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Efficient cysteine labelling of peptides with *N*-succinimidyl 4-[^{18}F]fluorobenzoate: stability study and *in vivo* biodistribution in rats by positron emission tomography (PET)†

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A rapid and high-yielding cysteine labelling of peptides has been observed with the specific labelling agent for amines, *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB). Interestingly, conjugation of the 4-fluorobenzoyl (FB) moiety is selectively achieved through a cysteine (Cys) thiol of the peptides with high yield (>80%) in short time (<5 min), while for a Cys amino acid derivative, a slow process has been observed. The large reactivity of these peptides for the conjugation reaction is rationalised on the basis of electrostatic interactions between the sulfhydril and the guanidinium groups of the amino acid side chains. Moreover, the stability of these novel conjugates and the biodistribution of the radiolabelled dodecapeptide by positron emission tomography (PET) in rats has been examined.

Introduction

In the past few decades, positron emission tomography (PET) has probably been the fastest growing area in molecular imaging.[1,2] This is reflected in the large number and nature of compounds that have been labelled with PET radionuclides. In addition to the variety of small organic compounds tagged, several radiolabelled biomolecules such as peptides, proteins, and antibodies have been employed for *in vivo* imaging of biological processes at the molecular and cellular level.[3,4]

Among the available isotopes for PET, ^{18}F is frequently considered to be the best due to its favorable nuclear and chemical properties. However, labelling of biomolecules with $^{18}F^-$ by direct reaction is generally not possible since the harsh conditions commonly required to obtain useful conjugation yields are not compatible with the stability of the sensitive molecule. To remedy this, preparation of bifunctional labelling agents (also referred to as prosthetic groups) is the approach used to label biomolecules. This strategy often involves multi-step synthetic pathways to prepare molecules which contain the label and active species that allow conjugation to nucleophiles of the sensitive molecule under mild conditions.[3–8] Despite the fact that tagging of peptides, proteins, antibodies, and oligonucleotides has been accomplished through several reaction mechanisms, *N*-succinimidyl (NHS) esters are likely the prosthetic groups more widely employed in bioconjugation chemistry.[9] These labelling agents are known to be specific to acylate amine groups, independent of the existence of other reacting groups in the biomolecule.

As an alternative strategy, the use of thiol-reactive prosthetic groups has emerged during the last few years.[10–15] They are based on the Michael addition of a thiol to a maleimide group. This labelling is considered to be more site-specific than the acylation of amine groups, since a free thiol functionality is not very common in most biomolecules and is only present in cysteine (Cys) residues, while lysines have a much higher relative abundance.

Aliphatic thiols do not react with NHS esters efficiently, and activation of the nucleophile is generally needed to increase reaction rates and yields.[16–19] However, the reactivity of Cys in peptides and proteins can be altered by nearby amino acids in order to carry out different biochemical roles.[20] For instance, the reactivity of Cys thiols has been reported to be tuned by electrostatic interactions of the thiolate moiety with charged residues.[21,22] Accordingly, activation of the thiol can be provided by the chemical surroundings in biomolecules.

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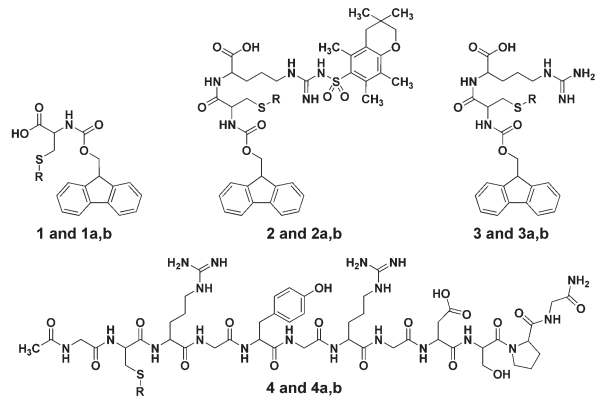


Chart 1 The chemical structures of **1–4** (where R = H), **1a–4a** (where R = 4-fluorobenzoyl), and **1b–4b** (where R = 4-[^{18}F]fluorobenzoyl).

As shown below, we have studied the rapid and high-yielding cysteine labelling of peptides **2–4** (*cf.* Chart 1), promoted by an adjacent arginine with the gold standard labelling agent *N*-succinimidyl 4-[^{18}F]-fluorobenzoate ([^{18}F])SFB) to perform ^{18}F-tagging through acylation of amine groups.[9,23–30] A part of these observations was the subject of a preliminary communication;[31] now we wish to report our results in full including a stability study of the radiolabelled peptides. Besides, the *in vivo* biodistribution in rats of the radiolabelled dodecapeptide **4b** (*cf.* Chart 1) has been examined *via* PET .

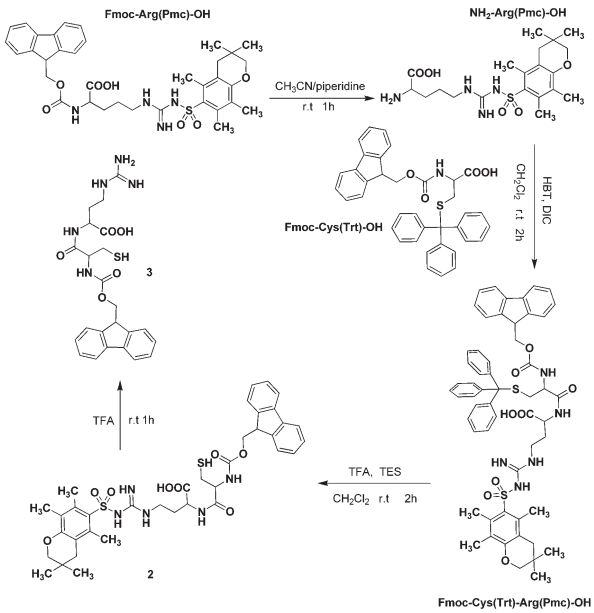
Results and discussion

Labelling of Cys thiols 1–4 with SFB

Compounds **1–3** were prepared (*cf.* Scheme 1 for the synthesis of peptides **2** and **3**) to investigate whether Cys labelling with NHS esters can occur. These compounds do not contain any free amino groups, but a Cys thiol with an adjacent arginine (Arg) residue in the case of the dipeptides **2** and **3**. In the first instance, the reaction was examined using the non-radioactive analogue of [^{18}F]SFB, *i.e.* SFB.

