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COMMUNICATION

Original synthesis of radiolabeling precursors for batch and on resin one-step/late stage radiofluorination of peptides

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Radiolabeling of peptides with fluorine-18 is hurdled by their chemical sensitivity and complicated processes. Original triflyl-pyridine intermediates afforded ammonium precursors that were radiolabeled at low temperature. From that study, a generic tag has been designed to allow a simple one-step/late-stage radiolabelling of peptides. The strategy has been transposed to an automated "on-resin" radiolabelling.

Radiolabeled peptides are increasingly demanded in molecular imaging and nuclear medicine[1]. They are used in both diagnosis[2] and therapy[3] depending on the disintegration properties of the selected radioisotope. Thanks to the synthesis options peptides offer, their affinity and stability can be tuned thus providing highly potent ligands with minimized toxicity for promising applications in precision medicine[4]. Positron emission tomography (PET) is one of the most sensitive functional nuclear molecular imaging technique for diagnosis. Among the available positron emitters, fluorine-18 meets numerous advantages in terms of ease of production in biomedical cyclotrons (at several GBq levels), half-life (109.8 min) and disintegration properties (low positron energy of 635 keV; 97% β+ branching) for optimal image generation. Peptides appeared in the molecular toolbox of nuclear imaging more than four decades ago and they were initially radiolabeled by use of the prosthetic approach[5]. The major strength of this sequential strategy is the dissociation of the preparation of a small radiofluorinated reagent, often necessitating harsh reaction conditions, from its conjugation with the peptide in mild and aqueous conditions. The reliability and robustness of this approach was illustrated for more than 30 years by the diversity of biologics that have been thus radiolabeled[6] and the regular upgrades, introducing click chemistry in the late 2000's[7] or the regioselective labeling of "zero size" radiofluorinated motifs[8] more recently. In return, regarding the constraints associated with the handling of short-lived positron emitters, a multistep radiosynthesis is a major drawback for a fully automated process, which is an important consideration in the development of radiotracers. Such limitations question whether a direct introduction of fluorine-18 in a single step could be considered for labeling peptides. Molecular fluorine $[^{18}F]F_2$ or reagents derived were used for the direct radiolabeling of peptides but with a limited extent due to the limitations associated with the production of these electrophilic species in terms of radioisotope supply or need of specific equipment[9]. Nucleophilic fluorine-18 sources, *e.g.* $[^{18}F]F^-$ in $H_2[^{18}O]$ or the classical $K[^{18}F]F/K_{222}$ complex, are preferred and have been extensively exploited for direct radiolabeling. This was illustrated either *via* silicon-[10], boron-[11] or aluminium-fluoride[12] bonds on a modified peptide or using adequate peptide-precursors to undergo site-specific deoxyfluorination[13] (Figure 1).

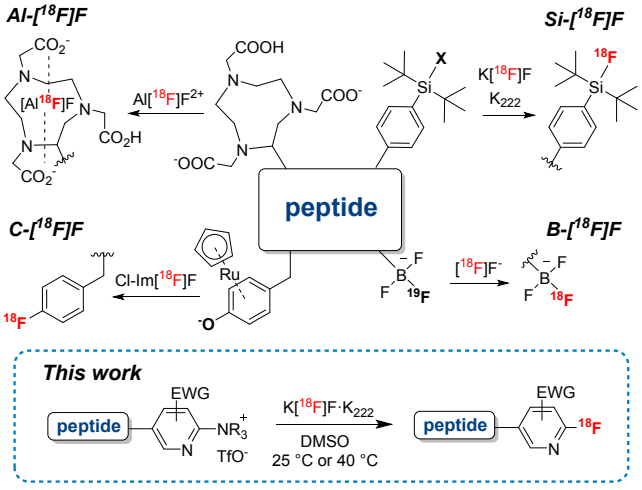

Figure 1. Late-stage fluorination of peptides.

Nevertheless, apart from the boron-fluoride bond formation, elevated temperatures are required and final molar activity remains a concern when isotopic exchange was exploited.

