeling using bioorthogonal Huisgen-Click

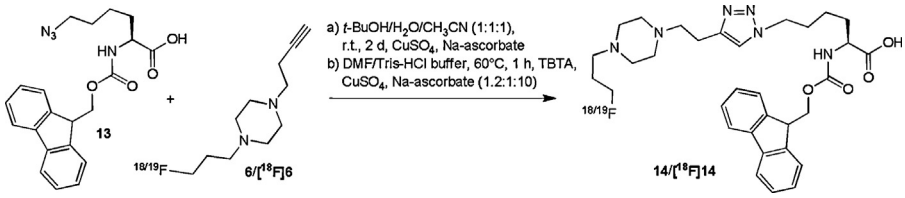
The Huisgen cycloaddition is highly attractive for the introduction of fluorine-18 into small organic compounds as well as in high molecular weight molecules like peptides due to the bioorthogonal character of this reaction. To investigate optimal conditions for the radiolabeling of the azide functionalized peptides, the new building block **[^{18}F]6** was first applied using *N*-Fmoc-ε-azido-L-norleucine (**13**) [37]. Compound **13** was chosen as sample amino acid due to its convenient availability. Furthermore, derivative **13** (and additionally **15**) can be used for the functionalization of respective SNEW peptides. Fluorine-19-containing reference **14** was prepared by the reaction of **6** with norleucine derivative **13** in a solution of *t*-BuOH/H_2O/acetonitrile with $CuSO_4$ and sodium ascorbate for 2 d in 55% yield. For the radiolabeling, solutions of TBTA, sodium ascorbate and $CuSO_4$ were added to a mixture of amino acid **13** (t_R = 22.4 min) in DMF and **[^{18}F]6** (t_R = 4.2 min) in Tris/HCl buffer at pH 8.0 (DMF was mandatory due to the high lipophilicity of **13**). In the beginning, the reaction was accomplished at ambient temperature and at 50 °C for 30 min according to literature conditions [38]. But it was found that higher temperatures (60 °C) and a longer reaction time (1 h) resulted in a better RCY (59% d.c., starting from **[^{18}F]6**) as outlined in Scheme 4. The conversion of **[^{18}F]6** into ^{18}F-labeled amino acid **[^{18}F]14** (t_R = 19.4 min) was controlled by radio-HPLC analyses (Fig. 8).

The second approach comprehends the functionalization of biologically active molecules with an alkyne moiety. A simple way to functionalize amino acid derivatives with alkyne residues consists of the reaction of Fmoc-L-lysine with 4-pentynoic acid to yield **15** [39]. As proof of concept, **[^{18}F]12** (t_R = 4.4 min) was applied for the radiofluorination of **15** (t_R = 22 min) using conditions (b). The click-labeled product **[^{18}F]16** (t_R = 19.8 min) was obtained in a RCY of 79% (d.c., starting from **[^{18}F]12**) under microwave conditions (50 watt, 1 h) (Scheme 5 and Fig. 9). For the confirmation of **[^{18}F]16**, the respective fluorine-19-containing reference **16** was prepared by the reaction of **12** with compound **15** in a solution of DMF/THF with CuI and DIPEA for 16 h in 27% yield according to conditions (a).

2.5. Application to peptides

The successful radiolabeling of sample amino acids prompted us to label the azide functionalized SNEW peptide **17** as pharmacologically relevant molecule with the alkyne containing building block **[^{18}F]6**. The SNEW key sequence is known to be highly affine to the EphB2 receptor. In this context, it is known that the EphB2 receptor is dysregulated in different tumor entities [31] in correlation with e.g. tumor metastasis [40].

Thus, a peptide with the sequence SNEWILPRLPQH was functionalized with (4*S*)-4-azido-L-proline [41] at the *C*-terminus to retain high affinity to its biological target, the EphB2 receptor, which necessitates an unmodified *N*-terminus [42]. Next, the non-radioactive reference peptide **18** was prepared by the click reaction of **6** with peptide **17** in Sørensen phosphate buffer and ethanol for 30 min at ambient temperature using sodium ascorbate and $CuSO_4 \cdot 5\ H_2O$ with 98% yield. Purification was performed via preparative HPLC and formation of **18** was confirmed using ESI-MS. Afterwards, the conditions found for the labeling of azide



Scheme 4. Reaction of **6**/**[^{18}F]6** with *N*-Fmoc-ε-azido-L-norleucine (**13**): (a) Normal conditions and (b) radiolabeling conditions.

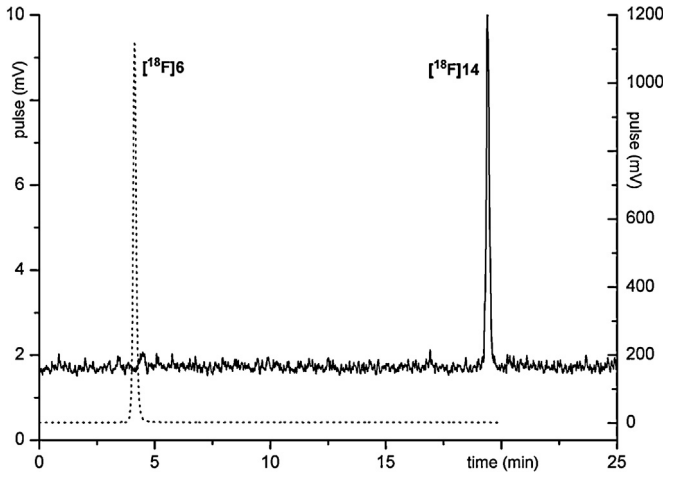


Fig. 8. Representative radio-HPLC chromatograms of **[^{18}F]6** (dotted line, γ-trace, t_R = 4.2 min) and of radiolabeled amino acid **[^{18}F]14** (solid line, γ-trace, t_R = 19.4 min).

