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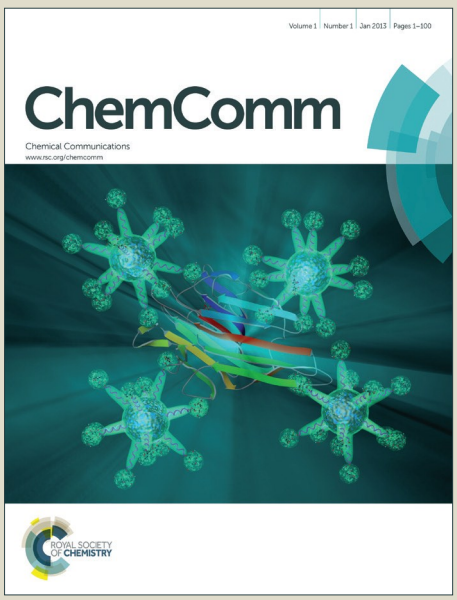

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Novel Chemoselective ^{18}F-Radiolabeling of Thiol-Containing Biomolecules under Mild Aqueous Conditions

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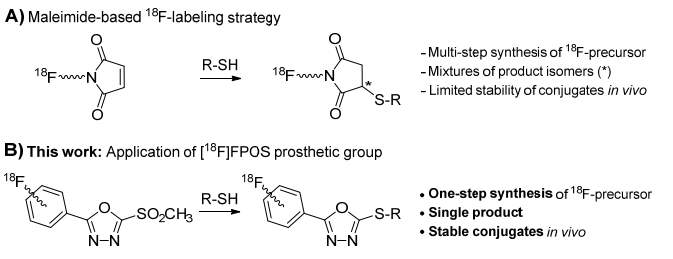
We report a novel prosthetic group based on a heterocyclic methylsulfone derivative for the rapid, stable, and chemoselective ^{18}F-labeling of thiol-containing (bio)molecules under mild aqueous reaction conditions. Compared to established maleimide approaches, the new methodology displays some clear advantages for imaging probe development.

The development of targeted imaging agents involves the incorporation of a reporter probe into a molecule of interest in a chemo- and/or site-specific manner so that the biological properties of the vector (e.g., target specificity and affinity) are not compromised. In the case of radionuclides, fast reaction kinetics are required at the very low concentration levels of radioactive substrates due to radioactive decay. In addition, mild aqueous reaction conditions become necessary when sensitive biologics (e.g., proteins) are employed. Such stringent requirements can make the development of radiotracers a challenging endeavour.

Due to its favourable physical properties, fluorine-18 (^{18}F; β^+ 0.635 MeV, 97% abundance, $t_{1/2}$ = 109.7 min) is the most frequently used radionuclide in nuclear medicine for imaging by positron emission tomography (PET).[1] The incorporation of a ^{18}F label into peptides and proteins is most commonly achieved by modifications of lysine (Lys) or cysteine (Cys) residues using a prosthetic group approach.[2] Prosthetic groups are small, bifunctional molecules, which can be readily radiolabeled with ^{18}F and subsequently conjugated selectively to the molecule of interest. In comparison to Lys, Cys residues are found less abundant in proteins, which permits conjugation chemistry in a more controlled fashion. In addition, proteins and peptides can be engineered to contain a free thiol group for site-specific labeling. As a consequence, a number of cysteine-selective conjugation methods have been reported[3] of which the prosthetic group approach based on maleimide chemistry remains the most widely used strategy for the ^{18}F-labeling of thiol-containing (bio)molecules. This is due to fast reaction kinetics, high selectivity, and the possibility of employing aqueous reaction conditions at room temperature and neutral pH. However, the Michael-type addition of thiols to maleimides without chiral auxiliaries results inevitably in the formation of racemic product mixtures (Scheme 1), which can impede with obtaining approval from regulatory authorities for clinical applications of the radioconjugate, in particular those derived from small molecules. In addition, the suitability of the thiol-maleimide condensation adduct in bioconjugates has recently been questioned because of its potential instability under physiologically relevant (e.g., reducing) conditions.[4] Clearly, there is a need for new bioconjugation approaches which provide an alternative to maleimide chemistry for the rapid, stable, as well as chemo- and/or site-selective (radio)labeling of cysteine residues.[3, 5]



Scheme 1. ^{18}F-Radiolabeling of (bio)molecules (R) *via* different thiol reactive prosthetic groups (A) maleimide derivatives and (B) phenyloxadiazole methylsulfones; squiggled lines represent different linkers or chemical entities for incorporation of the ^{18}F label.