The conjugation of SFB to **1–3** was studied in DMSO–phosphate buffer (pH = 8.5) as the reaction medium (*cf.* Experimental Section). Thus, while moderate basic buffers provide both enhanced nucleophilicity and high solubility of the biomolecule, the organic solvent favours solubility of SFB. Such combinations of a polar aprotic organic solvent (DMSO, DMF, or CH_3CN) and a basic buffer (phosphate or borate) are generally used to react biomolecules with SFB and other NHS esters.[9]

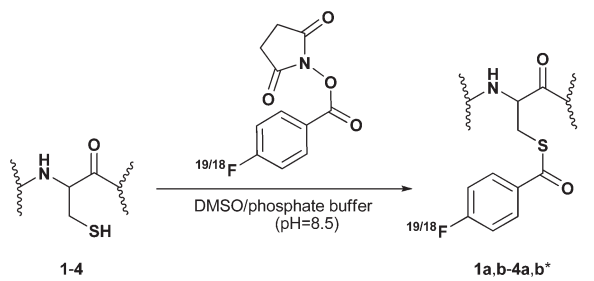
Analytical HPLC (UV and MS-ESI detection) was used to follow the progress of the reaction. Formation of new products **1a–3a** (*cf.* Chart 1 and Scheme 2) was observed concomitantly with consumption of **1–3**. The conjugates were HPLC-purified and characterised as the corresponding thiobenzoates by MS and NMR (*cf.* Experimental Section). As can been seen in Fig. 1, the reaction of **2** and **3** with SFB proceeded very fast with >90% yield of **2a** (91%) and **3a** (96%) after 70 min. However,



Scheme 1 Synthetic scheme outlining the preparation of peptides **2** and **3** (HBT = 1-hydroxybenzotriazole, DIC = diisopropylcarbodiimide, TFA = trifluoroacetic acid, TES = triethylsilane).

1a was obtained considerably more slowly (88% yield after 14 h), which strongly suggests that the presence of the Arg residue in peptides **2** and **3** promotes the faster reaction. This effect was observed to be somewhat smaller when the guanidino group was Pmc-protected, since **2** was slightly less reactive than **3**.

While there is no known consensus about any sequence leading to Cys hyper-reactivity in proteins,[20] it is reasonable to assume that in small peptides an adjacent Arg residue can stabilize the thiolate form through colombic interaction with the positively charged guanidino group of the Arg side-chain. As a result of this stabilization process, an increase in the acidity constants has to be expected, which would lead to a larger population of the most reactive species, thiolate, for a given pH (*cf.* Scheme 3). In fact, interactions between Cys and Arg residues within the active site pocket of proteins has been previously reported.[21]



* Letter codes (**a**= ^{19}F and **b**=^{18}F) after the product number indicate conjugate of the former Cys thiol

Scheme 2 Simplified overview of the reaction of Cys thiols **1–4** with SFB and [^{18}F]SFB.

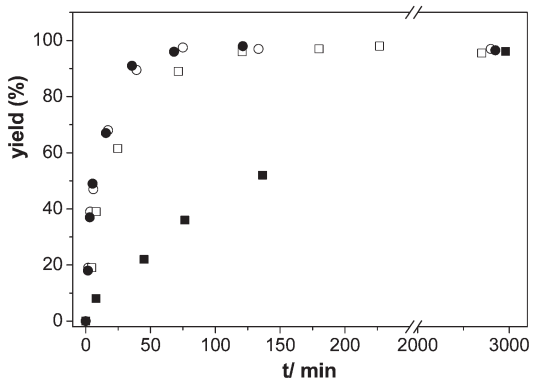


Fig. 1 Plot of the chemical yields for **1a** (■), **2a** (□), **3a** (●), and **4a** (○) against reaction time.

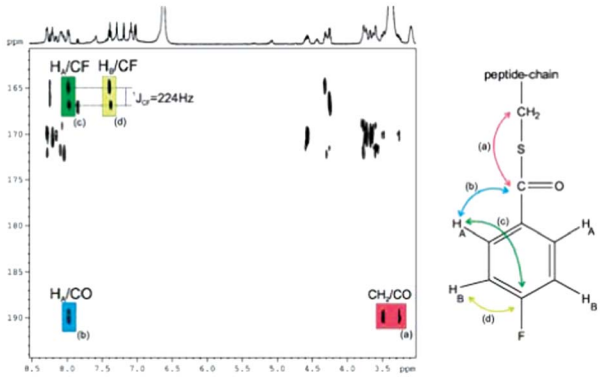
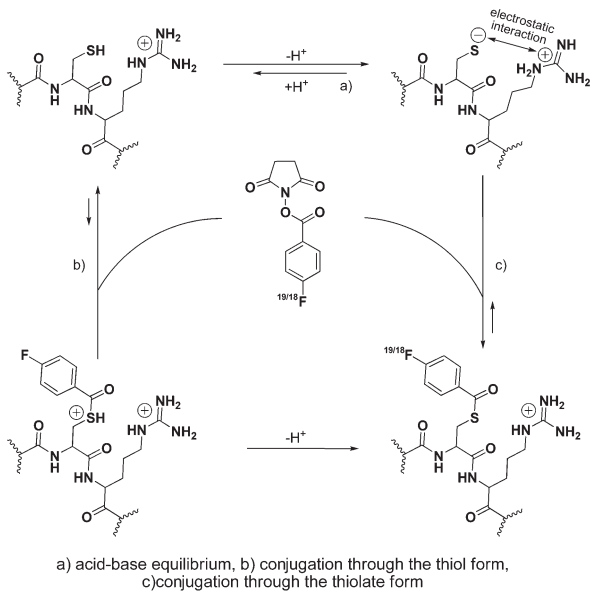


Fig. 2 $^1H/^{13}C$ HMBC NMR spectrum (DMSO) of **4a** which demonstrates that the 4-fluorobenzoyl group is linked to the Cys residue.