Easy-access to fluorine-18-radiolabeled peptides by late stage fluorination is still a challenge today. Shifting from the standard prosthetic concept of "radiolabel first and then conjugate" to

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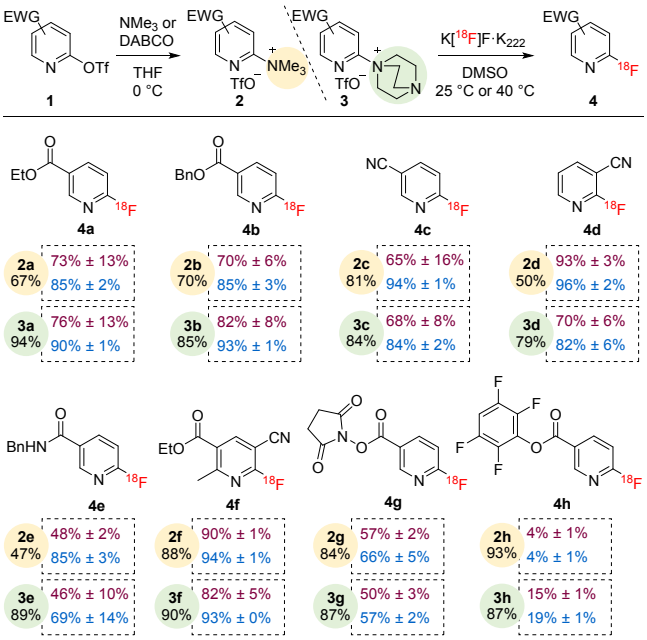
"conjugate first and then radiolabel", we report herein advances in the one-step/late stage toward "minimalist" approaches to radiolabel peptides at low temperature. Our approach is based on the higher reactivity of the pyridine ring[14], which is boosted in presence of electron-withdrawing groups[15] (Figure 1).

We wanted first to examine the influence of the electron-withdrawing groups (EWGs) on the kinetics of radiofluorination at low temperature by SNAr using trialkylammonium as a leaving group. However, we initially faced a synthetic obstacle to access these ammonium precursors **2**, as quaternarization of such electron-deficient pyridines proved to be highly challenging (*ESI: I.5-Preliminary studies*). Methylation of the corresponding dimethylamines was surprisingly completely ineffective, even with a single ethyl ester as EWG and using highly electrophilic methyl sources. We next tried to reproduce reported procedures involving the direct introduction of trimethylamine on the pyridine ring by chlorine substitution[14c, 15b, 16]. In our case, this method led to the observation of the desired ammonium chlorides as intermediates only, quickly evolving to the dimethylamine derivatives by chloride nucleophilic attack to one of the methyl group of the ammonium.

This last observation prompted us to investigate a new route to pyridine ammoniums **2** by the direct addition of trimethylamine to 2-triflyl pyridines **1**, the triflate group being a highly efficient nucleofuge and a very poor nucleophile. If the nucleophilic aromatic substitution of (hetero)aryl triflates by primary and secondary amines have been reported[17], addition of tertiary amines leading to the corresponding ammonium was only described for triazine triflates.[18]. By applying this strategy to our electron-deficient pyridines, a surprisingly good conversion was observed for the formation of **2** as a stable ammonium. With this new method in hand, we next designed a library of 16 pyridine derivatives displaying different EWGs and divided in two series of quaternary ammonium precursors: trimethylammoniums **2** and ammoniums **3** derived from DABCO amine, which are largely underexploited in radiochemistry (Scheme 1). As EWGs, we selected i) nitrile and ester (ethyl and benzyl) that are widely encountered in ^{18}F-radiochemistry, and a combination of both; ii) amide (benzyl) to mimic a peptide bond; and iii) *N*-hydroxysuccinimide (NHS) and tetrafluorophenyl (TFP) activated esters. Compound **4g**, acting as acylating prosthetic reagent, would thus be prepared in a simpler way than its ubiquitous benzene homologue 4-[^{18}F]fluorobenzoate ([^{18}F]SFB), prepared in a multistep time-consuming procedure.[19] Such a simplified approach was already proposed for the TFP activated ester.[15a] The triflate intermediates **1** were synthesized from the fairly accessible pyridones in good to excellent yields varying from 53 to 91 %. Triflates **1** were next treated with either trimethylamine or DABCO affording the final quaternary ammonium triflate precursors (**2** and **3**) in good to excellent yields.

The performance of each precursor toward radiofluorination was then evaluated at 40 °C and even at room temperature (*ESI: II.2-Kinetic studies*). As expected, the conversions were higher at 40 °C, whatever the EWG and no drastic differences could be observed between ammonium precursors **2** or **3** (*ESI: II.2-Table S1*). The kinetic profile shows a plateau reached as early as 5 to 10 min for the most reactive precursors.