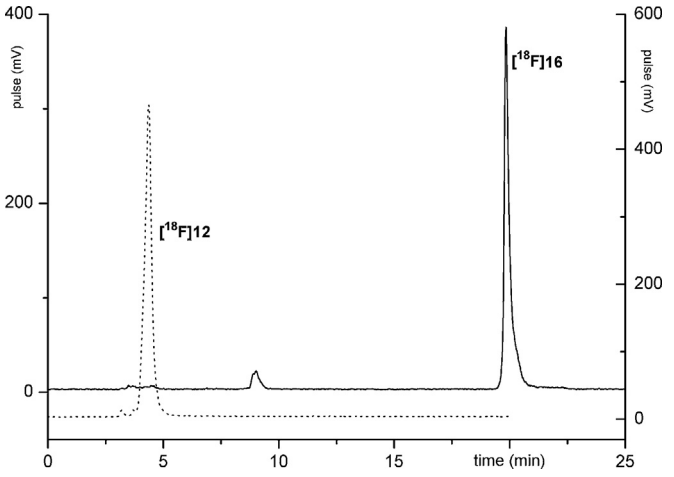
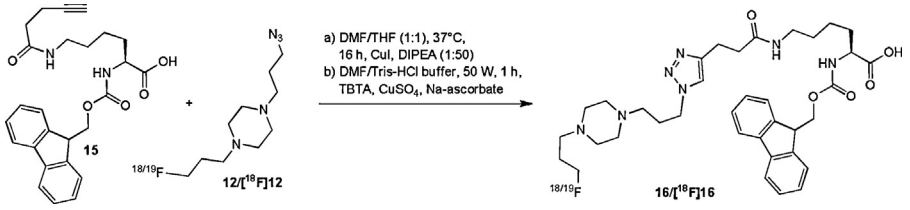


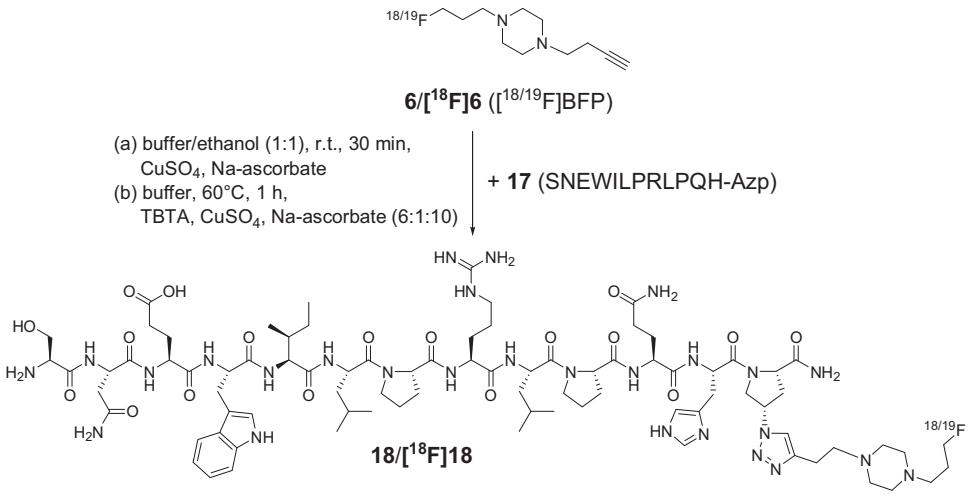
Fig. 9. Representative radio-HPLC chromatograms of **[^{18}F]12** (dotted line, γ-trace, t_R = 4.4 min) and of radiolabeled amino acid **[^{18}F]16** (solid line, γ-trace, t_R = 19.8 min).

containing amino acids were applied for the radiofluorination of azide functionalized peptide **17**. Thus, TBTA, $CuSO_4 \cdot 5\ H_2O$ and sodium ascorbate (6:1:10 eq) were added to a mixture of SNEW peptide **17** (1 eq.) and **[^{18}F]6** in Sørensen phosphate buffer at pH 7.2. For optimizations, the reaction time (10, 30, 60 min) as well as temperature (r.t., 40, 50, 60 °C) and amount of peptide precursor **17** (100–600 μg) were varied. An optimal peptide amount was found to be 0.3–0.5 mg/mL. More insoluble residues were found in the reaction mixture when higher peptide concentrations were used, which supposed to be an unknown triazole-copper species [43]. Best conditions were pointed out in (Scheme 6). The process of conversion of **[^{18}F]6** into ^{18}F-labeled peptide **[^{18}F]18** was controlled by radio-HPLC (Figs. 10 and 11).

A separation of labeled peptide **[^{18}F]18** from starting peptide **17** was possible via HPLC, but the separation of **[^{18}F]18** from **[^{18}F]6** was not amenable due to the similar t_R values (t_R = 3.2 min for **[^{18}F]6** and 4.0 min for **[^{18}F]18**) using various RP-18 columns and gradients. To overcome this problem, size-exclusion chromatography (SEC) using a HighTrap® desalting column with phosphate buffer as eluent at a flow rate of 0.5 mL/min was applied, which showed desirable separation properties due to the medium molar mass (M_W = 198.28 g/mol) of the introduced building block **6**/



Scheme 5. Reaction of **12**/**[^{18}F]12** with lysine derivative **15**: (a) Normal conditions and (b) radiolabeling conditions.



Scheme 6. Synthesis pathway to the $^{18/19}$F-labeled peptide **18**/**[^{18}F]18** with **6**/**[^{18}F]6**: (a) Normal conditions and (b) radiolabeling conditions.

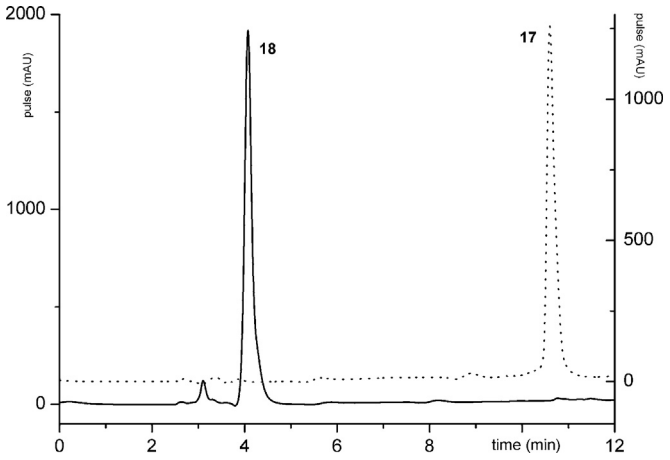


Fig. 10. Representative HPLC chromatograms of SNEWILPRLPQH-Azp **17** (dotted line, UV-trace, t_R = 10.8 min) and of the reaction mixture of reference peptide **18** (solid line, UV-trace, t_R = 4.1 min). Gradient: 0 min 10% ACN, 12 min 40% ACN (+0.1% TFA).