On the other hand, although the presence of the electron withdrawing Pmc-group increases to some extent, the positive charge delocalized in the guanidine moiety, steric hindrance associated with this bulky group is probably responsible for the lower reactivity observed for **2** compared to **3**.

To study, in a larger peptide, the pronounced reactivity of Cys thiols **2** and **3** as a consequence of an Arg-mediated stabilization of the thiolate form, Ac-GCRGYGRGDSPG-NH_2 (**4**) was synthesised (*cf.* Chart 1 and Experimental Section). The experimental conditions used to react SFB with dodecapeptide **4** were the same as those applied to **1–3**. Once again, consumption of **4** was accompanied by formation of a new product (**4a**) whose MS-ESI molecular ion peak ($[M + 2H]^{+2}$, m/z = 672.5) was in accordance with attachment of the 4-fluorobenzoyl moiety to **4**. Conjugation reaction was simply corroborated by direct visualization of the fluorine signal in the ^{19}F NMR spectrum of **4a** (isolated by semi-preparative HPLC). It is important to highlight that only one signal was observed (−73.6 ppm), therefore giving further evidence that a single position was altered according to the electrospray ionization mass spectrum. The $^1H/^{13}C$-HMBC NMR experiment finally confirmed that the Cys sulfhydryl group was acylated (*cf.* Fig. 2). The key point is the visualization of a cross peak between Cys–CH_2 β protons and the carbonyl carbon of the fluorobenzoyl group.

Akin to **2a** and **3a**, formation of **4a** was a rapid process with 96% yield in less than 70 min (*cf.* Fig. 1) that provided additional support to the proposed Arg-mediated enhanced reactivity of Cys.



Scheme 3 Depiction of the stabilization process of the thiolate form for Cys thiols **2–4**, and its participation in the major pathway of the conjugation process with SFB and [^{18}F]SFB.

Radiolabelling of Cys thiols 1–4 with [^{18}F]SFB

Noteworthily, the rapid and high yielding conjugation of **2–4** with SFB was observed under conditions of conventional reaction of NHS esters with biomolecules (*e.g.* conjugation of biomolecules with dyes or fluorophores for optical imaging). Once the concept had been established for the reaction of Cys thiols with SFB, the radiolabelling of **1–4** with [^{18}F]SFB was performed. The experimental conditions used for the reaction of Cys thiols with SFB and [^{18}F]SFB were necessarily different, since, in radiochemistry, the bifuctional labelling agent always has to be in large deficit in comparison to the biomolecule. As a consequence, the concentration of [^{18}F]SFB was very low (*ca.* 4 μM), whereas SFB could be used in excess (1.5 equivalents) in the conventional chemistry. Moreover, [**1–4**] was smaller when reacted with [^{18}F]SFB compared to that used with SFB (3 mM *vs.* 20 mM) to reproduce the typical concentration of biomolecules employed in the synthesis of radiotracers (1–5 mg mL^{-1}).

As shown in Fig. 3 (top) for peptide **4**, the HPLC-coupled radiograms of the reaction of **1–4** with [^{18}F]SFB at different reaction times showed the formation of thioester (**1b–4b**), unreacted [^{18}F]SFB and, depending on the Cys thiol, varying amounts of 4-[^{18}F]fluorobenzoic acid ([^{18}F]FB) from the hydrolysis of NHS ester, which competed with the labelling in aqueous basic media.[9] To confirm the identity of **1b–4b** (*cf.* Chart 1 and Scheme 2), the reaction mixtures were co-eluted with the corresponding [^{19}F]-derivatives, *i.e.*, **1a–4a**. The

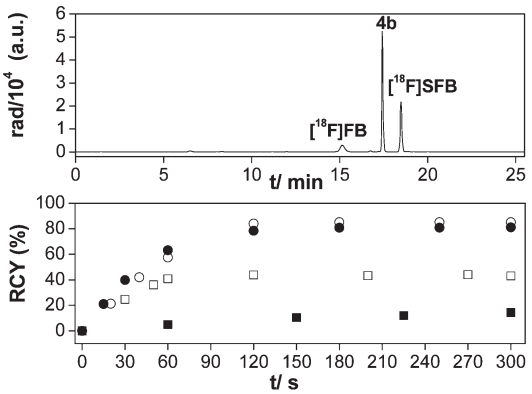


Fig. 3 Radiochromatogram for the reaction (60 s) of peptide **4** with [^{18}F]SFB (top) and plot of RCY's for conjugates **1b** (■), **2b** (□), **3b** (●), and **4b** (○) *versus* reaction time (bottom).