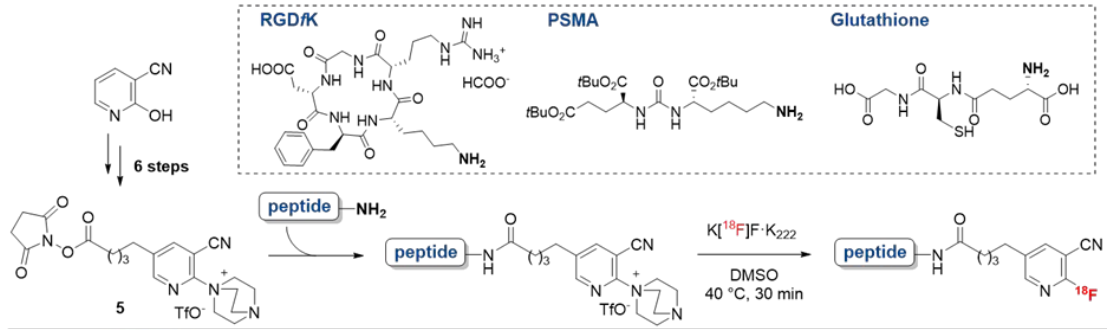
Scheme 1. Synthesis of precursors **2** and **3** and their radiofluorination at low temperatures. Conversions after 15 minutes at 25 or 40 °C (n = 3)

Nitriles and esters, or combination of both, gave the highest conversions reaching more than 90% even at room temperature after 15 min reaction for precursors **2d** and **2f** in particular. Due to the softer electron-withdrawing character of amide compared to ester or nitrile, a lower conversion was observed starting from precursors **2e** and **3e**. Activated esters (NHS and TFP) gave the lowest yields because of their relative instability under the radiofluorination conditions (Scheme 1). Non-radioactive by-products were characterized and correspond to 6-NHS and 6-TFP[15a] derivatives.

This kinetic study showed that EWG-substituted pyridines could be radiolabeled at low temperatures and demonstrated the relevance of designing a pyridine-based tag that could be conjugated to a peptide for a direct radiofluorination. In that end, the first step was to assess the chemical stability of radiolabeled pyridines **4** regarding their potential inclination to defluorination (*ESI: II.6-Stability studies*). The hydrolytic stability was checked by incubation of all labeled pyridines **4** in PBS for 30 min, but no trace of free fluoride-18 was observed in any case. A second control was performed by incubation of **4c** in foetal bovine serum up to 60 min, again without observable defluorination, indicating a presumptive satisfying chemical stability for future *in vivo* application. We then undertook the synthesis of a generic tag displaying a nitrile group as EWG for the functionalization of peptides. Compound **5** was prepared in 6 steps from commercial 3-cyano-2-pyridone. The "conjugate first and then radiolabel" strategy was then evaluated with three model compounds: two peptides (glutathione, c(RDGfK))

and a PSMA ligand, the latter being extensively used in nuclear medicine for theranostic applications. Each compound was functionalized with the cyanopyridine tag **5** providing selectively the precursors **3i**-**k**, resulting from the coupling of the peptides or PSMA with the NHS-ester. No aromatic substitution at the pyridine ring with the free amine was observed during this step. Precursors were then subjected to radiofluorination with K[^{18}F]F/K_{222} for 30 min at 40 °C (Scheme 2). Peptides **4i**, **4k** and **4j** were obtained with isolated yields ranging from 42 to 55 % and their identity was checked by radioHPLC (*ESI: II.4 Fig S2, Fig S3 and Fig S4*).

Scheme 2. Late-stage fluorination of peptides at low temperature via a pyridine tag.



To go one step further, the reactivity of ammonium precursors **2** and **3** was harnessed for the development of an advanced late-stage radiofluorination process for peptide labeling. By immobilization of the trialkylamine on a solid support, we interrogated whether our method allowed the loading of triflates **1** on a resin by SNAr and if the corresponding supported ammoniums would retain their reactivity toward radiofluorination. Such ammonium triflates could also act as an anion exchanger to directly trap the [^{18}F]fluorides from the aqueous solution produced by the cyclotron. This strategy would facilitate the whole radiolabeling process with an easy translation to automation by loading the solid support in a cartridge, as well as short synthesis time and selective release of the labeled pyridine from the resin. Although anion metathesis of [^{18}F]fluorides with a labeling precursor on a solid support is known,[20] immobilization of the precursor and its labeling on a resin with [^{18}F]F-/H_2O have never been reported yet. To evaluate this new concept, we prepared a supported quinuclidine amine **6** in a few steps from a commercial (aminomethyl)polystyrene resin (Figure 2).