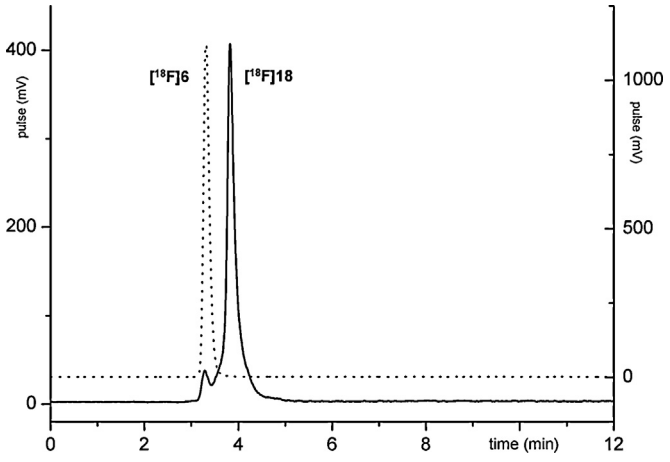


Fig. 11. Representative radio-HPLC chromatograms of **[^{18}F]6** (dotted line, γ-trace, t_R = 3.2 min) and of radiolabeled peptide **[^{18}F]18** (solid line, γ-trace, t_R = 4.0 min). Gradient: 0 min 10% ACN, 12 min 40% ACN (+0.1% TFA).

[^{18}F]6. Peptide **[^{18}F]18** was obtained in 17–25% RCY (d.c., starting from **[^{18}F]6**) with >99% RCP and A_S = 4 ± 2 GBq/μmol (S.D., n = 3).

Additionally, the stability of **[^{18}F]18** for an application as radiotracer was investigated *in vitro* by incubation using rat plasma for 1 h at 37 °C (samples were taken after 20, 40 and 60 min). After 1 h, more than 81% of **[^{18}F]18** were found to be intact as indicated by radio-HPLC analysis.

3. Conclusions

The radiolabeling of peptides and proteins still remains an outstanding challenge in radiopharmacy. Thus, the evaluation and preparation of various labeling building blocks were found in the literature. In general, their application highly depends on the functional groups of the respective biomacromolecule.

Herein, we demonstrated the evaluation of a fast and simple automated synthesis procedures of two novel efficient and robust building blocks 1-(3-azidopropyl)-4-(3-fluoropropyl)piperazine ([^{18}F]AFP) and 1-(but-3-ynyl)-4-(3-fluoropropyl)piperazine ([^{18}F]BFP) with good RCYs and high RCPs suitable for convenient radiofluorinations using the bioorthogonal Huisgen-click reaction. Further, the subsequent purification from the precursor was accomplished via cartridges. LogD determinations and *in vitro* tests revealed favorable properties and a high stability of both building blocks, no radiodefluorination was observed. The ^{19}F-references and the appropriate precursors were easily available in two and four preparation steps, respectively, starting from low cost starting material. For radiofluorination purposes, Fmoc-protected amino acid derivatives were modified and radiofluorinated using both novel building blocks under milder reaction conditions in contrast to harsh fluorine-18 labeling and with high RCY within the click reaction. This procedure was successfully applied to the radiolabeling of a SNEW peptide with high affinity to the EphB2 receptor. Due to the medium molar mass of both building blocks, an easy separation of the ^{18}F-peptide from the starting material was accessible using SEC.

4. Experimental

4.1. General

All reagents were purchased from commercial sources and were used without further purification. Anhydrous solvents (THF, acetonitrile, dichloromethane) were purchased from Sigma-Aldrich (over molecular sieves, 99.5%). 1-Fluoro-3-iodopropane was purchased from Apollo Scientific. Starting materials **1** and **7** were prepared according to the literature [32,33]. Analytical TLCs were performed on pre-coated Silica Gel 60 F_{254} plates or on F_{254}s plates for RP-18 (Merck) and visualized under UV-light (λ = 254 nm) or detected by staining the plates with ninhydrin spray reagent (5% in ethanol) followed by heating. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were recorded on a Varian Inova-400 spectrometer at 400, 101 and 376 MHz, respectively. Chemical shifts are reported downfield from tetramethylsilane (TMS) and trichlorofluoromethane as internal standard, respectively. MS spectra were obtained on a Micromass Quattro-LC spectrometer using electron spray as ionization method. Melting points were recorded on a Cambridge Instruments Galen III apparatus and are uncorrected. Microanalyses were carried out with a Hekatech CHNS elemental analyser EuroEA 3000. Analytical HPLC was performed on a Elite LaChrom/VWR Hitachi HPLC system, equipped with a reverse phase column (Phenomenex Aqua C18; 250 × 4.6 mm; 100 Å), a UV-diode array detector (220 nm) and a scintillation radiodetector (Raytest, Gabi Star). The elution was done using a gradient of acetonitrile:water (containing 0.1% TFA) (0–1 min: 10% ACN; 1–17 min: 10 → 45% ACN; 17–18 min: 45 → 90% ACN; 18–25 min: 90% ACN) unless otherwise stated at a flow rate of 1 mL/min.

All radioactive compounds were identified using analytical radio-HPLC by comparison of the retention time of the reference compound. Decay-corrected RCYs were quantified by integration of radioactive peaks on a radio-TLC using a radio-TLC scanner (Fuji, BAS2000).

[^{18}F]Fluoride was produced utilizing the PET cyclotron Cyclone 18/9 (IBA, Belgium). [^{18}O]H_2O was irradiated with protons (18 MeV, 30 μA) exploiting the ^{18}O(p,n)^{18}F nuclear reaction.

4.2. General procedure for the peptide synthesis

Peptide **17** was prepared on rink amide-MBHA resin (extent of labeling: 0.6 mmol/g loading) on a 0.05–0.10 M scale. The Fmoc protecting group was removed with 20% piperidine in DMF for 15 min. The carboxyl group of the incoming amino acid was activated with HBTU and HOBt. Fmoc-amino acid (5 eq), HBTU (5 eq), HOBt (5 eq), and DIPEA (10 eq) were dissolved in DMF and NMP, respectively, and added to the resin. The coupling was carried out twice for each residue in a coupling time of 2 h. The peptide was deprotected and cleaved from the solid support with a mixture of TFA/TIS/H_2O 95:2.5:2.5 for 4 h at ambient temperature.

Afterwards, the resin was filtered, the crude peptide was precipitated by adding cold diethyl ether and washed with ice-cold diethyl ether. The residual ether was removed by evaporation and the peptide was purified by preparative HPLC using a Varian Dynamax Microsorb 60-8 C18 column (250 × 21.4 mm) at a flow rate of 20 mL/min with gradients of water and acetonitrile with 0.1% TFA.