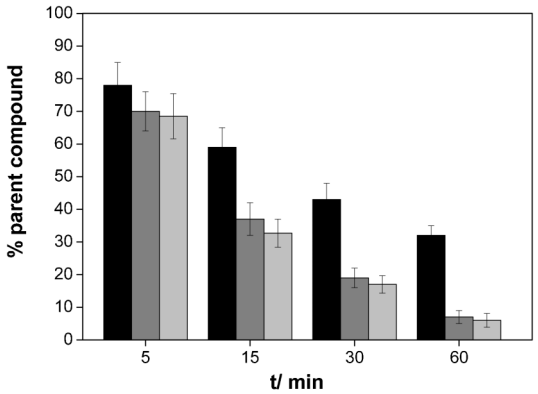


Fig. 4 Radio-HPLC analysis of the *in vitro* stability of compounds **1b** (black bars), **3b** (grey bars), and **4b** (light grey bars) where the percentage of parent compound is shown.

radiochemical yields (RCYs) of **1b–4b** obtained for each reaction time are plotted in Fig. 3 (bottom). Five minutes after additions of **3** and **4**, RCYs for the conjugation reactions were >80%, and no residual [^{18}F]SFB remained. Labelling of **2** gave RCYs below 40% (5 min), while **1** showed the lowest reactivity with a RCY of <15% in 5 min. Therefore, obtained RCYs correlated well with the results found using conventional chemistry. However, the reaction of Cys thiols **1–4** proceeds faster with [^{18}F]SFB (seconds) than with SFB (minutes) as can clearly be seen in the timescales of Fig. 1 and 3 (top). This difference arises from the large defect used in the case of the reaction with [^{18}F]SFB, which is observed as a pseudo-first order process with apparent faster kinetics. In fact, the difference in reactivity, in terms of product yields, observed between **2** and **3** as a consequence of the presence of the Pmc-group for **2**, was more pronounced when the prosthetic group was used in large defect compared to Cys thiols, *i.e.* for the conjugation with [^{18}F]SFB. This can be rationalised on the basis of the low concentration of [^{18}F]SFB and its above mentioned hydrolysis reaction.

These results indicate that an efficient Cys labelling promoted by adjacent Arg residues occurs under the conditions of standard peptide and protein labelling with [^{18}F]SFB.

In vitro stability of 1b, 3b, and 4b

The radiosynthesis of compounds **1b–4b** provided [^{18}F]-labelled thioesters with radiochemical purity over 95% for more than 8 h (*cf.* Experimental Section). In order to explore whether Cys labelling of peptides with NHS esters could be used as an alternative strategy to obtain probes for *in vivo* imaging, the stability of **4b** in plasma was studied. After a certain incubation time, samples were analyzed by analytical HPLC to determine the percentage of intact peptide. As can been seen in Fig. 4, only *ca.* 5% of **4b** was found after 1 h. This fast decomposition of **4b** was not unexpected since linear peptides are known to be very rapidly cleaved by proteases.[32] However, **4b** was found to be much less stable than other linear peptides labelled through 4-[^{18}F]-fluorobenzoylation of amine groups.[33] In fact, thioesters are expected to be less stable than amides (product of the labelling of amine groups) and, therefore, less suitable to be used as probes.

In order to gain further insight into the low stability of **4b**, compounds **1b** and **3b** were also incubated in plasma (*cf.* Fig. 4). While **3b** exhibited similar stability to that observed for **4b**, a lower *in vitro* degradation of **1b** was found (approximately 30% of parent compound in 1 h) where proteolytic cleavage can not be involved. The decomposition of **1b**, **3b**, and **4b** is not attributable to hydrolysis of the thioester linkage, since [^{18}F]FB was not observed in the analysis. These results indicate that degradation of peptide **4b** mostly occurred by proteolysis although other reaction pathways can not be discarded.

PET Biodistribution of peptide 4b in rats

Results showed that the biological half-life of **4b** was very short. In fact, just after injection, the uptake in the kidneys and bladder was very high. After only 20 min all the dose appeared in the urine, indicating the complete renal excretion of the radiolabelled peptide or its radiometabolites (*cf.* Fig. 5). Time activity curves showed the high radioactive clearance of the kidney and the progressive accumulation in the urinary bladder during the first few minutes of the study. When **4b**

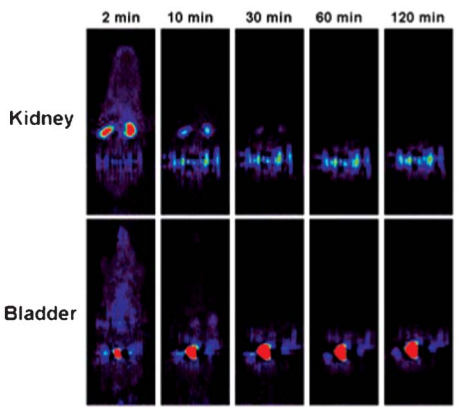


Fig. 5 Representative images obtained from the same animal at the level of the kidneys and bladder showing the biodistribution of **4b** during the time.

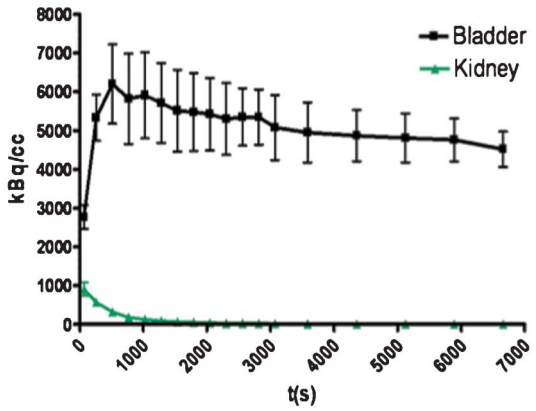


Fig. 6 Mean time activity curves obtained from all animals for the bladder and kidneys. The plasmatic clearance by renal excretion of **4b** could be appreciated.

was completely excreted, the radioactivity in the urinary bladder remained constant until the end of the exploration, as can be seen in Fig. 6.