The grafting of this resin by SNAr was studied with three selected 1-triflyl pyridines **1b**, **1c** and the PSMA derivative **1j**. Incubation in DMF at room temperature of these pyridines with resin **6** was quite efficient and provided resins **7b**, **7c** and **7j** in satisfying grafting yields with a final ammonium loading comprised between 0.46 and 0.90 mmol/g. The potential of these supported precursors for radiofluorination was explored by loading the resins in cartridges that were installed on a PET synthesizer *(ESI:II.5 Fig S6 and S7)*. Trapping efficiency of resins **7** was first determined, after elution of the [^{18}F]fluoride aqueous solution produced by the cyclotron, 23-35% of the starting activity remained on the cartridge, presumably by anion metathesis between [^{18}F]fluoride and triflate anion. After a short air-drying of the cartridge, a moderately slow elution with DMSO was performed. Analysis of the resulting eluate indicated a conversion of 75% in the desired labeled pyridine, with only [^{18}F]fluoride and pyridines **4** as radioactive species. This enabled the isolation of pyridines **4** with a high radiochemical purity after only a simple formulation on C18 or Al_2O_3 SPE cartridge. We however noted the presence of the corresponding 2-hydroxypyridines as the major non-radioactive byproduct, resulting from the competitive SNAr with water. With this process, pyridines **4b**, **4c** and **4j** were obtained with low to moderate radiochemical yield, demonstrating the proof of concept of the on resin radiolabeling. A longer air-drying, the use of other polar organic solvents or other types of resins are options for improvement. To further simplify this method, resin **7b** was also prepared *via* slow elution of a cartridge filled with resin **6** with a solution of 1-triflyl pyridine **1b** and radiolabeling was performed without any alteration of the yield of **4b**. This method radically differs from previous reports describing the use of resins either in a solid phase peptide synthesis approach[21] or in a solid phase organic radiosynthesis approach that always involved standard K[^{18}F]F-K_{222} complex, organic solvents, elevated temperatures [22].

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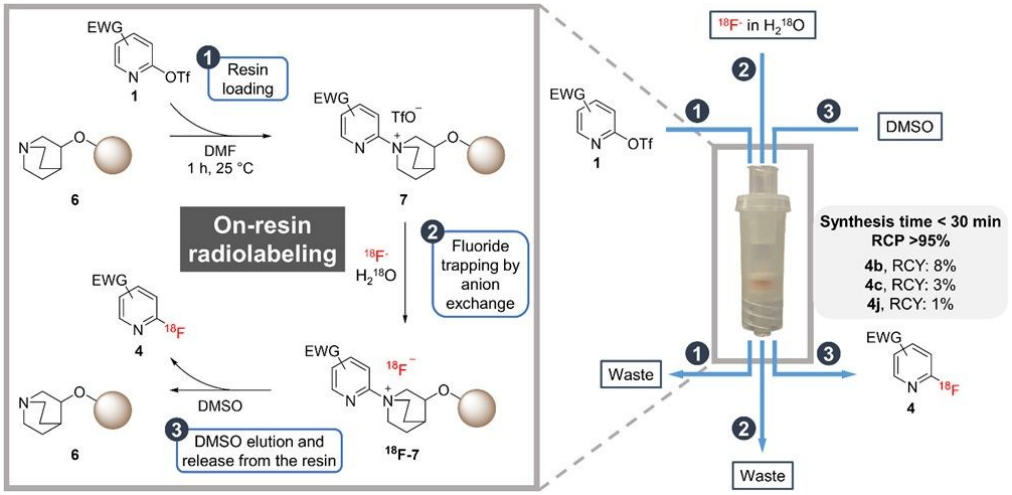

Figure 2. Automated on-resin radiolabeling at room temperature using aqueous $^{18}F^-$ ion performed by a home-made module.

In summary, we have developed an original synthesis exploiting a triflyl intermediate to prepare trimethylammonium and DABCO-derived precursors for the radiolabeling with fluorine-18. We have demonstrated that such precursors, displaying an appropriated electron-withdrawing group, could be radiolabeled at room temperature. Taking advantage of this, we have designed a generic tag that allowed for a one-step/late stage radiofluorination of model peptides and a PSMA ligand at moderate temperature with satisfactory yields. Inspired by the DABCO-derived ammonium precursor and the high reactivity of the triflyl intermediate, we established the proof of concept of an "on-resin" radiofluorination of peptides in an innovative, simple and automatable way. This approach may contribute to popularize radiolabeled peptides in molecular imaging and opens new opportunities to radiolabel compounds accepting an auxiliary group to incorporate fluorine-18.

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Conflicts of interest

There are no conflicts to declare.

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