4.3. Chemistry

4.3.1. 1-(But-3-yn-1-yl)piperazine (**2**)

Piperazine (7.21 g, 83.68 mmol) was dissolved in anhydrous THF (70 mL), but-3-yn-1-yl 4-methylbenzenesulfonate (**1**) [32] (5.38 g, 23.99 mmol) was added dropwise and the mixture was stirred at 60 °C for 3 h. The resulting precipitate was filtered and washed with THF (70 mL), the solvent was removed under reduced pressure and the crude product was purified by column chromatography (ethyl acetate → methanol) to obtain compound **2** as pale yellow oil (2.98 g, 90%). R_f = 0.13 (methanol); ^{1}H NMR (CD_3CN): δ 2.75–2.73 (m, 4H, Pip-H), 2.46 (t, ^3J = 7.8 Hz, 2H, $NC\underline{H}_2CH_2$), 2.36–2.31 (m, 4H, Pip-H), 2.31 (dd, ^3J = 7.8 Hz, ^4J = 2.7 Hz, 2H, $NCH_2C\underline{H}_2$), 2.15 (t, ^4J = 2.7 Hz, 1H, CH); ^{13}C NMR (CD_3CN): δ 84.1 (C_q), 70.1 (CH), 58.4 ($N\underline{C}H_2CH_2$), 55.0 (Pip-C), 46.8 (Pip-C), 17.1 ($NCH_2\underline{C}H_2$); MS (ESI+) *m/z*: 139 [M^++H]; Anal. calcd for $C_8H_{14}N_2$ (138.20): C, 69.42%; H, 10.21%; N, 20.27%; Found: C, 69.30%; H, 10.17%; N, 20.83%.

4.3.2. 3-(4-(But-3-ynyl)piperazin-1-yl)propan-1-ol (**3**)

3-Bromopropanol (3 mL, 35.92 mmol) was added to a solution of compound **2** (2.92 g, 21.13 mmol), NaI (320 mg, 2.11 mmol) and triethylamine (3.5 mL, 25.36 mmol) in anhydrous THF (20 mL) and the mixture was stirred at 50 °C for 16 h. Afterwards, the solvent was removed under reduced pressure and the residue was dissolved in water. The aqueous layer was extracted with dichloromethane (3 × 15 mL), the organic layers were dried over Na_2SO_4, the solvent was removed, and the residue was purified by column chromatography (methanol) to obtain compound **3** as a colorless solid (2.84 g, 68%). R_f = 0.42 (methanol); M.p. 94–97 °C; ^{1}H NMR (CD_3OD): δ 3.62 (t, ^3J = 5.2 Hz, 2H, $OC\underline{H}_2$), 2.59 (t, ^3J = 5.8 Hz, 2H, $NC\underline{H}_2CH_2$), 2.52 (t, ^3J = 7.2 Hz, 2H, $OCH_2CH_2C\underline{H}_2$), 2.67–2.45 (m, 8H, Pip-H), 2.39 (dt, ^3J = 7.5 Hz, ^4J = 2.7 Hz, 2H, $NCH_2C\underline{H}_2$), 2.28 (t, ^3J = 2.7 Hz, 1H, CH), 1.77–1.71 (m, 2H, $OCH_2C\underline{H}_2$); ^{13}C NMR (CD_3OD): δ 106.2 (C_q), 70.6 (CH), 61.7 (OCH_2), 58.0 (NCH_2), 56.8 ($OCH_2CH_2\underline{C}H_2$), 53.8 (Pip-C), 53.4 (Pip-C), 30.0 ($OCH_2\underline{C}H_2$), 17.2 ($NCH_2\underline{C}H_2$); MS (ESI+) *m/z*: 197 [M^++H]; Anal. calcd for $C_{11}H_{20}N_2O$ (196.29): C, 67.31%; H, 10.27%; N, 14.27%; Found: C 67.30%; H 10.01%; N 14.33%.

4.3.3. 3-(4-(But-3-ynyl)piperazin-1-yl)propyl 4-methylbenzenesulfonate (**4**)

4-Methylbenzenesulfonyl chloride (330 mg, 1.74 mmol) was added to a solution of compound **3** (310 mg, 1.58 mmol) and triethylamine (0.9 mL, 6.32 mmol) dissolved in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at ambient temperature for 1.5 h. Afterwards, the solvent was removed under reduced pressure at 25 °C and the residue was purified by column chromatography (ethyl acetate:methanol 10:1 → 6:1) to obtain **4** as pale yellow oil (240 mg, 43%). R_f = 0.65 (methanol). Little amounts of **5** were formed during the purification; ^{1}H NMR ($CDCl_3$): δ 7.79 (d, ^3J = 8.3 Hz, 2H, H-o), 7.34 (d, ^3J = 8.0 Hz, 2H, H-m), 4.09 (t, ^3J = 6.4 Hz, 2H, $OC\underline{H}_2$), 2.60 (t, ^3J = 7.4 Hz, 2H, $NC\underline{H}_2CH_2$), 2.72–2.57 (m, 8H, Pip-H), 2.48 (t, ^3J = 7.0 Hz, 2H, $OCH_2CH_2C\underline{H}_2$), 2.45 (s, 3H, CH_3), 2.38 (dt, ^3J = 7.8 Hz, ^4J = 2.6 Hz, 2H, $NCH_2C\underline{H}_2$), 1.97 (t, ^3J = 2.6 Hz, 1H, CH), 1.96–1.91 (m, 2H, $OCH_2C\underline{H}_2$); ^{13}C NMR ($CDCl_3$): δ 130.1 (C-p), 129.7 (C-i), 128.1 (C-m), 127.2 (C-o), 82.9 (C_q), 69.2 (CH), 57.1 ($O\underline{C}H_2$), 55.6 ($N\underline{C}H_2$), 53.3 (Pip-C), 53.0 (Pip-C), 43.4 ($OCH_2CH_2\underline{C}H_2$), 30.0 ($OCH_2\underline{C}H_2$), 24.5 ($CH_3$), 16.9 ($NCH_2\underline{C}H_2$); MS (ESI+) *m/z*: 179 [M^+−OTs]; Anal. calcd for $C_{18}H_{26}N_2O_3S$ (350.48): C, 61.69%; H, 7.48%; N, 7.99%; Found: C, 61.35%; H, 7.70%; N, 7.83%.