The obtained results were in accordance with the pharmacokinetic data reported for other linear peptides.[32,34] It is well-known that linear peptides are rapidly metabolised by peptidases providing a high excretion ratio that limits target exposure by complete plasmatic clearance. To improve their pharmacokinetics, different strategies have been successfully applied to stabilize such peptides towards proteolysis as incorporation of unnatural amino acids or cyclisation.[34,35]

Experimental section

General methods

All chemicals, solvents, and materials were commercially available and used as received without further purification. Silica gel (230–400 mesh) from Scharlau was used for column chromatography. Analytical and semi-preparative HPLC runs were performed using an Agilent 1100 Series coupled to a MS (API-ES positive ionization mode), UV-Vis diode array, and a Raytest Gina isotopic detector. As the stationary phase, analytical and semi-preparative Teknokroma Mediterranea Sea_{18} columns (5 μm) were employed. The cyclotron used for ^{18}F production was an 18/9 model from IBA. ^{1}H, ^{13}C and other routine NMR experiments (COSY, TOCSY, HSQC and HMBC) were usually obtained on a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm TBI probe with Z-gradients. For some samples a Bruker Avance 500 MHz spectrometer equipped with a 5 mm triple channel (^{1}H, ^{13}C, ^{15}N) cryoprobe with Z-gradients was used. ^{19}F NMR experiments were performed with a Bruker Avance III 400 spectrometer equipped with a 5 mm BBOF probe with Z-gradients.

Preparation of Fmoc-Cys-OH (1)

Compound **1** was synthesised according to a procedure previously described by Zhao *et al.*[36]

Preparation of Fmoc-Cys-Arg(Pmc)-OH (2)

Fmoc-Arg(Pmc)-OH (0.75 mmol) was dissolved in acetonitrile–piperidine (4/1 v/v, 5 mL) at room temperature. After one hour, the crude mixture was dried and purified by silica gel flash chromatography using ethyl acetate–methanol–trietylamine (7.9/2/0.1 v/v/v) as eluent. Then, the compound was dissolved (0.46 mmol) in dichloromethane (3 mL) and added drop-wise over 30 min to a solution of Fmoc-Cys(Trt)-OH (0.5 mmol), 1-hydroxybenzotriazole (0.5 mmol), and 1,3-diisopropylcarbodiimide (0.5 mmol) in dichloromethane (7 mL). The mixture was allowed to react for 2 h at room temperature. After washing the crude reaction mixture with water, the dipeptide was purified by silica gel chromatography using a solvent gradient from chloroform to chloroform–methanol (9/1 v/v). Finally, the dipeptide was reacted with TFA (1.0 mmol) and triethylsilane (1.0 mmol) in dichloromethane (0.6 mL) at room temperature for 2 h. Then, the crude was dried under vacuum and purified by flash chromatography using a solvent gradient from chloroform to chloroform–methanol (9/1 v/v). **2** was obtained as a white solid. ^{1}H NMR (500 MHz, DMSO) δ 7.76 (d, J = 7.8 Hz, 2H), 7.60 (d, J = 7.0 Hz, 1H), 7.59 (d, J = 7.3, 1H), 7.55 (b, 0.8H), 7.41 (t, J = 7.2 Hz, 1H), 7.40 (t, J = 7.3 Hz, 1H), 7.31 (d, J = 7.2, 1H), 7.30 (d, J = 7.2, 1H), 4.67 (b, 0.8H), 4.62 (b, 1H), 4.51 (b, 1H), 4.44 (b, 1H), 4.37 (b, 1H), 4.20 (t, J = 7.0, 1H), 3.21 (b, 2H), 2.99 (m, 1H), 2.88 (m, 1H), 2.61 (t, J = 6.4, 2H), 2.54 (s, 3H), 2.52 (s, 3H), 2.10 (s, 3H), 1.31 (s, 3H), 1.28 (s, 3H). Calculated mass for $C_{38}H_{47}N_5O_8S_2$, 765.3. MS-ESI (positive mode) for **2**: $[M + H]^+$, m/z = 766.

Preparation of Fmoc-Cys-Arg-OH (3)

2 (0.06 mmol) was reacted in TFA (0.5 mL) for one hour at room temperature. The reaction mixture was diluted with acetonitrile and purified by semi-preparative HPLC (25 × 1 cm, water–acetonitrile–TFA from 9/1/0.01 v/v/v to 6/4/0.01 v/v/v in 25 min, 5 mL min^{-1}). The collected fractions were dried under reduced pressure and compound **3** was obtained as a white solid. ^{1}H NMR (500 MHz, DMSO): δ 8.31 (t, J = 9.3, 1H), 7.91 (d, J = 7.1, 1H), 7.89 (d, J = 7.8, 1H), 7.74 (m, 2H), 7.65 (m, 0.8H), 7.63 (d, J = 7.2, 0.8H), 7.43 (t, J = 7.4, 1H), 7.39 (t, J = 7.2, 1H), 7.34 (t, J = 7.6, 1H), 4.32 (m, 1H), 4.24 (m, 1H), 4.23 (m, 1H), 4.21 (m, 1H), 4.20 (m, 1H), 3.09 (m, 2H), 2.82 (m, 1H), 2.67 (m, 1H), 1.78 (m, 1H), 1.62 (m, 1H), 1.51 (m, 2H). ^{13}C NMR (125 MHz, DMSO): δ 174.0, 171.2, 157.6, 156.8, 144.6, 141.6, 128.5, 128.0, 126.2, 121.0, 66.6, 58.0, 52.5, 47.5, 41.1, 28.8, 27.2, 26.0. Calculated mass for $C_{24}H_{29}N_5O_5S$, 499.2. MS-ESI (positive mode) for **2**: $[M + H]^+$, m/z = 500.

Preparation of peptide Ac-GCRGYGRGDSPG-NH_2 (4)

The synthesis of peptide **4** was accomplished according to a published procedure.[37]

Reaction of Cys thiols 1–4 with *N*-succinimidyl 4-fluorobenzoate (SFB)

To a solution of the cysteine thiols (0.01 mmol) in a 2/1 (v/v) mixture (300 μL) of DMSO–phosphate buffer (pH = 8.5, 0.2 M), SFB (0.015 mmol) dissolved in DMSO (200 μL) was added. The progress of the reactions was monitored by HPLC/UV/MS (15 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/

0.01 v/v/v in 30 min, 2 mL min^{-1}). The labelled compounds **1a**, **2a**, **3a**, and **4a** were purified by semi-preparative HPLC and the collected fractions were concentrated under vacuum to obtain white solids. Chemical yields were determined based on HPLC measurements by using isolate conjugates as standards.