In 2013, the group of late Barbas III reported a Julia-Kocieński-like reagent for the chemoselective labeling of thiol containing

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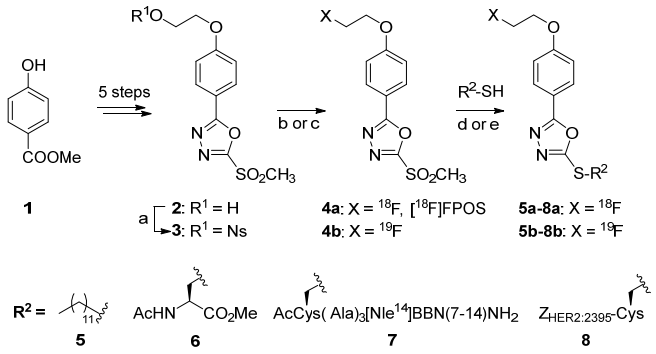
Electronic Supplementary Information (ESI) available: experimental details, characterisation of intermediates, and final compounds by NMR, MS, and HPLC. See DOI: 10.1039/x0xx00000x

proteins with a fluorescent probe by nucleophilic aromatic substitution.[6] They showed that a phenyloxadiazole methylsulfone reacts rapidly and selectively in aqueous media at neutral pH and room temperature in the presence of other potential nucleophiles with the sulfhydryl group of cysteine residues of various model compounds as well as proteins. The conjugates, obtained as single isomers, were more stable under physiological conditions in comparison to the corresponding fluorescent analogues obtained by maleimide chemistry. With the goal to develop this new chemistry in the context of radiotracer synthesis, we set out to investigate the utility of heterocyclic methylsulfone derivatives (Scheme 1) for the ^{18}F-radiolabeling of thiol-containing (bio)molecules.

The phenyloxadiazole methylsulfone scaffold offers different synthetically accessible positions for the incorporation of an ^{18}F label. For this work, we developed a prosthetic group bearing an appropriately functionalized alkoxy side chain attached to the aromatic moiety, which enables fluorination by aliphatic nucleophilic substitution (Scheme 2). Thus, fluorination precursor, nosylate **3**, was prepared from commercial methyl 4-hydroxybenzoate **1** in a total of 6 steps and good overall yield of approx. 20% (Supporting Information).[6] Compound **3** , isolated as a white solid, was found stable upon storage at -25 °C for a time period of >6 months.



Scheme 2. Synthesis of fluorinated phenyloxadiazole methylsulfones and their conjugation to thiol containing model- and biomolecules. a) Ns-Cl, DMAP, CH_2Cl_2, rt, 60%; b) for ^{18}F: nosylate **3**, [^{18}F]KF-$K_{2.2.2}$, CH_3CN/*t*BuOH (1:1), 85 °C, 10 min; c) for ^{19}F: alcohol **2**, DAST, CH_2Cl_2, rt, 40%; d) for ^{18}F: [^{18}F]FPOS **4a**, 0.1-20 mM thiol substrates in mixtures of PBS (0.2 M, pH 7,4) and different organic solvents (10-65% v/v), 37-55 °C, 15 min; e) for ^{19}F: [^{19}F]FPOS **4b**, thiol substrates in mixtures of PBS (0.2 M, pH 7,4) and THF (50% v/v) or THF/DMSO (each 10% v/v), 2 h, rt, 65-93%.

Radiofluorination of nosylate **3** (6 μmol, 10 mM) was accomplished with [^{18}F]KF-$K_{2.2.2}$ in a mixture of CH_3CN and *t*BuOH (1:1) at 85 °C for 10 min. After HPLC purification, ^{18}F-labeled prosthetic group **4a** (termed [^{18}F]FPOS; fluoro phenyloxadiazole methylsulfone) was obtained with an activity of 6-16 GBq in a radiochemical yield (RCY) of 27±6% (n=7) decay corrected (d.c.) from end of bombardment (EOB). Unlike reported maleimide-based prosthetic groups that require multistep synthesis due to their sensitivity to the basic reaction conditions applied for labeling reactions with [^{18}F]KF-$K_{2.2.2}$, [^{18}F]FPOS **4a** was obtained ready for conjugation chemistry in a single step and with a RCY comparable or improved to those reported for ^{18}F-labeled maleimide derivatives.[7] [^{18}F]FPOS **4a** was obtained in sufficient radiochemical purity so that it could be used for subsequent reactions with thiol substrates with or without purification by HPLC. Non-radioactive ^{19}F-analogue **4b** for the syntheses of cold reference compounds **5b**-**8b** (see Supporting Information) was prepared by the reaction of alcohol **2** with DAST.