4.3.4. 7-(But-3-ynyl)-7-aza-4-azoniaspiro[3.5]nonane 4-methylbenzene-sulfonate (**5**)

Compound **4** (240 mg, 0.68 mmol) dissolved in methanol (5 mL) was stirred at 65 °C for 2 d. The solvent mixture was concentrated and ice-cold toluene (3 mL) was added. The resulting precipitate was filtered, washed with ice-cold toluene (10 mL) and petroleum ether (10 mL), and the solid was dried in high vacuum to obtain **5** as pale yellow solid (100 mg, 43%). M.p. 150–151 °C; ^{1}H NMR (CD_3CN): δ 7.60 (d, ^3J = 8.1 Hz, 2H, H-o), 7.15 (d, ^3J = 8.0 Hz, 2H, H-m), 4.14 (t, ^3J = 8.3 Hz, 4H, H-1/3), 3.44–3.37 (m, 4H, Pip-H), 2.69–2.60 (m, 4H, Pip-H), 2.58 (t, ^3J = 7.3 Hz, 2H, $NC\underline{H}_2CH_2$), 2.56–2.48 (m, 2H, H-2), 2.34 (dt, ^3J = 7.5 Hz, ^4J = 2.7 Hz, 2H, $NCH_2C\underline{H}_2$), 2.33 (s, 3H, CH_3), 2.20 (t, ^3J = 2.7 Hz, 1H, CH); ^{13}C NMR (CD_3CN): δ 146.9 (C-p), 139.4 (C-i), 129.3 (C-m), 126.6 (C-o), 83.3 (C_q), 70.6 (CH), 60.2 ($N\underline{C}H_2$), 56.3 (C-1/3), 47.6 (C-5/6/8/9), 21.2 (CH_3), 17.3 ($NCH_2\underline{C}H_2$), 14.4 (C-2); MS (ESI+) *m/z*: 179 [M^+−OTs]; MS (ESI−) *m/z*: 171 [OTs]; Anal. calcd for $C_{18}H_{26}N_2O_3S$ (350.48): C, 61.69%; H, 7.48%; N, 7.99%; Found: C 61.54%; H 7.55%; N 8.09%; Analytical HPLC: t_R = 6.5 min.

4.3.5. 1-(But-3-ynyl)-4-(3-fluoropropyl)piperazine–BFP (**6**)

1-Fluoro-3-iodopropane (0.6 mL, 7.20 mmol) and triethylamine (1.0 mL, 7.20 mmol) were added to a solution of compound **2** (1.00 g, 7.23 mmol) in anhydrous THF (10 mL). The mixture was stirred at 50 °C for 16 h. Afterwards, the solvent was removed under reduced pressure and the residue was purified by column chromatography (ethyl acetate) to obtain **6** as a yellow oil (1.13 g, 79%). R_f = 0.58 (methanol); ^{1}H NMR ($CDCl_3$): δ 4.49 (dt, $^2J_{H,F}$ = 47.2 Hz, ^3J = 6.0 Hz, 2H, FCH_2), 2.60 (t, ^3J = 5.5 Hz, 2H, NCH_2), 2.57–2.38 (m, 8H, Pip-H), 2.44 (t, ^3J = 7.2 Hz, 2H, NCH_2), 2.38 (dt, ^3J = 7.0 Hz, ^4J = 2.6 Hz, 2H, $C\underline{H}_2$), 1.97 (t, ^3J = 2.7 Hz, 1H, CH), 1.94–1.81 (m, 2H, $FCH_2C\underline{H}_2$); ^{13}C NMR ($CDCl_3$): δ 82.9 (C_q), 82.7 (d, $^1J_{C,F}$ = 164.3 Hz, FCH_2), 69.1 (CH), 57.1 (NCH_2), 54.4 (d, $^3J_{C,F}$ = 5.5 Hz, NCH_2), 53.2, 53.0 (2 × Pip-C), 28.8 (d, $^2J_{C,F}$ = 19.8 Hz, $FCH_2\underline{C}H_2$), 16.9 (CH_2); ^{19}F NMR ($CDCl_3$): δ–220.4; MS (ESI+) *m/z*: 221 [M^++Na]; Anal. calcd for $C_{11}H_{19}FN_2$ (198.28): C, 66.63%; H, 9.66%; N, 14.13%; Found: C, 66.51%; H, 9.70%; N, 13.83%; Analytical HPLC: t_R = 4.1 min.

4.3.6. 1-(3-Azidopropyl)piperazine (**8**)

Piperazine (670 mg, 7.78 mmol) was dissolved in anhydrous THF (10 mL), 3-azidopropyl 4-methylbenzenesulfonate (**7**) [33] (904 mg, 3.54 mmol) was added dropwise and the mixture was stirred at 50 °C for 16 h. The resulting precipitate was filtered and washed with THF (10 mL), the solvent was removed under reduced pressure and the crude product was purified either by bulb tube distillation (100 °C, 5 × 10^{-2} mbar) or by column chromatography (ethyl acetate:ethanol 1:1) to yield compound **8** as pale yellow oil (325 mg, 54%). R_f = 0.14 (methanol); ^{1}H NMR ($CDCl_3$): δ 3.33 (t, ^3J = 6.8 Hz, 3H, CH_2N_3), 2.88 (t, ^3J = 4.9 Hz, 4H, NCH_2), 2.35–2.45 (m, 6H, NCH_2), 1.71–1.80 (m, 2H, $NCH_2C\underline{H}_2$), 1.61 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 56.0 ($N\underline{C}H_2$), 54.6 (Pip-NCH_2), 49.8 (CH_2N_3), 46.2 (Pip-NCH_2), 26.2 ($NCH_2\underline{C}H_2$); MS (ESI+) *m/z*: 170 (100) [M^++H]; Anal. calcd for $C_7H_{15}N_5$ (169.23): C, 49.68%; H, 8.93%; N, 41.38%. Found: C 49.77%; H 9.19%; N 41.33%.

4.3.7. 3-(4-(3-Azidopropyl)piperazin-1-yl)propan-1-ol (**9**)

NaI (10 mg, 0.06 mmol) and triethylamine (77 mg, 0.77 mmol) were added to a solution of compound **8** (108 mg, 0.64 mmol) in anhydrous THF (4 mL). 3-Bromopropanol (133 mg, 0.96 mmol) was added and the mixture was stirred at 50 °C for 16 h. Afterwards, the solvent was removed under reduced pressure

and the residue was dissolved in water. The aqueous layer was extracted three times with dichloromethane and the organic layer was dried over Na_2SO_4, the solvent was removed, and the residue was purified by column chromatography (ethanol) to yield compound **9** as a colorless syrup (139 mg, 96%). R_f = 0.34 (methanol); ^{1}H NMR ($CDCl_3$): δ 4.00 (br. s, 1H, OH), 3.67 (t, ^3J = 5.4 Hz, 2H, OCH_2), 3.30 (t, ^3J = 6.9 Hz, 2H, CH_2N_3), 2.79–2.25 (m, 12H, Pip-H, 2× NCH_2), 1.79–1.64 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$): δ 64.7 (OCH_2), 58.9 (NCH_2), 55.3 (NCH_2), 53.8, 53.4 (2× Pip-C), 49.7 (CH_2N_3), 27.2, 26.4 (2× CH_2); MS (ESI+): *m/z* (%) 228 (100) [M^++H], 143 (20) [$M^+-C_3H_3N_3$]. Anal. calcd for $C_{10}H_{21}N_5O$ (227.31): C, 52.84%; H, 9.31%; N, 30.81%; Found: C, 52.55%; H, 9.33%; N, 30.54%.