Purification of Fmoc-Cys(FB)-OH (1a) and Fmoc-Cys(FB)-Arg(Pmc)-OH (2a)

Compound **1a** and **2a** were purified by semi-preparative HPLC (25 × 1 cm, water–acetonitrile–TFA from 5/5/0.01 v/v/v to 3/7/0.01 v/v/v in 10 min, 5 mL min^{-1}). ^{1}H NMR (500 MHz, $CDCl_3$) for **1a**: δ 8.00 (d, 2H), 7.76 (d, J = 7.3 Hz, 2H), 7.57 (d, J = 7.6 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.40 (t, J = 6.7 Hz, 2H), 7.26 (t, J = 7.1 Hz, 2H), 7.14 (m, 2H), 5.82 (b, 1H), 4.69 (b, 1H), 4.41 (m, 1H), 4.36 (m, 1H), 4.23 (t, J = 6.2 Hz, 1H), 3.70 (m, 1H), 3.57 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) for **1a**: δ 167.7, 166.3, 144.0, 141.7, 132.9, 130.7, 130.6, 128.1, 127.5, 125.5, 120.4, 116.4, 116.2, 68.0, 54.6, 47.4, 31.0. Calculated mass for $C_{25}H_{20}FNO_5S$, 465.1. MS-ESI (positive mode) for **1a**: $[M + Na]^+$, m/z = 488.

^{1}H NMR (500 MHz, DMSO) for **2a**: δ 8.34 (d, J = 7.2, 0.9H), 7.99 (d, J = 8.5, 1H), 7.98 (d, J = 8.5, 1H), 7.89 (d, J = 7.2, 2H), 7.79 (d, J = 8.5, 0.8H), 7.71 (d, J = 7.0, 1H), 7.70 (d, J = 7.1, 1H), 7.41 (t, J = 7.2, 2H), 7.39 (t, J = 8.5, 2H), 7.29 (t, J = 7.2, 2H), 6.7 (b, 0.9H), 6.39 (b, 0.9H), 4.32 (m, 1H), 4.30 (m, 1H), 4.24 (m, 1H), 4.21 (m, 1H), 4.17 (m, 1H), 3.51 (dd, J = 13.3, J = 4.1, 1H), 3.23 (dd, 13.3, J = 9.5, 1H), 3.04 (m, 2H), 2.56 (t, J = 6.5, 2H), 2.48 (s, 3H), 2.47 (s, 3H), 2.02 (s, 3H), 1.76 (t, J = 6.5, 2H), 1.74 (m, 1H), 1.59 (m, 1H), 1.46 (m, 2H), 1.25 (s, 6H). ^{13}C NMR (125 MHz, DMSO) for **2a**: δ 190.2, 174.0, 170.7, 166.2, 156.8, 156.7, 153.2, 144.6, 141.5, 135.4, 135.0, 133.7, 130.7, 128.5, 127.9, 126.1, 123.5, 121.0, 118.6, 117.0, 74.3, 66.7, 54.5, 52.7, 47.4, 40.3, 33.0, 31.9, 29.1, 27.3, 26.4, 21.6, 19.0, 18.0, 12.8. Calculated mass for $C_{45}H_{50}FN_5O_9S_2$, 888.3. MS-ESI (positive mode) for **2a**: $[M + H]^+$, m/z = 888.

Purification of Fmoc-Cys(FB)-Arg-OH (3a)

Conjugate **3a** was isolated by injection of the reaction mixture into a semipreparative HPLC system (25 × 1 cm, water–acetonitrile–TFA from 6/4/0.01 v/v/v to 4.5/5.5/0.01 v/v/v in 15 min, 5 mL min^{-1}). ^{1}H NMR (500 MHz, DMSO): δ 8.28 (b, 0.6H), 7.99 (m, 2H), 7.90 (d, J = 7.3, 2H), 7.83 (d, J = 8.7, 1H), 7.71 (d, J = 7.3, 2H), 7.58 (b, 0.7H), 7.42 (t, J = 7.4, 2H), 7.39 (t, J = 7.3, 2H), 7.30 (m, 2H), 4.33 (m, 1H), 4.32 (m, 1H), 4.23 (m, 1H), 4.22 (m, 1H), 4.20 (m, 1H), 3.53 (dd, J = 13.4, J = 4.2, 1H), 3.10 (q, J = 6.1, 2H), 1.77 (m, 1H), 1.63 (m, 1H), 1.52 (q, J = 7.1, 2H). ^{13}C NMR (125 MHz, DMSO): δ 190.2, 157.5, 156.7, 144.6, 144.5, 145.6, 133.7, 130.6, 130.7, 128.5, 127.9, 126.2, 121.0, 117.2, 117.0, 66.7, 54.6, 52.9, 47.4, 41.2, 32.0, 29.1, 26.0. Calculated mass for $C_{31}H_{32}FN_5O_6S$, 621.2. MS-ESI (positive mode) for **3a**: $[M + H]^+$, m/z = 622.

Purification of the peptide–FB conjugate (4a)

The reaction crude was submitted to semi-preparative HPLC (25 × 1 cm, water–acetonitrile–TFA from 9/1/0.01 v/v/v to 6/4/0.01 v/v/v in 30 min, 5 mL min^{-1}). Calculated mass for $C_{55}H_{78}FN_{19}O_{18}S$, 1343.6. MS-ESI (positive mode) for **4a**: $[M + 2H]^{+2}$, m/z = 672.5. See ESI† for NMR characterisation.