With [^{18}F]FPOS **4a** in hand, we investigated its reactivity with model compounds n-dodecylthiol and the amino acid AcCysOMe in aqueous medium (PBS, pH 7.4) which contained some organic solvents for solubilizing the substrates and reagents (Table 1, entries 1-2). Complete conversion of crude [^{18}F]FPOS **4a** was observed (γ-HPLC) after 15 min at 37 °C. Thioether adducts **5a** and **6a** were obtained as single products and isolated by HPLC purification (0.6-0.8 GBq; not optimized). Their identity as well as those of the other radiolabeled products listed in Table 1 was confirmed by HPLC comparison with the corresponding non-radioactive ^{19}F-reference compounds (Supporting Information).

We next turned our attention to Cys-containing, tumor-targeting molecules. For proof of concept, we chose a derivative of the peptide bombesin (BBN) and the affibody $Z_{HER2:2395}$ as an example of a protein. Both biomolecules are currently under investigations in radiolabeled form as diagnostic probes and/or therapeutics in nuclear oncology.[8] Specifically, we used a stabilized analogue of the minimum binding sequence of BBS, [Nle^{14}]BBN(7–14), a peptide with high affinity and specificity towards the gastrin-releasing peptide receptor, which is over-expressed by, e.g., prostate, breast, and small-cell lung cancer.[9] The peptide was elongated N-terminally with a $(\beta Ala)_3$ spacer[10] and Nα-acetylated Cys to provide test compound AcCys$(\beta Ala)_3$[Nle^{14}]BBN(7-14)NH_2 (Table 1, entries 3-5). The affibody $Z_{HER2:2395}$ is a non-immunoglobulin-based, small targeting protein (approx. 7 kDa) with high specificity and sub-nanomolar affinity towards the human epidermal growth factor receptor-2 (HER2), a phosphoglycoprotein expressed in high incidence by breast, lung, and ovarian cancer as well as B-cell acute lymphoblastic leukemia. For the study described herein, an analogue of the protein bearing a Cys residue at the C-terminus, $Z_{HER2:2395}$-Cys (Table 1, entries 6-7), was used.[11]

Fluorine-18 labeling of AcCys$(\beta Ala)_3$[Nle^{14}]BBN(7-14)NH_2 and $Z_{HER2:2395}$-Cys was carried out with purified [^{18}F]FPOS **4a** to avoid competing reaction of excess nosylate **3** with the thiol substrates. In addition, the reactions were performed at a concentration range typically reported for the radiolabeling of these molecules (Table 1). ^{18}F-labeling of BBN derivative with [^{18}F]FPOS **4a** proceeded smoothly under the reaction conditions described above for the model compounds yielding, after HPLC purification, product **7a** with a specific activity (A_s) of 53 GBq/μmol and a d.c. RCY of 33% (Table 1, entry 3). Lowering the amount of the peptide substrate by a factor of four was possible but required slightly elevated reaction temperature (50 °C) for complete conversion of [^{18}F]FPOS **4** within 15 min (Table 1, entries 4-5). Reaction of $Z_{HER2:2395}$-Cys with [^{18}F]FPOS **4a** in PBS at 37 °C for 15 min resulted only in a very low conversion of the prosthetic group **4a** (approx. 5%) which could be improved at 50 °C to 40% as estimated by radio-TLC (Table 1, entries 6-7).

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Table 1. Investigated thiol-containing substrates and conditions of their reaction with [^{18}F]FPOS **4a**.

Entry	Thiol substrate / Product	Amount / Concentration [a]	Temperature / Time	Conversion of [^{18}F]FPOS **4a**
1	n-Dodecylthiol / **5a**	17 µmol/12 mM	37 °C/15 min	quant.[b], [c]
2	AcCysOMe / **6a**	28 µmol/20 mM	37 °C/15 min	quant.[b], [c]
3	AcCys(βAla)$_3$[Nle^{14}]BBN(7-14)NH$_2$ / **7a**	1.6 µmol/3.5 mM	37 °C/15 min	quant.[b], [d]
4	AcCys(βAla)$_3$[Nle^{14}]BBN(7-14)NH$_2$ / **7a**	0.4 µmol/0.9 mM	37 °C/15 min	ca. 33% [b], [d]
5	AcCys(βAla)$_3$[Nle^{14}]BBN(7-14)NH$_2$ / **7a**	0.4 µmol/0.9 mM	50 °C/15 min	quant.[b], [d]
6	Z$_{HER2:2395}$-Cys / **8a**	0.14 µmol/0.14 mM	37 °C/15 min	<5%[d], [e]
7	Z$_{HER2:2395}$-Cys / **8a**	0.14 µmol/0.14 mM	50 °C/15 min	ca. 40%[d], [e]

[a] solvent systems (% v/v); entries 1-2: 35% PBS (0.2 M, pH 7.4), 65% CH_3CN/*t*BuOH (1:1); entries 3-5: 45% PBS (0.2 M, pH 7.4), 45% CH_3CN, 10% DMSO; entries 6-7: 90% PBS 0.2 M/Na ascorbate 0.02 M (pH 7.4), 10% EtOH; [b] determined by γ-HPLC; [c] reaction with crude [^{18}F]FPOS **4a**; [d] reaction with HPLC purified [^{18}F]FPOS **4a**; [e] estimated by radio-TLC.