4.3.8. 3-(4-(3-Azidopropyl)piperazin-1-yl)propyl 4-methylbenzenesulfonate (**10**) and 7-(3-azidopropyl)-7-aza-4-azoniaspiro[3.5]nonane 4-methylbenzenesulfonate (**11**)

4-Methylbenzenesulfonyl chloride (1.13 g, 5.94 mmol) was added to a solution of compound **9** (900 mg, 3.96 mmol) and Et_3N (802 mg, 7.92 mmol) dissolved in anhydrous CH_2Cl_2 (20 mL). The mixture was stirred at ambient temperature for 16 h. Afterwards, the solvent was replaced by methanol (20 mL) and the mixture was heated at 60 °C for 6 h. After cooling to r.t., the solvent was removed under reduced pressure and the residue was purified via RP-18 column chromatography (LiChroprep: 5–20 μm; water:methanol 8:2 → 1:1) and lyophilization to yield **11** as colorless solid (240 mg, 43%). R_f = 0.10 (RP-TLC, methanol). M.p. 215–216 °C. ^{1}H NMR (CD_3CN): δ 7.64 (d, ^3J = 8.1 Hz, 2H, H-o), 7.21 (d, ^3J = 7.9 Hz, 2H, H-m), 4.26 (t, ^3J = 8.2 Hz, 4H, H-1/3), 3.78–3.67 (m, 4H, Pip-H), 3.62 (t, ^3J = 6.5 Hz, 2H, CH_2N_3), 3.39–3.34 (m, 4H, Pip-H), 3.11–3.07 (m, 2H, NCH_2), 2.55 (dt, ^2J = 17.0 Hz, ^3J = 8.3 Hz, 2H, H-2), 2.35 (s, 3H, CH_3), 1.90–1.88 (m, 2H, $NCH_2C\underline{H}_2$). ^{13}C NMR (CD_3CN): δ 145.0 (C-p), 140.6 (C-i), 129.6 (C-m), 126.6 (C-o), 66.3 ($N\underline{C}H_2$), 54.7 (C-1/3), 49.3 (CH_2N_3), 47.6 (C-5/6/8/9), 25.0 ($NCH_2\underline{C}H_2$), 21.3 (CH_3), 14.4 (C-2). MS (ESI+): *m/z* (%) 210 (100) [M^+−OTs]. MS (ESI−): *m/z* (%) 171 (100) [OTs]. Anal. calcd for $C_{17}H_{27}N_5O_3S$ (381.49): C 53.52%; H 7.13%; N 18.36%. Found: C 53.50%; H 7.42%; N 18.18%. Analytical HPLC: t_R = 5.3 min.

4.3.9. 1-(3-Azidopropyl)-4-(3-fluoropropyl)piperazine – AFP (**12**)

1-Fluoro-3-iodopropane (471 mg, 2.50 mmol) was added to a solution of compound **8** (424 mg, 2.50 mmol) and Et_3N (380 mg, 3.76 mmol) in anhydrous THF (8 mL). The mixture was stirred at 50 °C for 16 h. Thereafter, the solvent was removed under reduced pressure and the residue was purified by column chromatography (ethyl acetate) to yield **12** as a pale yellow syrup (424 mg, 74%). R_f = 0.46 (methanol). ^{1}H NMR ($CDCl_3$): δ 4.50 (dt, $^2J_{H,F}$ = 47.3 Hz, ^3J = 5.9 Hz, 2H, FCH_2), 3.33 (t, ^3J = 6.7 Hz, 2H, CH_2N_3), 2.63–2.37 (m, 12H, Pip-H, 2× NCH_2), 1.95–1.72 (m, 4H, CH_2). ^{13}C NMR ($CDCl_3$): δ 82.7 (d, $^1J_{C,F}$ = 164.6 Hz, FCH_2), 55.4 (NCH_2), 54.4 (d, $^3J_{C,F}$ = 5.5 Hz, NCH_2), 53.3, 53.2 (Pip-C), 49.7 (CH_2N_3), 28.0 (d, $^2J_{C,F}$ = 19.8 Hz, $FCH_2\underline{C}H_2$), 26.4 (CH_2). ^{19}F NMR ($CDCl_3$): δ–220.4. MS (ESI+): *m/z* (%) 252 (10) [M^++Na], 230 (100) [M^++H], 159 (25) [$M^+-C_2H_4N_3$]. Anal. calcd for $C_{10}H_{20}FN_5$ (229.30): C, 52.38%; H, 8.79%; N, 30.54%; Found: C, 52.33%; H, 8.89%; N, 30.66%; Analytical HPLC: t_R = 4.2 min.

4.3.10. (S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-6-(4-(2-(4-(3-fluoropropyl)piperazin-1-yl)ethyl)-1H-1,2,3-triazol-1-yl)hexanoic acid (**14**)