Radiosynthesis of *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB)

[^{18}F]SFB was synthesised according to the well established three-step one-pot procedure with some modifications by using assembled Eckert & Ziegler modules (Isotope Products, Inc.). Thus, $^{18}F[F^-]$ was produced in a cyclotron by bombardment of $[^{18}O]H_2O$ with high energy protons (18 MeV). Then, radioactivity was delivered to the automatic synthesiser system where $^{18}F[F^-]$ aqueous solution was passed through a Sep-Pak light QMA cartridge (Waters, Inc.) to trap the fluoride. The $^{18}F[F^-]$ was eluted from the trapping cartridge to the reaction vessel with a solution of potassium carbonate (5.5 μmol) and 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (K_{222}, 18.7 μmol) in acetonitrile–water (1/1 v/v, 0.8 mL). The solvent was evaporated by applying He flow and vacuum at 110 °C. To ensure that the drying process was done successfully, additional anhydrous acetonitrile (1 mL) was added and the mixture was dried as before. To the anhydrous K_{222}–[^{18}F]KF complex, a solution of ethyl 4-(trimethylammonium triflate) benzoate (20 mmol) in anhydrous acetonitrile (1 mL) was added and the mixture was heated to 90 °C for 10 min. Then, tetrabutylammonium hydroxide (1 M in methanol, 25 μL) in anhydrous acetonitrile (0.4 mL) was added and the reaction vessel was heated to 120 °C for 5 min. Subsequently, a solution of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (0.05 mmol) in dry acetonitrile (0.6 mL) was added and the reaction was heated to 90 °C for 2 min. The crude mixture was cooled down to 40 °C and neutralised with aqueous acetic acid (5%, 3 mL). The final solution was purified by semipreparative HPLC (25 × 1 cm, water–acetonitrile–TFA 6/4/0.01, 7.5 mL min^{-1}). The desired fraction (t_r ∼ 9–11 min) was collected over 0.9% saline solution (20 ml) and passed through a Sep-Pak C18 cartridge (Waters, Inc.). The cartridge was rinsed with water (10 mL) and [^{18}F]SFB was eluted with acetonitrile (2 mL). The solvent was removed by bubbling N_2 at room temperature to provide a dry residue. Chemical and radiochemical purity was determined by analytical HPLC (15 × 0.46 cm, water–acetonitrile–TFA from 9/1/0.01 v/v/v to 6/4/0.01 v/v/v in 30 min, 2 mL min^{-1}). [^{18}F]SFB was obtained in a radiochemical yield (RCY) of 37 ± 5% (decay-corrected), with chemical and radiochemical purity exceeding 98%. A specific activity of 102 ± 7 GBq μmol^{-1} was estimated. [^{18}F]SFB was prepared in 74 ± 5 min.

Radiolabelling of cysteine thiols 1–4 with [^{18}F]SFB

To a solution of the thiol (0.15 μmol) in a 2/1 (v/v) mixture (30 μL) of DMSO–phosphate buffer (pH = 8.5, 0.2 M), [^{18}F]SFB (*ca.* 20 MBq) in DMSO (20 μL) was added. The reaction was kept at room temperature and analysed at different reaction times. An analytical HPLC system (15 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 30 min, 2 mL min^{-1}) was used to identify the thioesters (by co-elution with the corresponding ^{19}F-standards) and to determine the RCY obtained for the conjugates.

Radiosynthesis of [^{18}F]Fmoc-Cys(FB)-OH (1b)

The amino acid derivative **1** (1.5 μmol) was dissolved in a 2/1 (v/v) mixture (150 μL) of DMSO–phosphate buffer (pH 8.5, 0.2

M) and added to a solution of [^{18}F]SFB (~2 GBq) in DMSO (100 μL). After reacting at room temperature for 30 min, the crude mixture was diluted with aqueous TFA (0.1%, 2.6 mL) and injected into a semipreparative HPLC (25 × 1 cm, water/acetonitrile/TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 15 min, 5 mL min^{-1}). The fraction with product (t_r ~ 16–18 min) was collected and passed through a Sep-Pak light C18 cartridge (Waters, Inc.) after dilution with saline (0.9%, 20 ml). The cartridge was rinsed with water (10 mL) and compound **1b** was eluted with ethanol (1 mL) over a phosphate buffer solution (PBS, pH = 7.4, 9 mL). Chemical and radiochemical purity were determined by analytical HPLC (25 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 30 min, 2 mL min^{-1}) to be >95% for >8 h with a specific activity of 59 ± 9 GBq μmol^{-1}. **1b** was obtained in a RCY of 9 ± 3% (decay-corrected, 56 ± 6 min).

Radiosynthesis of [^{18}F]Fmoc-Cys(FB)-Arg(Pmc)-OH (2b)

[^{18}F]SFB dry residue (~2 GBq) was dissolved in DMSO (100 μL) and added to a solution of **2** (0.75 μmol) in a 2/1 (v/v) mixture (150 μL) of DMSO–phosphate buffer (pH 8.5, 0.2 M). After reacting at room temperature for 10 min, the reaction mixture was diluted with aqueous TFA (0.1%, 2.6 mL) and purified by semipreparative HPLC (25 × 1 cm, water–acetonitrile–TFA 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 15 min, 5 mL min^{-1}). The fraction of interest (t_r ~ 17–20 min) was collected and diluted with saline solution (0.9%, 30 ml) and passed through a Sep-Pak light C18 cartridge (Waters, Inc.). The cartridge was rinsed with water (10 mL) and **2b** was eluted with ethanol (1 mL) over a phosphate buffer solution (PBS, pH = 7.4, 9 mL). Chemical and radiochemical purity were determined by analytical HPLC (25 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 30 min, 2 mL min^{-1}). Peptide **2b** was obtained in a RCY of 26 ± 3% (decay-corrected, 37 ± 3 min). Chemical and radiochemical purity exceed 95% for >8 h. A specific activity of 76 ± 8 GBq μmol^{-1} was obtained.