Neither longer reaction time nor higher temperature led to a significant improvement of the conversion rate of [^{18}F]FPOS **4a**. Based on literature data, elevated temperature appears to be a prerequisite for the modification of the Cys-modified affibody presumably by making the C-terminus more accessible as the result of reversible partial unfolding of the protein.[12] [13] In contrast to the studied affibody, it is likely that increased temperature are not necessary for the modification of other proteins with more exposed Cys residues.[6] Despite the relative low conversion of [^{18}F]FPOS **4a**, [^{18}F]Z$_{HER2:2395}$-Cys **8a** was isolated after purification using a size exclusion cartridge within a total synthesis time 160 min and up to 17 GBq/µmol (n=3), which is among the highest A_s reported for ^{18}F-labeled formats of the affibody. [7a, 12-14]

The tumor targeting properties of the radiolabeled affibody [^{18}F]Z$_{HER2:2395}$-Cys **8a** was studied *in vivo* by PET/CT imaging of CD1 nude mice bearing HER2-positive SCOV3 xenografts (see Supporting Information for details). Nude mice with HER2-negative RAMOS xenografts were used as a control. Images were acquired 2 h and 4 h post injection of the radiotracer (7-20 MBq/15 µg per mouse; n=2 for each mouse model). Already 2 h post injection, the ^{18}F-labeled affibody has cleared from the blood pool and showed a markedly high accumulation in HER2-positive xenografts resulting in a favorably high tumor-to-background ratio (Figure 1). Negligible uptake of radioactivity was observed in the RAMOS-cell derived tumors of the control group hence proving the specificity of the radiolabeled protein towards HER2 receptors. No accumulation of radioactivity was found in the bones thus demonstrating the *in vivo* stability of the radiotracer. Albeit used as a model protein for the current study, [^{18}F]Z$_{HER2:2395}$-Cys **8a** exhibits very promising *in vivo* characteristics as a PET imaging agent for the diagnosis of HER2-positive tumors which warrants further investigations.

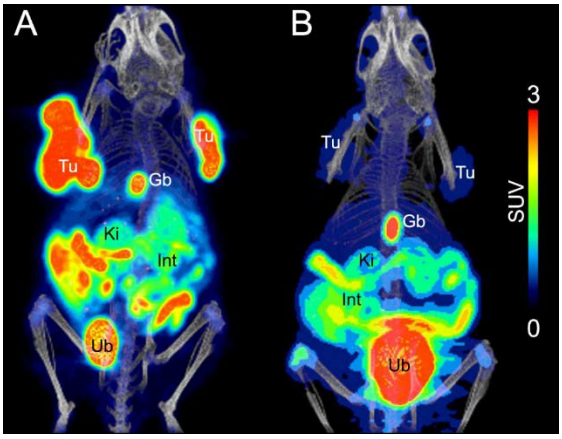

Figure 1. Representative 3-dimensional maximal intensity projections of PET/CT scans performed with HER2-positive SKOV3 (A) and HER2-negative RAMOS (B) tumor-bearing mice 2 hours p.i. of [^{18}F]Z$_{HER2:2395}$-Cys **8a** (5-10 MBq, 15 µg). The standardized uptake value (SUV, color bar) is scaled for both animals. Tu: tumors; Gb: gall bladder; Ki: kidneys, Int: intestine; Ub: urinary bladder.

In summary, we have developed an alternative to the maleimide-based conjugation strategy for the chemoselective ^{18}F-radiolabeling of peptides and proteins *via* modifications of Cys residues. The novel prosthetic group based on a ^{18}F-labeled derivative of phenyloxadiazole methylsulfone ([^{18}F]FPOS) is prepared in a single step from the corresponding nosylate precursor in high radiochemical yield and purity. [^{18}F]FPOS reacts selectively and fast (within minutes) with thiol-containing

substrates in aqueous medium (pH 7.4) at low to moderate temperature providing uniform radioconjugates with high specific activities that are stable *in vivo*. The new methodology presented herein meets all criteria of an effective and chemoselective radiolabeling strategy and thus, may have the potential to become the method of choice for the development of radiotracers derived from thiol-containing (bio)molecules. We are currently investigating applications of the novel radiolabeling strategy to different diagnostic and/or therapeutic radionuclides as well as other peptides and proteins of medical interest.

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