To a solution of *N*-Fmoc-ε-azido-L-norleucine (**13**) (370 mg, 0.97 mmol) in a mixture of *t*-butanol, water and acetonitrile (15 mL, v/v/v = 1:1:1) **6** (192 mg, 0.97 mmol), $CuSO_4$ (24 mg, 0.09 mmol) and sodium ascorbate (192 mg, 0.97 mmol) were added and the mixture was stirred at ambient temperature for 48 h. The solvent was removed under reduced pressure and the residue was triturated with ethyl acetate. The solid was filtered and washed with ethyl acetate and the filtrate was purified via column chromatography (ethyl acetate → ethyl acetate:methanol 2:1 → 1:2) to yield **14** as an orange oil. Yield: 310 mg (55%). R_f = 0.35 (methanol). ^{1}H NMR (acetone-D6, 400 MHz): δ 7.86 (d, ^3J = 7.5 Hz, 2H, Fmoc-H-4,5), 7.82 (s, 1H, H-triazole), 7.71 (t, ^3J = 6.6 Hz, 2H, Fmoc-H-1/8), 7.41 (t, ^3J = 7.4 Hz, 2H, Fmoc-H-3/6), 7.33 (t, ^3J = 7.4 Hz, 2H, Fmoc-H-2/7), 6.94–6.90 (m, 1H, NH), 4.55 (dt, $^3J_{H,F}$ = 47.4 Hz, ^3J = 5.6 Hz, 2H, FCH_2), 4.42–4.33 (m, 3H, CH_2O, H-α), 4.23 (t, ^3J = 6.9 Hz, 1H, Fmoc-C-9), 3.80–3.76 (m, 2H, H-ε), 3.46–3.21 (m, 8H, Pip-H, $C\underline{H}_2CH_2C$=), 3.13–3.03 (m, 4H, NCH_2, CH_2C=), 1.95–1.84 (m, 6H, FCH_2CH_2, H-β,δ), 1.42–1.37 (m, 2H, H-γ). ^{13}C NMR (acetone-D6, 101 MHz): δ 161.6 (O(C=O)N), 145.1 (Fmoc-C-1′/8′), 144.2 (Fmoc-C-4′/5′), 142.1 ($CH_2\underline{C}$=C), 128.5 (Fmoc-C-3/4/5/6), 128.0 (Fmoc-C-2,7), 126.2 (CH_2C=$\underline{C}$), 120.8 (Fmoc-C-1/8), 82.2 (d, $^1J_{C,F}$ = 163.6 Hz, FCH_2), 67.1 (CH_2O), 56.6 (C-α), 53.9 (d, $^3J_{C,F}$ = 5.7 Hz, $FCH_2CH_2\underline{C}H_2$), 50.7 (Pip-C), 50.3 ($NCH_2$), 48.1 (C-ε), 32.6 (Fmoc-C-9), 29.1 (C-β), 28.9 ($\underline{C}H_2C$=C), 26.7 (d, $^2J_{C,F}$ = 20.4 Hz, $FCH_2\underline{C}H_2$), 23.3 (C-δ), 22.2 (C-γ). ^{19}F NMR (acetone-D_6, 376 MHz): δ-222.0. MS (ESI+): *m/z* (%) 593 (40) [M^++H]. Analytical HPLC: t_R = 19.3 min.

4.3.11. (S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-6-(3-(1-(3-(4-(3-fluoropropyl)piperazin-1-yl)propyl)-1H-1,2,3-triazol-4-yl)propanamido)hexanoic acid (**16**)

To a solution of *N*-Fmoc-ε-pentyneamido-L-lysine **15** (52 mg, 0.11 mmol) in a mixture of DMF (1 mL) and THF (1 mL) CuI (4.2 mg, 0.01 mmol), **12** (12.8 mg, 0.06 mmol), and DIPEA (95 μL, 0.56 mmol) were added and the mixture was stirred 16 h at 37 °C. Then water (5 mL) was added and the product was extracted with ethyl acetate (5 mL) twice. The organic phase was concentrated and purified via preparative HPLC. The product was obtained via lyophilization as pale yellow, hygroscopic solid. Yield: 10 mg (27%). R_f = 0.51 (methanol). ^{1}H NMR (acetone-D_6, 400 MHz): δ 7.86 (d, ^3J = 7.5 Hz, 2H, Fmoc-H-4/5), 7.73 (dd, ^3J = 7.1 Hz, ^3J = 4.0 Hz, 2H, Fmoc-H-1/8), 7.68 (s, 1H, H-triazole), 7.41 (t, ^3J = 7.4 Hz, 2H, Fmoc-H-3/6), 7.32 (t, ^3J = 7.4 Hz, 2H, Fmoc-H-2/7), 7.22 (s, 1H, NHCO), 6.84 (d, ^3J = 7.7 Hz, 1H, NHCOO), 4.54 (dt, $^3J_{H,F}$ = 47.4 Hz, ^3J = 5.8 Hz, 2H, FCH_2), 4.46 (t, ^3J = 7.0 Hz, 2H, CH_2O), 4.34 (d, *J* = 7.0 Hz, 1H, H-α), 4.26–4.19 (m, 3H, CH_2N_3, Fmoc-H-9), 3.26–3.05 (m, 12H, Pip-H, 2× NCH_2), 2.95 (t, ^3J = 6.8 Hz, 2H, H-ε), 2.80 (t, ^3J = 6.0 Hz, 2H, CH_2C=), 2.51 (t, ^3J = 6.9 Hz, 2H, CH_2CO), 1.89–1.77 (m, 4H, H-β, $C\underline{H}_2CH_2F$), 1.49–1.40 (m, 6H, H-γ/δ/$C\underline{H}_2CH_2N_3$). ^{13}C NMR (acetone-D6, 101 MHz): δ 157.2 (O(C=O)N), 145.1 (Fmoc-C-1′/8′), 145.0 (Fmoc-C-4′/5′), 142.1 ($CH_2\underline{C}$=C), 128.5 (Fmoc-C-3/4/5/6), 128.0 (Fmoc-C-2/7), 126.2 (CH_2C=$\underline{C}$), 120.8 (Fmoc-C-1/8), 81.4 (d, $^1J_{C,F}$ = 163.9 Hz, FCH_2), 67.2 (CH_2O), 54.5 (C-α), 53.2 (d, $^3J_{C,F}$ = 6.0 Hz, $FCH_2CH_2\underline{C}H_2$), 51.2 ($\underline{C}H_2CH_2CH_2N_3$), 50.7 ($CH_2N_3$), 48.0 (Pip-C), 39.1 (C-ε), 35.4 (Fmoc-C-9), 31.9 ($\underline{C}H_2CO$), 30.5 (C-β,δ), 29.1 ($\underline{C}H_2CH_2N_3$), 26.6 ($\underline{C}H_2C$=C), 26.5 (d, $^2J_{C,F}$ = 20.9 Hz, $FCH_2\underline{C}H_2$), 23.5 (C-γ). ^{19}F NMR (acetone-D_6, 376 MHz): δ -222.2. MS (ESI+): *m/z* (%) 678 (100) [M^++H]. Analytical HPLC: t_R = 19.7 min.

4.3.12. SNEWILPRLPQH-Azp (**17**)

Peptide **17** was prepared according to the general procedure for peptides using 275 mg of resin preloaded with *N*-Fmoc-(*S*)-4-azido-L-proline. Peptide **17** was obtained as a white powder after purification by preparative HPLC (10–40% ACN+ 0.1% TFA within 12 min, t_R = 14.4 min; Analytical HPLC: 10–45% ACN+ 0.1% TFA within 17 min, t_R = 16.3 min) and lyophilization. Yield: 14.3 mg, 9%, M_W $C_{73}H_{111}N_{25}O_{18}$ calculated 1626 $[M]^+$, found 814 $[M+2H]^{2+}$.

4.3.13. SNEWILPRLPQH-triazolyl-FP (**18**)

Compound **6** (164 μg, 0.83 μmol) in ethanol was added to a solution of SNEWILPRLPQH-Azp (**17**) (900 μg, 0.55 μmol) in Sørensen phosphate buffer (400 μL, 0.1 M, pH 7.2) into a vial.