Radiosynthesis of [^{18}F]Fmoc-Cys(FB)-Arg-OH (3b)

Peptide **3** (0.75 μmol) was solved in a 2/1 (v/v) mixture (150 μL) of DMSO/phosphate buffer (pH 8.5, 0.2 M) and added to a solution of [^{18}F]SFB (~2 GBq) in DMSO (100 μL). After reacting at room temperature for 10 min, the crude mixture was diluted with aqueous TFA (0.1%, 2.6 mL) and injected into a semipreparative HPLC (25 × 1 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 15 min, 5 mL min^{-1}). The fraction with product (t_r ~ 12–14 min) was collected and passed through a Sep-Pak light C18 cartridge (Waters, Inc.) after dilution with saline (0.9%, 20 ml). The cartridge was rinsed with water (10 mL) and compound **3b** was eluted with ethanol (1 mL) over a phosphate buffer solution (PBS, pH = 7.4, 9 mL). Chemical and radiochemical purity were determined by analytical HPLC (25 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 30 min, 2 mL min^{-1}) to be >95% for >8 h with a specific activity of 79 ± 9 GBq/μmol. **3b** was obtained in a RCY of 57 ± 4% (decay-corrected, 31 ± 3 min).

Radiosynthesis of the [^{18}F]peptide–FB conjugate (4b)

[^{18}F]SFB dry residue (~2 GBq) was dissolved in DMSO (100 μL) and added to a solution of **4** (0.75 μmol) in a 2/1 (v/v) mixture (150 μL) of DMSO–phosphate buffer (pH 8.5, 0.2 M). After reacting at room temperature for 10 min, the reaction mixture was diluted with aqueous TFA (0.1%, 2.6 mL) and purified by semipreparative HPLC (25 × 1 cm, water–acetonitrile–TFA from 9/1/0.01 v/v/v to 6/4/0.01 v/v/v in 30 min, 5 mL min^{-1}). The desired fraction (t_r ~ 18–20 min) was diluted with saline solution (0.9%, 30 ml) and passed through a Sep-Pak light C18 cartridge (Waters, Inc.). The cartridge was rinsed with water (10 mL) and **4b** was eluted with ethanol (1 mL) over PBS (pH = 7.4) solution (9 mL). Chemical and radiochemical purity were estimated by analytical HPLC (25 × 0.46 cm, water–acetonitrile–TFA from 8/2/0.01 v/v/v to 2/8/0.01 v/v/v in 30 min, 2 mL min^{-1}). **4b** was obtained in a RCY of 54 ± 4% (decay-corrected, 38 ± 3 min), with chemical and radiochemical purity over 95% for >8 h. A specific activity of 70 ± 5 GBq μmol^{-1} was estimated.

In vitro stability of 1b, 3b, and 4b

In vitro stabilities of **1b**, **3b**, and **4b** were studied in plasma by incubation of saline solutions (100 μL) of thioesters (10 MBq) in plasma (500 μL) for 5, 15, 30, and 60 min at 37 °C. After incubation, samples were treated with urea (1.0 g, 5 min) for protein precipitation and subsequently passed through 0.22 μm hydrophilic filters (Waters, Inc.). The filtrates were analyzed by the semipreparative HPLC methods described above in their respective radiochemical synthesis. This procedure was repeated 3 times.

Animals

PET biodistribution study was performed in four adult male Sprague–Dawley rats weighting 364 ± 22 g. Animals were housed in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and humidity at 55 ± 10%. Food and water were available *ad libitum*. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

PET acquisition

Animals were anesthetized with isoflurane and received an intravenous bolus injection of 25.3 ± 3.3 MBq of **4b**. Immediately after injection, rats were placed in an animal dedicated camera (microPET R4; Concorde, Siemens, Knoxville, TN, USA) for dynamic whole body image acquisition. Whole body data were acquired for 120 min. During all the acquisition procedure anaesthesia was maintained with a facial mask and a concentration of 2.5% of isoflurane vaporized in O_2.

Image quantification analysis

To obtain the dynamic whole body images (frames = 18), PET data were corrected for nonuniformity, random coincidences, and radionuclide decay and reconstructed with a filtered backprojection algorithm into a matrix size of 128 × 128, a voxel size of 0.85 × 0.85 mm, a slice thickness of 1.21 mm,

and an axial field of view covering the full length of the animal (approximately 20 cm). After reconstruction, volumes of interest (VOIs) were manually drawn in the kidneys and urinary bladder and the individual time activity curves were obtained.

Conclusions

In summary, Cys thiol-containing peptides were radiollabeled with [^{18}F]SFB, a widely used prosthetic group to tag biomolecules with ^{18}F, under standard conditions to give stable conjugates to be used for PET. [^{18}F]SFB is considered to be chemo-selective for amine labelling. Our results demonstrate that labelling can also occur rapidly at the Cys residue, when thiol reactivity is tuned by nearby residues, such as Arg in the case of **2–4**. Here we show that [^{18}F]SFB can not only randomly react rapidly with Lys but also with Cys residues if their reactivities are altered by the chemical surroundings. This approach could be used for labelling of certain peptides lacking free amino groups (such as **4**) using NHS esters. Biodistribution and/or *in vitro* stability studies of these Cys labelling peptides provided pharmacokinetic data in accordance with other linear peptides. Any decomposition of the labelled Cys thiols *via* hydrolysis of the thioester linkage was observed.

Moreover, thiol reactivity enhancement in peptides through electrostatic interactions with other vicinal residues may be advantageously employed to prepare peptides with improved features for being labelled with alternative prosthetic groups, *e.g. via* site-selective Michael addition of Cys to maleimides. Peptides can be chemically engineered to increase the reactivity of the thiol group (through stabilization of the thiolate with vicinal residues), thus providing more efficient conjugation reactions. This possibility is currently being investigated by our group.

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