Na-ascorbate (50 μL, 0.6 M, 30 μmol) and $CuSO_4 \cdot 5\ H_2O$ (50 μL, 0.4 M, 20 μmol) were added and the solution was stirred at ambient temperature for 30 min. Peptide **18** was obtained as a powder after purification by preparative HPLC (10–40% ACN+ 0.1% TFA within 12 min, t_R = 5.4 min; Analytical HPLC: 10–45% ACN+ 0.1% TFA within 17 min, t_R = 4.3 min) and lyophilization. Yield: 983 μg (98%). M_W $C_{84}H_{130}FN_{27}O_{18}$ calculated 1824 $[M]^+$, found 913 $[M+2H]^{2+}$.

4.4. Radiochemistry

4.4.1. 1-(But-3-ynyl)-4-(3-[^{18}F]fluoropropyl)piperazine–[^{18}F]BFP (**[^{18}F]6**)

[^{18}F]Fluoride (4 ± 2 GBq) was trapped on a Waters QMA cartridge in the remotely controlled synthesis apparatus TRACERlab Fx_{FN}. [^{18}F]Fluoride was eluted with a Kryptofix® K 2.2.2./K_2CO_3 solution (1.5 mL) into the reaction vessel and dried via azeotropic distillation with anhydrous acetonitrile (3 mL) for approx. 20 min. Afterwards, **5** (3 mg, 8.56 μmol) dissolved in acetonitrile (500 μL) was added, and the reaction mixture was stirred at 100 °C for 15 min. Acetonitrile (2 mL) was added, the solution was passed through a LiChrolut® Si cartridge, the cartridge was washed with acetonitrile (3 mL) and **[^{18}F]6** was eluted with ACN/H_2O (2 mL, v/v = 1:1). The solvent was evaporated at 90 °C in a gentle stream of nitrogen for 4–5 min to afford 1 ± 0.5 GBq (RCY = 31 ± 9, d.c.) **[^{18}F]6** with a RCP >98% within approx. 40 min and a A_S = 7 ± 4 GBq/μmol (S.D., n = 8). Radio-HPLC: t_R = 4.2 min. Radio-TLC: R_f = 0.56 (methanol).

4.4.2. 1-(3-Azidopropyl)-4-(3-[^{18}F]fluoropropyl)piperazine–[^{18}F]AFP (**[^{18}F]12**)

[^{18}F]Fluoride (1–12 GBq) was trapped on a Waters QMA cartridge in the remotely controlled synthesis apparatus TRACERlab Fx_{FN}. [^{18}F]Fluoride was eluted with a Kryptofix® K 2.2.2./K_2CO_3 solution (1.5 mL) into the reaction vessel and dried via azeotropic distillation with anhydrous acetonitrile (3 mL) for approx. 20 min. Afterwards, **11** (3 mg, 7.86 μmol) dissolved in acetonitrile (500 μL) was added, and the reaction mixture was stirred at 100 °C for 15 min. Acetonitrile (1 mL) was added, the solution was passed through a LiChrolut® Si cartridge, the cartridge was washed with acetonitrile (4 mL) and **[^{18}F]12** was eluted with ACN/H_2O (1.5 mL, v/v = 1:1). The solvent was evaporated at 90 °C for 4–5 min in a gentle stream of nitrogen to afford 2.5 ± 1.0 GBq (RCY = 29 ± 5%, d.c.) **[^{18}F]12** with a RCP >97% within approx. 40 min and a A_S = 10 ± 8 GBq/μmol (S.D., n = 19). Radio-HPLC: t_R = 4.4 min. Radio-TLC: R_f = 0.47 (methanol).

4.4.3. (S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-6-(4-(2-(4-(3-[^{18}F]fluoropropyl)piperazin-1-yl)ethyl)-1H-1,2,3-triazol-1-yl)hexanoic acid (**[^{18}F]14**)

TBTA (3.4 mg, 6.4 μmol) in DMF (100 μL), $CuSO_4 \cdot 5\ H_2O$ (1.3 mg, 5.2 μmol) in H_2O (100 μL) and Na-ascorbate (21.4 mg, 10.8 μmol) in H_2O (100 μL) were added to a solution of **13** (4.45 mg, 6.2 μmol) in DMF (100 μL) in this order. Subsequently, **[^{18}F]6** (~500 MBq) dissolved in Tris/HCl buffer (200 μL, pH 8.0) was added and the reaction was maintained at 60 °C for 1 h. **[^{18}F]14** was obtained in a radiochemical yield of 59% (d.c.) with a RCP >99% by means of HPLC. Radio-HPLC: t_R = 19.4 min. Radio-TLC: R_f = 0.65 (methanol).

4.4.4. (S)-2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-6-(3-(1-(3-(4-(3-[^{18}F]fluoropropyl)piperazin-1-yl)propyl)-1H-1,2,3-triazol-4-yl)propanamido)hexanoic acid (**[^{18}F]16**)

TBTA (1.06 mg, 2.0 μmol) in DMF (42 μL), $CuSO_4 \cdot 5\ H_2O$ (0.13 mg, 0.5 μmol) in H_2O (25 μL) and Na-ascorbate (0.99 mg, 5.0 μmol) in H_2O (21 μL) were added to a solution of **15** (4.95 mg, 5.0 μmol) in DMF (100 μL) in this order. Subsequently, **[^{18}F]12** (~1 GBq) dissolved in Tris/HCl buffer (200 μL, pH 8.0) was added and the reaction was irradiated at 50 W for 1 h. **[^{18}F]16** was obtained in a RCY of 79% (d.c.) with a RCP >91% by means of HPLC. Radio-HPLC: t_R = 19.8 min. Radio-TLC: R_f = 0.57 (methanol).

4.4.5. SNEWILPRLPQH-triazolyl-[^{18}F]FP (**[^{18}F]18**)

A solution of **[^{18}F]6** (250–850 MBq) in Sørensen phosphate buffer (500 μL, 0.1 M, pH 7.2) with Na-ascorbate (1.25 mg, 6 μmol) and TBTA (900 μg, 2 μmol) was added to SNEWILPRLPQH-Azp **17** (120–170 μg, 74–105 nmol). $CuSO_4 \cdot 5\ H_2O$ (1 mg, 4 μmol) was added immediately and the solution was stirred at 60 °C for 1 h. Purification of the reaction mixture was accomplished by size-exclusion chromatography (SEC). The solvent was evaporated *in vacuo* at 50 °C to afford 30–150 MBq (RCY: 17–25%, d.c.) of **[^{18}F]18** with RCP >99% within 120 min (starting from [^{18}F]fluoride drying). Radio-HPLC: t_R = 4.3 min.

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